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Isolation and culture of dental pulp stem cells from permanent and deciduous teeth

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ABSTRACT
Objective: To isolate dental pulp mesenchymal stem cells (MSCs) from non-infected human permanent and deciduous teeth.
Methods: It was an in-vitro experimental study. Human teeth were collected from 13 apparently healthy subjects including nine adults and four children. After decoronation dental pulps were extirpated from teeth and cultured via explant method in a stem cell defined media. Data was analyzed by descriptive statistics.
Results: As above MSCs emerged exhibiting fibroblast-like morphology. In vitro culture was positive for 100% (9/9) and 75% (3/4) of the permanent and deciduous teeth respectively. First cell appeared from deciduous tooth pulp in 10±6.2 days while permanent teeth pulp took 12.4±3.7 days. Together, 26.6±3.6 and 24.5±3.5 days were required for permanent and deciduous tooth pulp stem cells to be ready for further assays.
Conclusions: The protocol we developed is easy and consistent and can be used to generate reliable source of MSCs for engineering of calcified and non-calcified tissue for regenerative medicine approaches.

KEYWORDS: Dental pulp, Tissue explant, Mesenchymal stem cells, Permanent teeth, Primary / Deciduous teeth, Tissue engineering.


INTRODUCTION

Mesenchymal stem cells (MSCs) are attractive tools for tissue repair because of their differentiation capability and abundance in tissues.1 Virtually every tissue has tissue specific stem cells.2 Although, MSCs were first isolated in bone marrow,3 a decline is observed in using bone marrow MSCs while interestingly, an increasing trend is evident regarding exploration of various postnatal tissues as a source of MSCs.4 Growing evidence suggests a remarkable regenerative potential of MSCs from dental derived tissue such as Dental pulp stem cells (DPSCs) from impacted third molar,5 stem cells from apical papilla (SCAP)6 and stem cells from human exfoliated deciduous teeth (SHED).7

Permanent and deciduous (primary) teeth are considered as an easily accessible source for isolation and subsequent expansion of dental pulp cells for
the purposes of tissue engineering. The collection of
dental pulp is an easier and safer undertaking than
the collection from bone marrow.⁸

Since the discovery of dental pulp stem cells in 2000,
these have been investigated extensively through in-
vitro and in-vivo approaches. Very recently, research-
ners in Japan have conducted a phase I clinical trial to
evaluate the safety of autologous dental pulp stem
cells for regenerating dental pulp (DP).⁹

In Pakistan, the potential of dental pulp stem cells
has yet to be explored in pre-clinical and clinical
trials. Therefore, in the present study, we aim to
develop a protocol for the isolation and culture of
dental pulp stem cells from permanent and
deciduous teeth with a long term goal of their use
in regenerative medicine applications.

METHODS

Recruitment and Collection of Teeth Samples: This
was an in vitro study on human samples. Patients
were recruited from dental clinics at Aga Khan
University Hospital, Karachi. Inclusion criteria
for permanent teeth selection were healthy adult
subjects; aged between 18 to 85 years, presenting
for extraction of un-diseased third molars or
orthodontic patients needing extraction of sound
premolars for braces treatment. The inclusion
criteria for deciduous teeth were healthy children;
aged between 9 to 12 years, presenting with teeth
near to physiologic tooth exfoliation. Only those
deciduous teeth were selected whose dental pulp
was sound and without any carious exposure.
Written informed consent was obtained from
adult subjects and assents from parents of children
in English, or their native language. The study
was approved by institutional Ethics Review
Committee (ERC), Ref number 4-1997-BBS-ERC-12.
The exodontia of teeth was performed under local
anesthesia and one child subject shed off tooth by
gentle manipulation without any need for local
anesthesia.

Sample Processing: A total of nine (n=9) permanent
and four (n=4) deciduous teeth were collected.
After disinfecting with 3% sodium hypochlorite
solution for two minutes, tooth was rinsed with 1X
phosphate buffer saline (PBS) and dried using cotton
gauze. A cut around the cemento-enamel junction
was made using a sterilized dental diamond fissure
burs (Mani, Inc. USA) along with high speed hand
piece ( NSK, USA) under copious water supply to
decorate the tooth to expose the pulp chamber as
shown in Fig.1A & 1B.

Sectioned teeth were placed into the transport
media (TM) containing basic medium Dulbecco
modified essential medium F12 (DMEM-F12) sup-
plemented with 20% fetal bovine serum (FBS) and
penicillin 500U/mL, streptomycin 500µg/mL, am-
photericin B 1.25µg/mL) (Sigma Aldrich, Merck,
USA). Samples then placed on ice were transferred
to Juma Research Laboratories at Aga Khan Uni-
versity Hospital for subsequent processing and
culture. Using aseptic condition, 100 mm petri plate
(Sterilin) were set up for processing of each tooth in
a biohazard laminar flow hood. Extracted tooth was
decanted in a petri plate. Tooth was hold with the
help of a sterile forceps and gently extirpated out
dP tissue using endodontic H-file # 30 (MANI, Inc,
USA). DP tissue was placed in 1X PBS containing
1% antibiotic antymycotic solution (Sigma Aldrich)
in a petri plate for 10 to 20 minutes as seen in Fig. 2A
and was washed twice with 1X PBS (Sigma Aldrich,
Merck, USA) each for 10 minutes. Then were trans-
ferred into a new petri plate containing DMEM-F12
with 20% FBS. Minced into 1- 2mm³ pieces using
surgical blade # 20 (Feather, WAPI, USA) as demon-

Fig.1: Collection of dental pulp tissue from extracted tooth. (1A) Cutting procedure of extracted tooth after disinfection;
with the help of a stable finger support using dental fissure burs, the tooth was decoronated till cemento-enamel junction.
Decoration is an exothermic process therefore, an ample amount of sterile water was sprayed to reduce the heat.
(1B) Removal of crown exposed the pulp chamber. (1C) Extirpated dental pulp from adult tooth on the tip of dental file.
strated in Fig. 2B. DP minced fragments were plated in a T-25 flask (Thermo Scientific, USA) containing DMEM-F12 supplemented with 20% FBS, penicillin 100U/mL, streptomycin 100µg/mL, amphotericin B 0.25µg/mL, 1mM sodium pyruvate and 2mM L-glutamine (Sigma Aldrich). Explants were cultured at 37°C in a humidified incubator with 5% CO₂. Cultures were observed daily under inverted microscope (Olympus Corp, USA) for any contamination and cell growth via migration from explant. Micrographs were captured using DSL3 standalone microscope camera controller (Nikon, Japan) at different magnifications.

**Passage:** When cells reached 70 to 80% confluency they were either used for an assay or cryopreserved for later use. Cells were thoroughly washed with 1X PBS twice, trypsinized with 0.05% Trypsin-EDTA (Sigma Aldrich) for two to five minutes, neutralized by adding 10% FBS containing DMEM-F12. Detached cells were transferred in a tube, centrifuged at 500g for five minutes. Carefully decanted the supernatant, make sure not to dislodge the cell pellet. Cells were resuspended either in growth media for an intended assay or stored using freezing media containing 90% FBS and 10% DMSO (Sigma Aldrich) in liquid nitrogen at -196°C for later use or long term storage.

**Viability Assay:** Cells were grown till reached 60-65% confluency. Briefly, cells were trypsinized and resuspended in complete media. Equal volumes of cell suspension and 0.4% trypan blue (Gibco) were mixed, 10µL of prepared sample were loaded in both chamber of hemocytometer. Viable and non-viable cells were counted within five minutes of preparing sample. Counting was performed in duplicate.

**Statistical Analysis:** Data was analyzed using mean ± SD.

**RESULTS**

DP tissues were extirpated from tooth by decoronation as shown in Fig. 1C and were cultured via outgrowth / tissue explant method. In terms of cellular morphology, cells migrated out of the tissue explant, exhibited homogenous morphology having a typical fibroblast-like shape, with long cytoplasmic processes as shown in Fig. 3A & 3B.

The in vitro explant culture was positive for 100% (9/9) and 75% (3/4) permanent and deciduous teeth respectively. One pulp tissue from deciduous teeth didn’t show cell grow at all. The reason might be the osmotic shock to the pulp tissue due to sample transportation without transport media. The other pulp tissue grew into small colonies however, got contaminated during long primary culture. Both

<table>
<thead>
<tr>
<th>Type of Teeth</th>
<th>In-vitro Culture</th>
<th>First Cell Appearance (days)</th>
<th>Sub-culture (days)</th>
<th>Primary culture Duration (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Permanent (n=9)</td>
<td>Positive (100%)</td>
<td>12.4 ± 3.7</td>
<td>13.9 ± 4.2</td>
<td>26.6 ± 3.6</td>
</tr>
<tr>
<td>Primary (n=4)</td>
<td>Positive (75%)</td>
<td>10 ± 6.2</td>
<td>13.5 ± 4.9</td>
<td>24.5 ± 3.5</td>
</tr>
</tbody>
</table>

Table-I: Tabular summary of isolation and culture of dental pulp stem cells derived from permanent and deciduous teeth.
cell type exhibited low proliferative potential. DPSCs and SHED slightly differ in their growth rate. Deciduous teeth pulp gave rise first cell in 10 ± 6.2 days of culture while pulp from permanent teeth took 12.4 ± 3.7 days for the emergence of first cells. Together, 26.6 ± 3.6 and 24.5 ± 3.5 days were required for DPSC and SHED to be ready for further assays Table-I. Two pulp tissues, derived from a permanent and a deciduous tooth, were subjected to delayed processing of 21 and 24 hours respectively. In contrast to an average of 10 ± 6.2 days, the first cell appeared from deciduous teeth pulp tissue was increased to 17 days. However, the change due to delay in processing was not significant in case of pulp from permanent teeth.

Cellular viability of DPSC and SHED was observed using trypan blue dye exclusion test indicating the cell membrane integrity was preserved during cryopreservation as shown in Fig.4. We found 99.4±.09% and 94.19±1.65% viability for DPSC and SHED respectively.

DISCUSSION

We report a protocol that describes the isolation and culture of human dental pulp mesenchymal stem cells from permanent and deciduous teeth.
using explant method. DPSCs obtained from enzymatic and explant methods were reported to be comparable in terms of mesenchymal stem cell marker expression and multi-lineage differentiation thus suggested to be used as suitable autologous source to regenerate bone and cartilage.11

The emerging cells from tissue explant form loose colonies, comprised of cells with characteristic spindle shaped morphology, unlike epithelial cells which forms compact colonies. Our data are consistent with the results of other studies in which a homogenous population of cells were observed using tissue explant method.12,13 Interestingly, the protocol we developed is equally useful for teeth samples stored for up to 24 hours at 4°C. This is especially important, where a delay in sample processing is unavoidable. We presume such a strategy may be employed in setting up a tooth bank as reported elsewhere.14

The explant method has been extensively used for studying the dental pulp cells physiology, cell subpopulations capable of differentiating into odontoblasts or mineral-forming cells in vitro15 and dentin-like structure in vitro.16 DPSCs and SHED obtained from enzyme digestion were also capable of differentiating into odontoblast-like cells and produced dentin in vitro.6,7 Furthermore, an immature DPSC (iDPSC) population was obtained from SHED via explant method. These cells expressed embryonic stem cell markers and showed dense engraftment.17 Although both methods yield similar cell populations, probably, the advantage of employing enzyme is that it releases all different types of cells present in the DP tissue.15

Primary culture of DPSCs grows slowly,15 our cells also took longer time to grow in culture. We also performed enzymatic digestion by treating minced DP tissue with 3 mg/mL collagenase type 1and dispase type II. However, it gave low cell yield which remained unable to grow. In our experiments, the undigested tissue fragments seemed to be the sole source of cells in culture (data not shown). Further, the use of undigested tissue to obtain dental stem cells has also been mentioned in literature.18 Cryopreservation protocol if not followed properly may result in reduced viability of stored cells. The protocol we used was effective for maintaining more than 90% viability of dental MSCs.

Extracted teeth, considered as clinical waste, offer a promising source of autologous cells. These cells can potentially be used for regeneration of musculoskeletal19 chondrocytes, adipocytes, cornea, hair follicle, and endothelial cells.20,21 Previous studies demonstrated the transdifferentiation of human dental pulp cells into neuron-like cells,22 oligoprogenitors,23 cardiomyocytes,24 and insulin producing cells.25 Above mentioned studies provide evidence in favor of regenerative potential of dental pulp MSCs.

Considering the most accessible and feasible cell source, the usefulness of dental pulp stem cells or dental cells in generating iPSCs has also been documented in literature. SHED, SCAP,26 and DPSCS can easily be reprogrammed into iPSCs at relatively higher rates.27 Very recently, human dental stem cell derived transgene-free iPSCs were successfully used to generate functional neurons, exhibiting sodium and potassium currents, action potential, or spontaneous excitatory postsynaptic potential.18

We report a protocol for the extirpation of dental pulp tissue, in-vitro explant culture and propagation of dental pulpMSCs from permanent and deciduous human teeth. MSCs are comprised of heterogeneous population thus it is important to characterize them for the presence of stem cell sub-populations. Therefore, we plan to characterize these cells using classical MSCs markers and their regeneration potential in-vitro. We believe, the major benefits from the protocol that it could provide a reliable source of stem cells to be used in regeneration of damaged or diseased tissue or organs, generation of patient specific stem cells and iPSCs banking.


REFERENCES


Author’s Contribution:

SN contributed in sample collection and transportation to research lab, designed and performed the experiments, literature search, statistical analysis, manuscript writing and editing of manuscript.

FRK conceived the idea, designed study, obtained specimens, wrote manuscript and reviewed the manuscript.

RRZ critically reviewed the manuscript.

SSL carried out ordering, conducted pilot experiment, literature search.

MSK carried out ordering, conducted pilot experiment, and literature search.

NM participated in study design, interpreted data and reviewed manuscript.

TA supervised the project, wrote manuscript and critically reviewed the manuscript.

All authors have approved the final version of the manuscript and disclose no conflict of interest.