

In Vitro Characterization of Stem Cells from Human Exfoliated Deciduous Teeth (SHED)

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Abstract

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Stem cells from human exfoliated deciduous teeth (SHED) provide a new attractive source for stem cells; in this study we further characterize SHED. SHED were isolated, differentiated using osteogenic/odontogenic differentiation media, characterized using light microscope, SEM and immunocytochemistry using CD44. Also, Immunohistochemistry using CD44 was performed on extirpated pulp tissues. We found that a naturally exfoliated human tooth contains a population of stem cells that attain morphological homogeneity after the first passage, on adding the osteogenic/odontogenic medium, sporadic nodule-shaped structures were observed after two weeks that were positively stained with alizarin red and von Kossa stains. SHED stained with H& E showed a basophilic, eccentric nucleus with an eosinophilic cytoplasm in which two differently stained areas were clearly distinguishable. Also we found using SEM that SHED spread on the UBM scaffolds surfaces showing multiple filopodia and formed collagen-like structures by the seventh day. After three weeks, seeded scaffolds incubated in osteogenic/odontogenic media showed many mineralized nodules in the ECM. Cultured SHED revealed positive immunoreactivity when treated with CD44. Also, sections of pulp tissue treated with CD44 depict positively stained cells situated mainly in the perivascular areas reinforcing the hypothesis that pericytes may be the origin of SHED.

Introduction

Stem cells are defined as clonogenic cells that are capable of both selfrenewal and multilineage differentiation [1]. There are two broad types of mammalian stem cells; embryonic (ESCs) and adult stem cells (ASCs). ESCs face many ethical and technical considerations that limit their availability [2, 3]. ASCs are either multipotent or pluripotent. The later type is rare

and generally small in number but can be found in some tissues including umbilical cord blood [4]. Multipotent cells are generally referred to by their tissue of origin (mesenchymal stem cell, adipose-derived stem cell, etc.).

Mesenchymal stem cells (MSCs) have a diverse origin. It could be obtained from various types of connective tissues such as fat [5], bone [6], cord blood

and more attractively from both permanent [7] and deciduous dental pulp [8]. Different stem cell markers are used for identification and isolation of various types of mesenchymal stem cells, such as Bone morphogenetic, CD44, c-Kit, Muc-18, Stem cell antigen (Sca-1) and Stro-1 antigen. Unfortunately specific markers that could uniquely identify MSCs have yet to be found. Identification of human MSCs derived from deciduous dental pulp (SHED) has been one of the most promising fields in MSCs studies, considering that they are a feasible source for stem cells that provides a fascinating hope in cell transplantation and tissue engineering in both medical and dental fields.

The aim of the current study is to further characterize the putative stem cells in human exfoliated deciduous teeth *in vitro*.

Materials and Methods

Isolation and proliferation of SHED

Ten normally exfoliated human deciduous incisors were collected under complete aseptic conditions from seven to eight years-old children. The parent of the child was asked to sign a consent form. The pulp was separated from the remnant of each crown and then digested in a solution of 3 mg/ml collagenase type I and 4 mg/ml dispase for 45 minutes at 37°C. Single-cell suspensions from each dental pulp were cultured in a regular medium as previously reported [8], the media consisted of Dulbecco's modified Eagle's media (DMEM), containing 20% fetal bovine serum, 50 µg/ml L-ascorbic acid, 1% L-glutamine, 100 units/ml penicillin-100 µg/ml streptomycin and 2% Hepes buffer. These techniques resulted in a population of cells that can be termed as stem cells from human exfoliated deciduous teeth (SHED).

Differentiation of the putative SHED

The 2nd passage SHED was re-cultured in 6-well-culture-plates (1 x 10⁵ cell/well). After that the osteogenic/ odontogenic differentiation media, which consist of the plane media with 100 mM L-ascorbate-2-phosphate, 10⁻⁷ M dexamethasone and 3 mM inorganic phosphate, was added in half of the wells while the other half was incubated in plane pulp media as a control [9]. We evaluated the formation of calcified ECM by SHED after 14 days, using von Kossa and Alizarin red special stains.

Characterization of SHED

The second passage SHED was examined by light microscope, immunocytochemistry and scanning electron microscope (SEM). SEM was done after SHED was seeded on rabbit urinary bladder matrix (UBM), a biological scaffold used to observe the behavior of these cells when seeded *in vitro* in a 3D system before proceeding with the *in vivo* studies.

• **Light microscope:** Cells were fixed and stained with hematoxylin and eosin (H&E).

• **SEM:** To examine the topographical features of putative pulp stem cells, the second passage SHED was seeded on UBM scaffolds then the scaffolds were divided into two groups; one group was incubated in osteogenic/odontogenic inductive media, while the other group was incubated in plane pulp media, then samples from both groups were collected after four, seven and 21 days and examined using a Jeol SEM microscope, in Tissue Engineering Laboratories, Alexandria University.

• **By immunocytochemistry:** Mouse monoclonal CD44 (*Labvision*) antibody was used as a primary antibody and the avidin-biotin system (*Labvision*) was used as a detection system with ready to use biotinylated goat secondary antibody to complete the procedures.

• **CD44:** It is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration. Also, it has been used as a marker for MSCs [10, 11]. The following protocol was applied: 1. Positively charged slides with 2nd passage SHED were incubated overnight in the CO₂ incubator (37°C, 5% CO₂ and 95% humidity), washed with PBS three times then they were fixed on ice cold methanol (-20°C). 2. Reduce nonspecific background staining due to endogenous peroxidase, by incubation the slides in hydrogen peroxide for 10-15 minutes. 3. Wash twice in PBS. 4. Slides were placed in Ultra V Block and incubated for 5-10 minutes at room temperature. 5. Apply primary antibody at 1-2 µg/ml concentration and incubate overnight at 4°C. 6. Wash 4 times in PBS. 7. Apply ready to use secondary antibody and incubate for 10-15 minutes at room temperature. 8. Wash 4 times in PBS. 9. Apply enzyme labeled streptavidin and incubate for 10 minutes. 10. Rinse 4 times in PBS. 11. Apply appropriate percentage of DAB substrate with DAB chromogen and incubate until desired reaction is achieved. 12. Counterstained with hematoxylin and coverslip. The results were examined using light microscope.

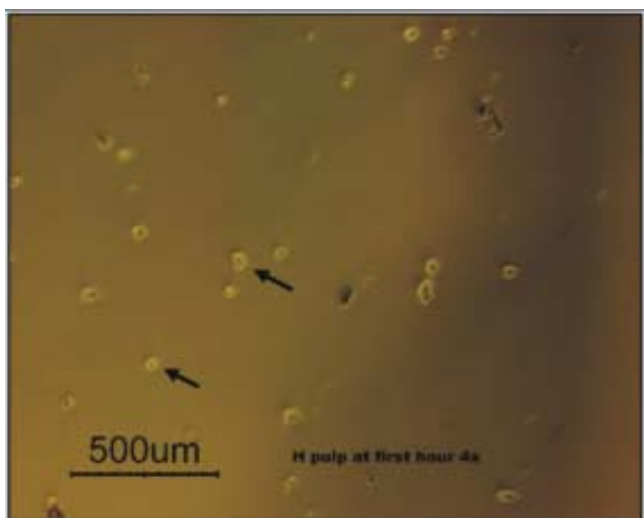


Figure 1a: Inverted microscope imaging of cultured pulp cells, one hour after culturing reveals unattached cells (black arrows) [original magnification x4].

B-Immunohistochemical study

Specimens of the extirpated pulp tissues were fixed in 4% paraformaldehyde for 1-2 hours, embedded in paraffin, sectioned at 5 µm thickness, placed on positively charged slides then stained with CD44 using the same immunocytochemistry procedures, except that in the first step we deparaffinized and rehydrated tissue sections.

Results

It is noteworthy that the successful cultures were established from only the upper not the lower

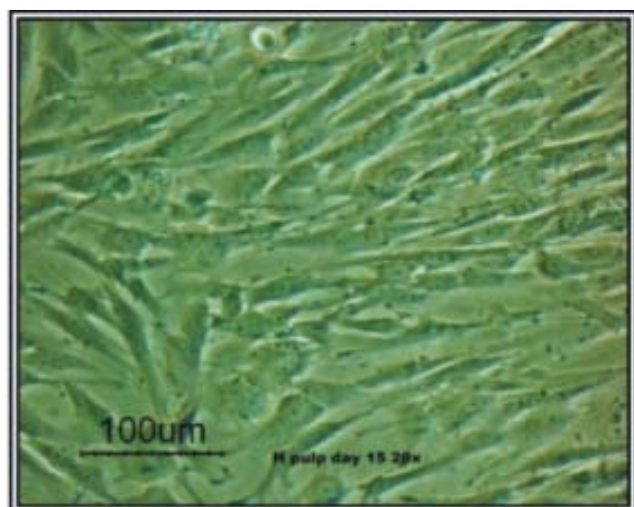


Figure 1b: Inverted microscope imaging illustrates proliferating pulp cells at day 15 [original magnification x20].

incisors of patients with high socioeconomic state.

Isolation and proliferation of SHED

Immediately one-hour post-culture, cells were seen emerging from the pulp tissue as numerous large round cells having a thin rim of cytoplasm and a large nucleus (Fig. 1 a).

Many cells were spindle-like; others appeared stellate-shaped while some appeared polygonal, thus reflecting the diversity of proliferating pulp cells. By the 14th day, 90% of the flask area was covered with cells, indicating the highly clonogenic and proliferative cell population of the dental pulp of human exfoliated deciduous teeth (Fig. 1 b).

After the primary passage, flat cells bearing a large nucleoli-rich nucleus appeared to predominate and attain morphological homogeneity (Fig. 1 c, d)

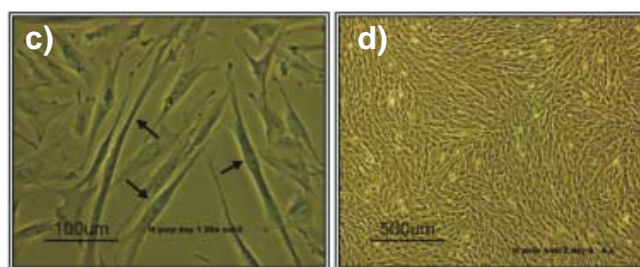


Figure 1c,d: Inverted microscope imaging of second passage SHED. Notice, flat cells bearing a large nucleoli-rich nucleus continue to dominate (black arrows) [original magnification x20] (c). Also, morphological homogeneity of the cultured cells seemed to expand [original magnification x4] (d).

Differentiation of the putative SHED

Postconfluency, the second passage cells continued growing and forming multilayers in culture (Fig.2 a). On adding the osteogenic/odontogenic medium, sporadic nodule-shaped structures were observed after two weeks. These nodules were positively stained with alizarin red (Fig.2 b) and von Kossa stains (Fig.2 c) respectively, indicating calcium accumulation *in vitro*. Unlike the control groups which showed no signs of *in vitro* mineralization (Fig. 2 d, e).

Characterization of SHED

Characterization of the second passage SHED was done by light microscope, scanning electron microscope and immunocytochemistry.

• **Light microscope:** Second passage SHED stained with H&E showed a basophilic, eccentric nucleus

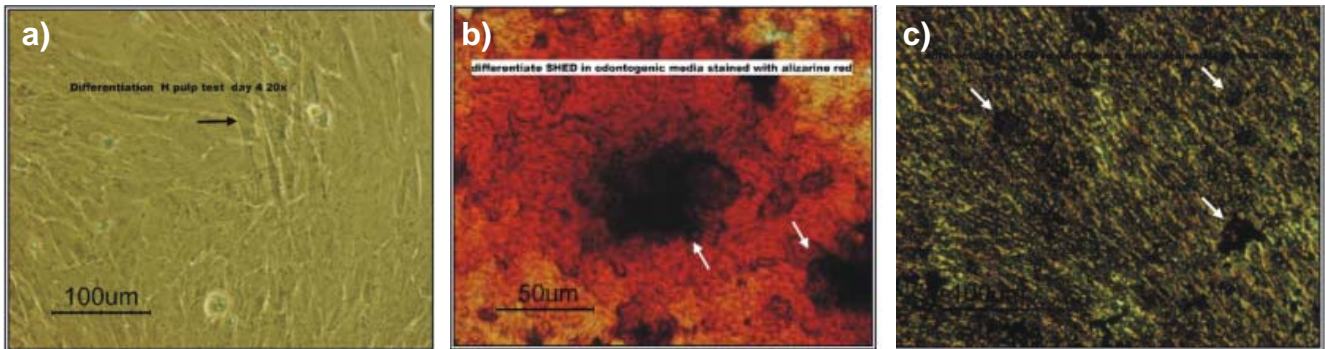


Figure 2: a) Inverted microscope imaging of cultured 2nd passage SHED. Postconfluency, cells generate multilayers in culture plates (black arrow) [original magnification x20]. b) Inverted microscope imaging shows higher magnification of the previous image. Alizarin red-positive nodules are evident (white arrows) [alizarin red stain, original magnification x40]. c) Inverted microscope imaging of differentiated 2nd passage SHED showing formation of black nodules indicating calcium formation (white arrows) [von Kossa stain, original magnification x20].

with an eosinophilic cytoplasm in which two differently stained areas were clearly distinguishable: an intensely

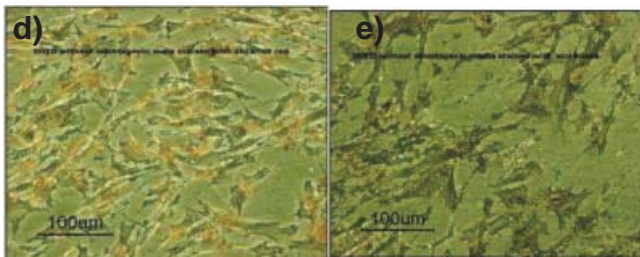


Figure 2d,e: Inverted microscope images show the lack of mineralized nodules in control culture after staining with alizarin red (d), von Kossa (e) [original magnification x20].

stained inner zone and a thin, relatively pale peripheral zone that encircle irregular plasma membrane (Fig. 3a).

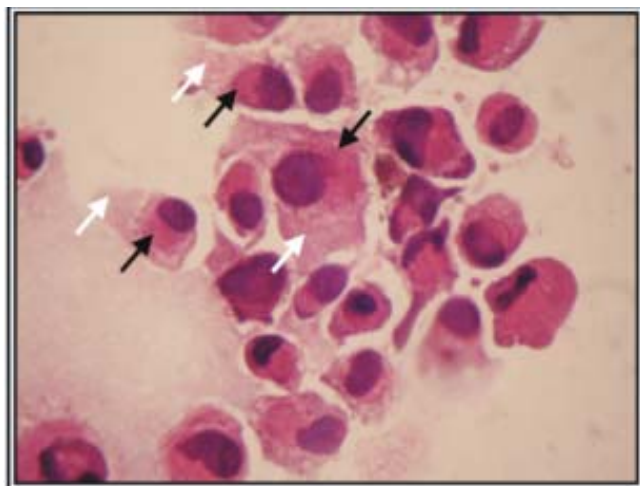


Figure 3a: Light microscope imaging showing attached cells with eccentric nucleus, and the cytoplasm is divided into two differently stained areas: an intensely stained inner zone (black arrows) and a less stained thin peripheral zone (white arrows). The plasma membrane showed an irregular profile [H&E stain, original magnification x100].

• **Scanning electron microscope:** Upon culturing on the UBM scaffolds, SHED appeared to spread on the scaffold surface showing multiple filopodia. Also, SHED started to form abundant collagenlike structures by the seventh day (Fig.3 b). After 3 weeks, the group of seeded scaffolds incubated in osteogenic/odontogenic inductive media showed many mineralized nodules in the ECM (Fig. 3 c), while the other group showed no signs of mineralization (Fig. 3 d).

• **Immunocytochemistry:** Immunophotyping of the cultured SHED treated with CD44 revealed positive immunoreactivity (Fig. 3e). This was shown by the evidence of strongly DAB stained cells with large oval-shaped nuclei. As for control specimens no discernible stain could be seen.

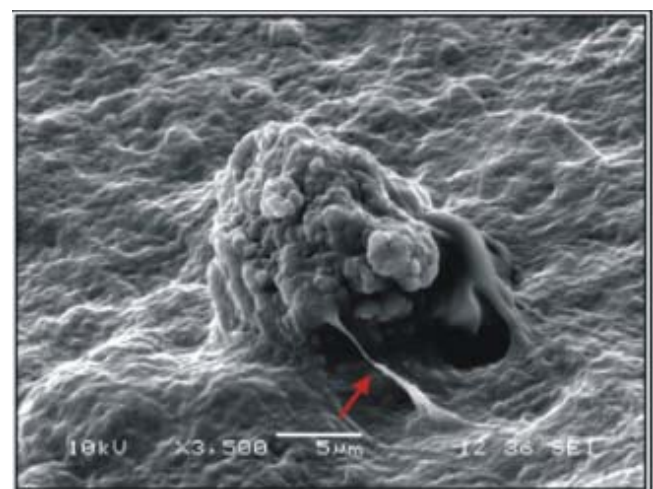


Figure 3b: SEM observation of UBM loaded with 2nd passage SHED cultured in osteogenic/odontogenic media for 7 days shows collagen like structures attach cells to the scaffolds (red arrow) [original magnification x3500].

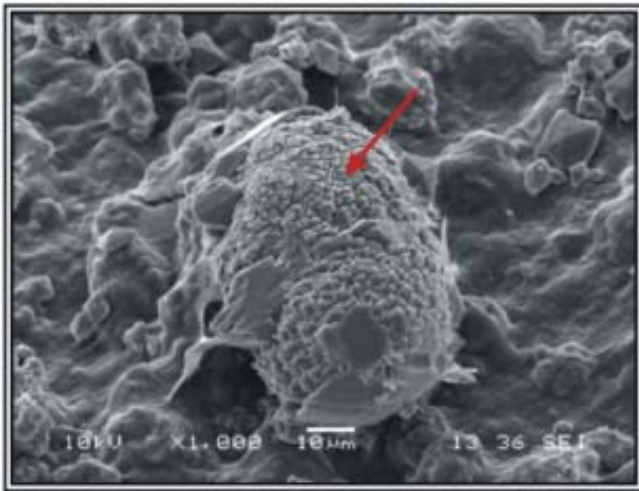


Figure 3c: SEM observation of UBM loaded with 2nd passage SHED cultured in osteogenic/odontogenic media for 21 days shows mineralization of the extracellular matrix (red arrows) [original magnification x1000].

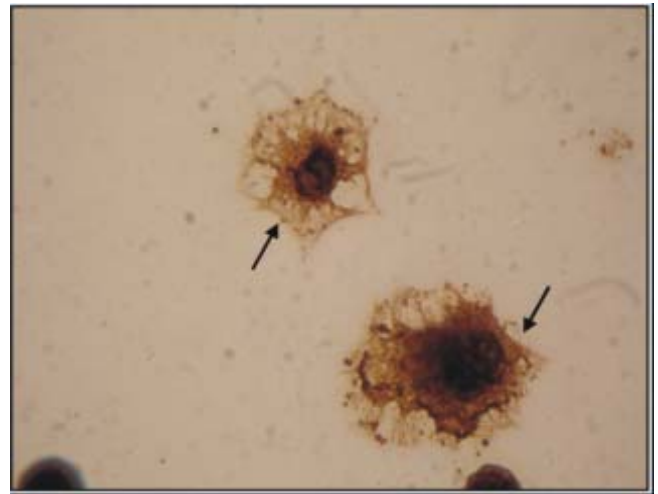


Figure 3e: Light microscope imaging shows positively stained cell membrane (black arrows). The nucleus is counterstained with hematoxylin [Immunoperoxidase staining to CD44, original magnification x100].

Immunohistochemistry

Sections of pulp tissue that was extirpated from exfoliated deciduous incisor and treated with the marker CD44 depicts some areas stained positively with DAB while other unstained areas, stained cells were situated in the perivascular areas and occasionally in the

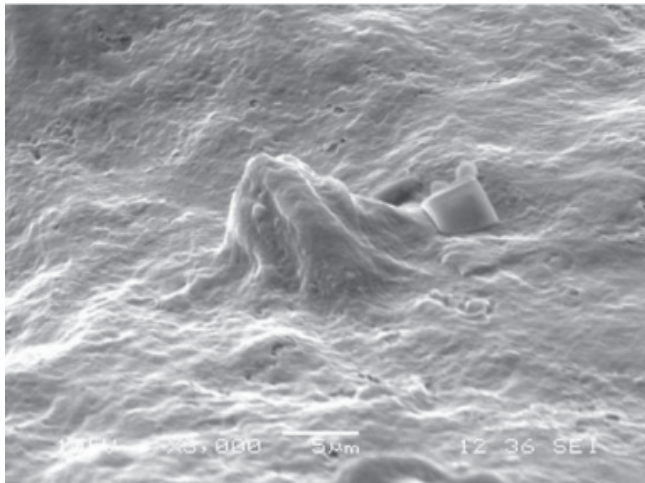


Figure 3d: SEM observation of UBM loaded with 2nd passage SHED cultured in normal pulp media for 20 days shows no mineralization signs of the ECM [magnification x 3000].

extravascular areas (Fig. 4 a). These cells exhibited large ovalshaped nuclei strongly with hematoxylin, similar to those of cultured SHED cells (Fig. 4 b).

Discussion

In the present study isolation of the pulp cells was done using the enzyme digestion method not the outgrowth method. As George T et al., 2006 [12] found, using bromodeoxyuridine (BrdU) incorporation and Zymed BrdU staining, that the cells isolated by enzyme digestion had a higher proliferation rate than those isolated by outgrowth method. Also, the digestion method releases all cell types in the pulp tissues, so the observation of cells of different morphologies is unsurprising [7, 13]. To date, different types of adherent

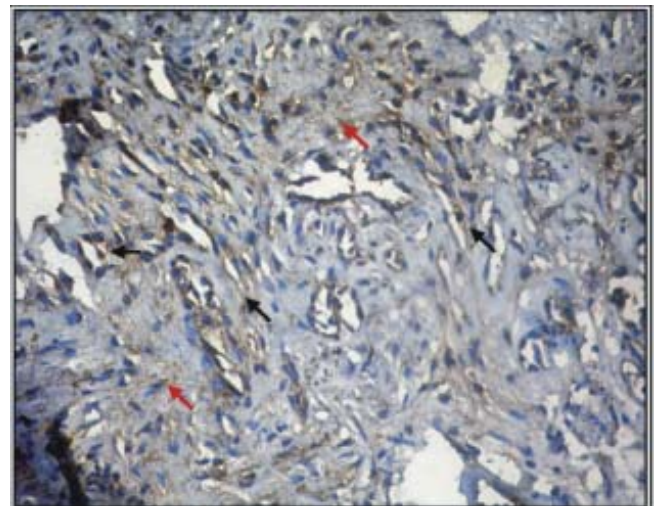


Figure 4a: Light microscope image shows positively stained cells in perivascular areas (black arrows), and extravascular areas (red arrows) [Immunoperoxidase staining to CD44 and hematoxylin stains, original magnification x40].

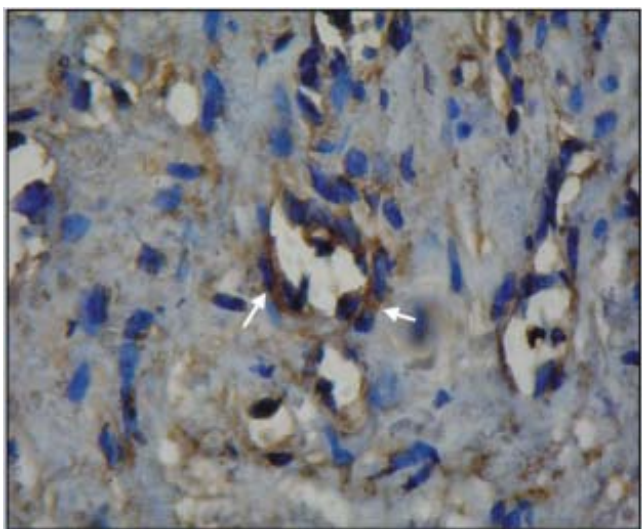


Figure 4b: A higher magnification of the previous image illustrates positively stained cells with oval shaped nuclei strongly stained with hematoxylin located mainly in perivascular areas (white arrows) [Immunoperoxidase staining to CD44 and hematoxylin stains, original magnification x100].

cells in pulp tissue seem to reveal diverse dividing ability; these include mesenchymal cells, pulp fibroblasts, endothelial cells and pericytes.

Also in the current work, DMEM, a variation of Eagle's minimal essential media (MEM) which contains approximately four times as much of the vitamins and amino acids present in the original formula and two to four times as much glucose, was used. It enhances the proportion of \pm smooth muscle actin positive cells, which represent a putative source of odontoblast-like cells [8, 14, 15] compared the effect of MEM (1.8 mM Ca and 1mM Pi) and RPMI 1640 (0.8 mM Ca and 5 mM Pi) on the behavior of human dental pulp cells and declared that MEM significantly increased cell proliferation, ^3H thymidine incorporation, strongly stimulated alkaline phosphatase activity and induced expression of transcripts encoding DSPP. In conclusion, these observations demonstrated that not only proliferation but also differentiation into odontoblast-like cells was induced by the rich calcium and poor phosphate MEM media and its modifications as compared to RPMI 1640. These observations are concurrent with a recent study by [16], indicating that a gradual increase (0-4 mM) in calcium concentration promotes osteoblastic cell proliferation. Thus, it is reasonable to speculate that the higher concentration of calcium in DMEM could favour odontoblast-like differentiation of human pulp cells.

We used deciduous incisors not molars because

during shedding of molars the gingival tissue tends to migrate within the pulp chambers more pronouncedly making the extracted pulpal tissue less pure [17]. It was very interesting to find that only pulps from the upper not the lower incisors yielded successful cultures, we think that this could be attributed to the fact that the remaining pulp tissue in the upper incisors is larger. Also, we found that cultures from patients with high socioeconomic level were more successful this may be due to the good nutritional level.

In this study, we used CD44 surface marker, both in immunocytochemistry and immunohistochemistry. This marker was used to identify and isolate MSCs from different tissues like bone marrow [18, 19], human term placenta [20], periodontal ligaments [10] and dental pulp [7, 21, 22].

On culturing the isolated pulp cells, SHED reached confluency within 14 days, forming an adherent monolayer of visible fibroblast-like colonies in the culture flask, with numerous dividing stem cells [7, 23, 24]. We used the second passage cultures because as the cultures were passaged, morphological homogeneity was gradually achieved in that flat cells bearing a large nucleoli-rich nucleus predominated, this in accordance with a study by Gronthos et al., 2000 [7].

Interestingly, we found that SHED as other MSCs predominantly has a bipolar fibroblastic, stellar or flat morphology, rather than a polygonal endothelial-like appearance which makes them share the same morphological characters with pericytes cultured *in vitro* [7, 25, 26].

Also, we found that post-confluent second passage SHED continued growing and forming multilayers in tissue culture. Then, after two weeks of incubation in osteogenic/odontogenic media, we noticed the production of a dense ECM and sporadic nodular structures were found. These results have been reported by other studies [27-30]. To detect the mineralization of the formed nodules, both Alizarin red and von Kossa stains were used.

We observed Alizarin red-positive nodules formed in the SHED cultures indicating calcium accumulation *in vitro*. These results are in accordance with other studies [7, 8, 31]. Also, we noticed that the calcified deposits were sparsely scattered throughout the adherent layer as single mineralized nodules rather than mineralization of the whole monolayer [7, 32]. Also, after von Kossa staining we observed the formation of black stained nodules [27-30].

It is possible that the nature of these mineralized nodules could be bone or dentin, however both [8, 29, 30] found that various bone markers CBFA1 (Core-Binding Factor, Runt Domain, Alpha Subunit 1), ALP, MEPE (Matrix extracellular phosphoglycoprotein with ASARM motif) and bone sialoprotein were up-regulated under the osteogenic/odontogenic induction of the secondary cultured SHED. In addition, DSPP was induced by the mineralizing induction. These data indicated that SHED possessed the ability to differentiate into functional odontoblast like cells *in vitro*. However, in the two previous studies although all the cell cultures were DSPP positive; it was not possible to observe the typical column-shaped morphology of an odontoblast. As to obtain specific odontoblast like appearance, the cells would require the guidance of a surrounding tissue or biomimetically designed scaffold material [29].

Morphologically, after haematoxylin and eosin staining, second passage SHED showed a basophilic, eccentric nucleus and an eosinophilic cytoplasm in which two differently stained areas were clearly distinguishable: an intensely stained inner zone and a thin, relatively pale peripheral zone, the plasma membrane showed an irregular profile. These observations resembled those of rats bone marrow MSCs in which the differently stained areas of cytoplasm were related to the higher amount of organelles present in the inner zone in relation to the peripheral one [33].

In vitro analysis of UBM scaffolds, seeded with SHED, by SEM showed that cells had spread well on their surfaces. Most of the cells had multiple filopodia. This is in agreement with the results of Yang et al., 2007 [34]. In addition, after 3 weeks UBM scaffolds loaded with SHED and incubated in osteogenic/odontogenic differentiation media revealed abundant formation of collagen-like structures together with many mineralized nodules in the ECM. On the other hand, the UBM scaffolds loaded with SHED and incubated in plane pulp media revealed absence of any mineralization signs of the ECM. This indicates the unique qualities of SHED.

By immunocytochemistry, we found that by using CD44 surface marker in characterization of second passage SHED cultured on positively charged slides, strongly DAB stained cells with oval-shaped nuclei and strong haematoxylin staining were identified.

By immunohistochemistry, we tried to determine the location of SHED *in situ* within the pulp tissue sections using CD44 surface marker. We found that by using CD44 surface marker to treat pulp paraffin sections,

some areas were stained positively while others were not; cells with oval-shaped nuclei and strong haematoxylin staining were identified *in-situ*. These cells were observed mainly in the perivascular areas as well as in some extravascular areas; these results were observed in human periodontal ligaments in an earlier study by Chen et al., 2006 [10].

This indicates that SHED *in-situ* is reactive with the mesenchymal stem cell marker CD44, and that they located primarily in the perivascular areas.

Interestingly, Crisan et al., 2008 [35] found that in many body organs (skeletal muscle, pancreas, adipose tissue, placenta, and bone marrow) pericytes, *in-situ*, express MSCs marker CD44.

In addition, Tecles et al., 2005 [36] used BrdU labeling to observe the proliferation and migration of stem cells from perivascular areas to the site of pulp injury, following deep cavity preparation, suggesting the perivascular origin of pulp stem cells.

Thus collectively, our findings using cell culture, immunohistochemistry and SEM studies suggest that the remnant pulps from the crowns of human exfoliated deciduous teeth contain a population of MSCs that have proliferative and differentiative potentials similar to other MSCs isolated from other body organs and reinforce previous assumption in other studies, that pericytes are a very powerful candidate to be the source of SHED in the pulps of human deciduous teeth.

Acknowledgments

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