EXPRESSION OF STAGE SPECIFIC EMBRYONIC ANTIGEN-4 (SSEA-4), IN DENTAL PULP ISOLATED FROM HUMAN PERMANENT TEETH – AN IMMUNOCYTOCHEMICAL STUDY

Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

In partial fulfilment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH VI

ORAL PATHOLOGY AND MICROBIOLOGY

APRIL 2016

CERTIFICATE

This is to certify that this dissertation titled **"EXPRESSION OF STAGE SPECIFIC EMBRYONIC ANTIGEN-4 (SSEA-4), IN DENTAL PULP ISOLATED FROM HUMAN PERMANENT TEETH – AN IMMUNOCYTOCHEMICAL STUDY"** is a bonafide dissertation performed by **V. SARANYA** under our guidance during the postgraduate period 2013-2016.

This dissertation is submitted to **THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY,** in partial fulfilment for the degree of **MASTER OF DENTAL SURGERY** in **ORAL PATHOLOGY AND MICROBIOLOGY, BRANCH VI.** It has not been submitted (partial or full) for the award of any other degree or diploma.

RAGAS DENTAL COLLEGE * Chennai

Dr. K. Ranganathan, M.D.S., MS (Ohio), PhD.,
Professor and HOD,
Dept. of Oral & Maxillofacial Pathology
Ragas Dental College & Hospital,
Chennai.

Dr. Umadevi K. Rao, M.D.S.,
Professor,
Dept. of Oral & Maxillofacial Pathology
Ragas Dental College & Hospital,
Chennai.

Dr. K. Ranganathan, MDS,MS(Ohio),Ph.D.
Professor and Head
Department of
Oral and Maxillofacial Pathology
Ragas Dental College and Hospital
Chennai

Dr.Umadevi K Rao, MDS
Professor
Department of
Oral and Maxillofacial Pathology
Ragas Dental College and Hospital
Chennai

Dr. S. Ramachandran, M.D.S.,
Principal,
Ragas Dental College & Hospital,
Chennai.

PRINCIPAL
RAGAS DENTAL COLLEGE AND HOSPITAL
UTHANDI, CHENNAI - 600 119.

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY CHENNAI

DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation titled **"EXPRESSION OF STAGE SPECIFIC EMBRYONIC ANTIGEN-4 (SSEA-4), IN DENTAL PULP ISOLATED FROM HUMAN PERMANENT TEETH – AN IMMUNOCYTOCHEMICAL STUDY"** is a bonafide and genuine research work carried out by me under the guidance of **Prof. Dr. K. Ranganathan, M.D.S., MS (Ohio), PhD.,** Professor and Head and **Prof. Dr. Umadevi K. Rao, M.D.S,** Professor, Department of Oral & Maxillofacial Pathology, Ragas Dental College and Hospital, Chennai.

V. Saranya

Dr. V. Saranya
Post Graduate Student
Department of Oral & Maxillofacial Pathology,
Ragas Dental College and Hospital,
Chennai.

Date: 07. 01. 2016

Place: Chennai

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY CHENNAI

DECLARATION BY THE GUIDE

I hereby declare that this dissertation titled **"EXPRESSION OF STAGE SPECIFIC EMBRYONIC ANTIGEN-4 (SSEA-4), IN DENTAL PULP ISOLATED FROM HUMAN PERMANENT TEETH – AN IMMUNOCYTOCHEMICAL STUDY"** is a bonafide and genuine research work carried out by **Dr. V. Saranya**, Post Graduate Student in the Department of Oral & Maxillofacial Pathology, Ragas Dental College and Hospital, Chennai under our guidance in partial fulfillment for the requirement of the degree of **Master of Dental Surgery** (Oral Pathology and Microbiology).

Prof. Dr. K. Ranganathan, M.D.S., MS (Ohio), PhD.,
Professor and HOD,
Department of Oral & Maxillofacial Pathology
Ragas Dental College & Hospital,
Chennai.

Date: 07.01.2016
Place: Chennai

Dr. K. Ranganathan, MDS,MS(Ohio),Ph.D,
Professor and Head
Department of
Oral and Maxillofacial Pathology
Ragas Dental College and Hospital
Che

Dr. Umadevi K. Rao, M.D.S.,
Professor,
Dept. of Oral & Maxillofacial Pathology
Ragas Dental College & Hospital,
Chennai.

Date: 08.01.2016
Place: Chennai

Dr Umadevi K Rao, MDS
Professor
Department of
Oral and Maxillofacial Pathology
Ragas Dental College and Hospital
Chennai

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY CHENNAI

ENDORSEMENT BY THE HEAD OF THE DEPARTMENT AND HEAD OF THE INSTITUTION

This is to certify that this dissertation titled **"EXPRESSION OF STAGE SPECIFIC EMBRYONIC ANTIGEN-4 (SSEA-4), IN DENTAL PULP ISOLATED FROM HUMAN PERMANENT TEETH – AN IMMUNOCYTOCHEMICAL STUDY"** is a bonafide dissertation performed by **Dr. V. Saranya** under our guidance during the postgraduate period 2013-2016. It has not been submitted (partial or full) for the award of any other degree or diploma.

Dr. K. Ranganathan, M.D.S., MS (Ohio), PhD.,
Professor and HOD
Department of Oral & Maxillofacial Pathology
Ragas Dental College & Hospital
Chennai.

Dr. K. Ranganathan, MDS,MS(Ohio),Ph.D.
Professor and Head
Department of
Oral and Maxillofacial Pathology
Ragas Dental College and Hospital
Chennai

Date: 07.01.2016
Place: Chennai

Dr. S. Ramachandran, M.D.S.,
Head of the Institution
Ragas Dental College & Hospital
Chennai.

PRINCIPAL
RAGAS DENTAL COLLEGE AND HOSPITAL
UTHANDI, CHENNAI - 600 119.

Date: 08.01.2016
Place: Chennai

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY
CHENNAI

COPYRIGHT

DECLARATION BY THE CANDIDATE

I hereby declare that the **The Tamilnadu Dr. M.G.R. Medical University, Tamilnadu** shall have the right to preserve, use and disseminate this research work in print or electronic format for academic / research purpose.

V. Saranya

Date: 07.01.2016

Signature of the Candidate

Place: Chennai

Dr. V. Saranya

Acknowledgment

I would like to take this opportunity to express my gratitude to everyone who have helped me and supported me through this journey.

I am dedicating this work to my beloved dad ***Mr. A. Vijayakumar*** *and my loving mom* ***Mrs. Sridevi Vijayakumar*** *and my brother* ***Mr. V. Karthik kumar*** *for their endless sacrifice, dedication and prayers that made me fortunate enough to have such an education and whatever I am today. Without you three I am nothing. Whatever happened is all because of you.*

My heartfelt gratitude and respect to my teacher ***Dr. K. Ranganathan****, Professor and Head of Department, Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, for his patient guidance, support and encouragement throughout my study. I am really blessed for having a dedicated teacher like him, who has always been my inspiration.*

Words are inadequate to express my gratitude to ***Dr. Uma Devi. K. Rao****, Professor, Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, for her valuable guidance and support all through my study. Your patience and perfection is one thing I want to learn. Thank you so much mam for all the support and guidance.*

My sincere gratitude and respect to ***Dr. Elizabeth Joshua****, Professor, Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, for her guidance and support. She was approachable for any help and always made me feel at home by her caring nature. Thank you mam.*

I express my sincere heartfelt gratitude and thanks to Professor ***Dr. T. Rooban****, Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, for his valuable and constructive suggestions and useful critiques that helped me to complete the study. Thank you for the support, motivation whenever I was down and the confidence you had in me all through my post graduate programme. Thank you sir.*

I am extremely thankful to the Principal ***Dr. S. Ramachandran*** *and Chairman* ***Mr. Kanakaraj****, Ragas Dental College and Hospital for providing all the facilities in our institution.*

I am extremely grateful to Readers ***Dr. N. Lavanya****,* ***Dr. C. Lavanya****, Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, for their continuous encouragement and support. I also thank Senior Lecturers* ***Dr. S. Sudharsan*** *and* ***Dr. Kavitha Shankar*** *for supporting me in all possible ways.*

I extend my sincere gratitude and respect to ***Ms Kavitha Wilson****, Geneticist and Lab manager, who helped me a lot in every step of my study.*

I am extremely thankful for my seniors ***Dr. A. Vasaki*** *and* ***Dr. P. Gowri Shankar*** *who helped me in all ways with their valuable suggestions to complete this study.*

*My sincere thanks to **Mr. Rajan,** Lab technician for his support in completing my study. I also thank **Mrs. Vasanthi**, Attender for her prayers and support that helped me a lot in completion of my study. I thank **Ms. Aarthi** for her help in statistics throughout my post graduate programme. I sincerely thank **Mr. Krishnakumar** Ragas General hospital for his support in helping me to work in the cell culture lab beyond the college hours.*

*I earnestly thank all my batch mates **Dr. Angaiyarkkanni**, **Dr. Deepasri**, **Dr. Divya**, **Dr. Joseph** and **Dr. Malarvizhi** for all their support throughout my post-graduation. I also thank all **my seniors** and **juniors** for all their help and support. A special thanks to my junior **Dr. Saranya Manikandan** who helped me in all possible ways throughout my study.*

*It would not be fine if I forget to mention the main reason for my study to happen. **"MY CELLS"** The ones who made this study possible, who travelled with me all through my study, who taught me what is patience, who were with me during my failures, who sacrificed their lives for my happiness and success. Thank you my dear ones.*

*Above all I thank **Almighty** for everything which happened to me so far. Without you and your blessings am nothing.*

Lastly, with deepest gratitude I wish to thank every person who came into my life, inspired, touched and illuminated me through their presence. Thank you.

ABSTRACT

Background

Dental Pulp Cells (DPCs) are unique and viable source of adult mesenchymal stem cells. These cells have a capacity to differentiate into multiple lineage.

Aim

To isolate and characterize mesenchymal stem cells from dental pulp in α-Modified Minimum essential medium (α-MEM) and to study the expression of Stage Specific Embryonic Antigen-4 (SSEA-4) - an embryonic marker in their 1^{st}, 2^{nd} and 3^{rd} passages.

Materials and Methods

Isolation of pulp tissue was done from 30 permanent teeth samples. Among 30 samples, growth characteristics and morphology was assessed and immunocytochemistry was also done for SSEA-4 for two different samples in the 1^{st}, 2^{nd} and 3^{rd} passages of the culture.

Results

4 successful cultures yielded sufficient cells for characterization. Among 4, growth characteristics and morphology was assessed for 2 samples. There was gradual increase in the mitotic to post mitotic phenotype. The average Population Doubling Time (PDT) and seeding efficiency was 3.26 (days) and 70.15%

respectively. Immunocyotochemical analysis was done using SSEA-4 which showed negative expression in the cells of all the passages.

Conclusion

Dental Pulp Stem Cells (DPSCs) are the excellent source of stem cells. The stem cells were successfully isolated from dental pulp of 4 different third molars. The growth characteristics and morphology was assessed for two samples. SSEA-4 expression was not seen in all the 3 passages of the two samples. But the expression was seen in positive controls. We believe that cells of the later passages could turn out to be positive for SSEA-4, which would comprise of differentiated cells.

Keywords: Stem cells, Stage Specific Embryonic Antigen-4 (SSEA-4), Immunocytochemistry.

CONTENTS

Introduction

Cell culture is the method of isolation and growing of prokaryotic, eukaryotic or plant cells under controlled conditions. Stem cell is a "clonogenic, undifferentiated cell that is capable of self-renewal and multi-lineage differentiation"[1].

The term "stem cell" was coined by Russian histologist **Maximov A** in 1909 and the existence of hematopoietic stem cells (HSCs) was first described by him. In 1998, **Thomson J** and **Gearhart J** for the first time grew human embryonic stem cells *in vitro*[2, 3]. In 2000, **Gronthos S** and **Shi S** cultured cells from permanent tooth pulp[4]. In 2003, **Miura M**, **Gronthos S** and **Zhao M** used human primary teeth as one of the new sources of stem cells[5].

Three major developments that have facilitated cell culture in the lab are: (i) the use of antibiotics to inhibit the growth of contaminating microorganisms, (ii) the use of trypsin to facilitate the subculture of cells and (iii) the use of chemically defined culture medium for specific types of cell.

Stem Cells:

Stem cells have the potential to divide indefinitely and can give rise to different types of cells that make up an organism. A stem cell should fulfill three basic criteria: Self-renewal, multilineage differentiation and functional reconstitution[6].

Stem cells are classified according to their origin and differentiation potential. Based on their differentiation potential, they can be totipotent,

pluripotent or multipotent stem cells. According to their origin, they are little embryonic or adult stem cells.

Embryonic stem cells are thus undifferentiated cells present in the inner cell mass of the embryo. Adult stem cells exist as undifferentiated cells in differentiated tissue niches. Adult stem cells are of two types: Hematopoietic stem cells (HSCs) and Mesenchymal stem cells (MSCs) [7].

As the name implies, mesenchymal stem cells are non-hematopoietic in origin and have the ability to differentiate into tissues of mesenchymal and non-mesenchymal in origin. These cells reside in various tissues including bone marrow, skin, eyes, neuron, intestine and tooth[8].

The dental pulp from human permanent teeth, periodontal ligament, dental follicle, apical papilla and exfoliated deciduous teeth are sources of multipotent MSCs which have the potential for self-renewal and multilineage differentiation[9].

The expression of stem cell markers helps in studying and understanding the characteristics of stem cells. Stem cell markers include embryonic stem cell markers like Oct-4, Nanog and Stage Specific Embryonic Antigen-4 (SSEA-4) and mesenchymal stem cell markers like CD29, CD44, CD146, CD271, STRO-1 and CD 106 [9, 10].

Among these markers, Stage Specific Embryonic Antigen-4 (SSEA-4) is an embryonic stem cell marker present on the surface of cell membrane. It is

an early embryonic glycolipid antigen. SSEA-4 specifically marks human embryonic stem cells, cells in early stage of embryogenesis and human teratocarcinoma cells. SSEA-4 is also used to identify induced pluripotent stem cells[11]. SSEA-4 is a glycolipid epitope present in the cell membrane of embryonic stem cells and is responsible for cell proliferation and differentiation[12].

Thus, SSEA-4 is used as a marker to identify pluripotent stem cells (induced pluripotent stem cells and human embryonic stem cells). SSEA-4 is also used to identify adult stem cells with embryonic stem cell properties from various sites. However, there are only very few studies of their expression in DPSCs[13].

The aim of this study is to identify and isolate the stem cell population in mesenchymal cells isolated from the pulpal tissue of permanent teeth and to examine the expression of SSEA-4 by immunostaining using anti – SSEA-4 antibodies.

Aim and Objectives

AIM

To isolate and characterize Mesenchymal Stem Cells (MSCs) from dental pulp in α-modified minimum essential medium (α-MEM) and to study the expression of Stage Specific Embryonic Antigen - 4 (SSEA – 4) in the 1st, 2nd and 3rd passages of the culture.

OBJECTIVES

1. To isolate and culture MSCs from permanent teeth using enzyme disaggregation technique in α-MEM growth medium.

2. To study the phenotypic and growth characteristics of cells isolated from the dental pulp of permanent teeth in the 2nd and 3rd passage of the culture respectively.

3. To study the population doubling time of cells isolated from the dental pulp of permanent teeth in the 3rd passage of the culture.

4. To study the expression of SSEA-4 in duplicate in the cells of 1st, 2nd and 3rd passages of the culture.

Materials and Methods

MATERIALS FOR TISSUE CULTURE

Reagents:

1. Growth medium: α-modified minimal essential medium (*α*-MEM)
2. Fetal bovine serum (Invitrogen ™)
3. Antibiotics:
    a. Penicillin-100 IU/ml.
    b. Streptomycin-100µg/ml.
4. Dulbecco's - phosphate buffered saline (D-PBS) (potassium chloride-0.2g/l, potassium phosphate monobasic - 0.2g/l, sodium chloride-8g/l, sodium phosphate dibasic-1.15g/l)
5. De-ionized water
6. Distilled water
7. Collagenase (type I, filtered) (Hi Media ™)
8. Dispase (neutral protease, grade II) (Roche ™)
9. Ethylene-di-amine-tetra-acetic acid (Hi Media ™)
10. Trypsin 1:125 (Tissue culture grade, Hi media ™)

Equipment:

1. Culture plates (Tarsons ™)
2. 24-well plates (Cell star ™)
3. Glass pipettes
4. Disposable pipettes and pipette tips.

5. Leak-proof screw-cap vials.
6. BP blade no. 15.
7. Centrifuge tubes.
8. Laminar flow cabinet
9. Scott duran bottles
10. Carbon dioxide incubator. (Thermo electron Corporation. Forma series II water jacketed-HEPA class 100)
11. Phase contrast microscope. (Olympus CKX41 ™)
12. Digital camera. (Kodak AF3X, 8.2 mega pixels, 3x optical zoom)
13. Improved neubauer counting chamber
14. Laboratory centrifuge (R-86 Remi ™)
15. Cyclomixer (C101 Remi ™)
16. Prabivac vacuum pump
17. Cellulose acetate filter (pore size 0.2µm)
18. Electronic balance (Dhona 200D ™)
19. Hot air oven
20. Autoclave
21. Micromotor (Marathon ™)
22. Contra-angled Hand piece (NSK ™)
23. Chisel
24. Mallet
25. Carborundum discs

MATERIALS FOR IMMUNOCYTOCHEMISTRY

Reagents:

1. Antibodies – (Abcam)
    a) Monoclonal mouse antihuman antibody to SSEA-4 [Annexure - I]
    b) Rabbit polyclonal secondary antibody to anti - mouse IgG (HRP) [Annexure - II]
2. Bovine Serum Albumin (BSA) (Hi media ^{TM})
3. Phosphate buffered saline (sodium chloride 8g/l, disodium hydrogen ortho phosphate 1.15g/l, potassium dihydrogen orthophosphate 0.2g/l, potassium chloride 0.2g/l)
4. Acetone (Merck ^{TM})
5. APES (3-aminopropyl-triethoxy-silane)
6. Sodium hydroxide
7. Hydrochloric acid (Merck ^{TM})
8. DPX (distrene, dibutyl phthalate, xylene)

Equipments:

1. Glass slides
2. Micro centrifuge tubes (Tarsons ^{TM})
3. Cryo boxes (Tarsons ^{TM})
4. Coplin jars

5. Humidified chamber
6. Electronic timer
7. Light microscope
8. Cover slips

METHODOLOGY

STUDY DESIGN

In vitro study.

ETHICAL APPROVAL

Approval for the project was obtained from the Institutional Review Board of Ragas Dental College, India [Annexure - III].

SAMPLE SIZE

30 permanent teeth samples obtained from the Department of Oral surgery, Ragas Dental College. Informed consent was obtained from all the patients in a pre-approved format (English and Tamil). [Annexure – V]

ELIGIBILITY CRITERIA

Inclusion criteria:

- Impacted third molars
- Teeth with no caries and with vital pulpal tissue

Exclusion criteria*:*

- Teeth with evidence of decay or pulpal necrosis.
- Extracted third molars that have not been transferred to transport media immediately within 15 minutes of extraction.

Transport Media:

Extracted teeth were transferred immediately to serum - free α-Minimal Essential Medium (α-MEM), with added antibiotics (Penicillin - 100 IU, Streptomycin - 100µg/ml) twice the strength, at a pH of 7.2 to 7.4 and maintained at 4°C with the help of ice-packs. They were transported in leak-proof, sterilized culture vials.

ISOLATION AND PROCESSING OF TISSUE

a. Tooth surface was cleaned by immersing the tooth in povidone - iodine solution for 30 seconds and by washing with cold phosphate buffered saline for three times (PBS).
b. Grooves were placed around the cemento-enamel junction with a carborundum disc and cold PBS irrigation to avoid heating while cutting.
c. The tooth was split using chisel and mallet to expose the pulp chamber.

d. The pulp tissue was obtained from the pulp chamber with the help of forceps and spoon excavator and put into 2ml of α-minimal essential medium (α-MEM) on a petri plate (60mm diameter) to prevent the tissue from drying.

PRIMARY CULTURE OF DENTAL PULP CELLS

a. The dental pulp tissue was minced into tiny pieces (approx. $1mm^3$ in size) with a surgical blade.
b. The tissue was immersed into 1 ml of α-minimal essential medium (α-MEM) containing collagenase (3mg) and dispase (1mg) in 3:1 ratio.
c. It was incubated at 37^0C and 2% CO_2 for up to 3.5 to 4 hours for permanent teeth. Mechanical tapping was done to facilitate enzymatic disaggregation.
d. Cells were centrifuged at 2500 rpm for 5 minutes.
e. The supernatant was removed and the pellet were suspended in α-minimal essential medium (α-MEM) containing 15% fetal bovine serum (FBS) and 1x antibiotics and plated in a 60mm petri plate.
f. The cells were maintained at 37^0C and 2 % CO_2 in the incubator.
g. Forty-eight hours after the cell isolation, the culture media was discarded and fresh media was added to the petri plate. Media was changed every alternate day until cell reached confluency.

SUBCULTURE

Five to seven days after the cell isolation, colonies were identified in the culture plates, cells with a long spindle / fibroblastoid shape (*Figure 1*). The cells were sub-cultured after they reached 70-80% confluency on a 60mm culture grade petri plate. The number of days taken for the primary culture to reach confluency was recorded for each sample. After centrifugation, the obtained cell pellet was plated at a density of ~12 x 10^3 cells/plate.

a. The culture was examined carefully for signs of deterioration or contamination.
b. The media was discarded from the petri plate.
c. Two washes with 2ml PBS was done to remove any residual serum.
d. 1ml trypsin 0.25% with EDTA 0.05% was added to the petri plate (60mm diameter) and kept in the CO_2 incubator for 1 min.
e. The monolayer was checked under the microscope to check for detachment and rounding-up of the cells.
f. The plate was tapped at the bottom until all the cells were detached.
g. Cells suspended in trypsin was collected in a centrifuge tube and centrifuged at 2500 rpm for 3 minutes.

h. Supernatant obtained after centrifugation was discarded. Medium was added to the remaining cell pellet. After repeated pipetting, cells were dispersed in the petri plate.

i. The cells were counted in a haemocytometer.

j. The cell suspension was diluted to appropriate seeding concentration by adding adequate volume of medium.

k. Split ratios for subculture were 1:2 in which one half was suspended on APES coated slides.

l. The petri plate was closed and returned to the incubator and media was changed twice a week.

PHENOTYPIC CHARACTERIZATION

F - I, F - II, F – III (mitotic) and F - IV, F - V, F – VI, F - VII (post-mitotic) phenotypes

a. Cell lines from the second to third passage were plated on three 60 mm tissue culture petri plates at a concentration of 5 x 10^3 cells /ml.

b. Using a phase contrast microscope (20x magnification), 30 cells from each plate (90 cells in total) were observed and counted for eight consecutive days. As described by **Bayreuther *et al*** (1988)[61], the cells were classified into two groups based on their morphologically, as mitotic (F I, F II, F III) (*Figure 7 - 9*) and post-mitotic (F IV, F V, F VI, F VII) (*Figure 10 – 13*) phenotypes.

Crystal Violet Staining:

- Fix the cells with ice-cold methanol for 10 – 15 minutes
- Then cells are washed in PBS (3 x 5 mins)
- Add 0.5% crystal violet solution to the cells. Incubate for 30 minutes.
- Wash the cells in distilled water several times, until the dye stops coming off.
- The cells were allowed to dry at room temperature and viewed under microscope.

Giemsa Staining:

- Fix the cells with ice-cold methanol for 10 – 15 minutes
- Then cells are washed in PBS (3 x 5 mins)
- Add giemsa working solution to the cells. Incubate for 30 minutes.
- Wash the cells in distilled water several times
- The cells were allowed to dry at room temperature and viewed under microscope.

ESTIMATION OF GROWTH CURVE AND ITS DERIVATIVES

a. Cells were inoculated at 12 x 10^3 cells/ml/well on 24-well plates

b. After overnight attachment, cells from 3 randomly selected wells were trypsinized and counted using a haemocytometer.

c. The medium was changed on the 3rd and 6th day.

d. The count was repeated every 24 hours for 8 consecutive days.

e. Cells from each well were counted thrice to avoid error.

f. The daily average of cell counts of each well were used to plot the growth curve.

g. The total seeding cell count and the cell count in one well on the first day (i.e. after 12 hours of seeding) was used to estimate the seeding efficiency in percentage by using the equation,

$$\frac{\text{Cell count/well/ml after 12 hours x 100}}{\text{Seeding cell count/well/ml}}$$

h. The growth curve was plotted and population doubling time (PDT) derived from the exponential growth phase.

i. Population doubling time was calculated using the formula,

$$\frac{0.301 \times t}{\log (nt) - \log (n0)}$$

t - time, nt - final count of cells, n0 - initial count of cells.

IMMUNOCYTOCHEMISTRY

Cells were fixed on APES coated slides using methanol and immunostaining was done for SSEA-4 in 1st, 2nd and 3rd passage.

Protocol for growing and fixing of cells on APES coated slides

APES coating

a. Slides soaked in soap-water for 2 hours
b. Slides washed thrice in tap water
c. Soaked overnight in 1/10 N hydrochloric acid
d. Slides washed thrice in distilled water
e. Slides baked in the hot-air oven for 4 hours at 60ºC
f. Slides dipped in 50ml acetone for 2 minutes
g. In 2% APES for 5 minutes
h. Two dips in distilled water
i. Slides autoclaved

APES COATING PROCEDURE FLOW CHART

Slides soaked in soap-water for 2 hours

⇩

Slides washed thrice in tap water

⇩

Soaked overnight in 1/10 N hydrochloric acid

⇩

Slides washed thrice in distilled water

⇩

Slides baked in the hot-air oven for 4 hours at 60ºC

⇩

Slides dipped in 50ml acetone for 2 minutes

⇩

In 2% APES for 5 minutes

⇩

Two dips in distilled water

⇩

Slides autoclaved

Growing cells on a slide

a. Autoclaved APES coated slides were transferred to a 90mm petri plate.
b. Cells in the 1st, 2nd and 3rd passage were trypsinized, resuspended and plated.
c. With the addition of fresh media the cells were allowed to grow on the slides till it reaches confluency.

Fixation

a. The cells were thoroughly washed in PBS (5 x 3 mins) and fixed with methanol for 10 – 15 minutes.
b. The slides were rinsed in PBS (3 x 5 mins) and stored at -4°C.

Blocking of non-specific binding

Protein block was done with 1% BSA (Bovine Serum Albumin). (Alternatively, 2 - 5% normal serum in PBS for 1 hour is sometimes used as blocking agent. Normal serum should be the same species from which the secondary antibody was raised).

Primary antibody incubation

The primary antibody was diluted to the recommended concentration in 1% BSA and PBS. The primary antibody was added to each slide and incubated overnight at 4°C. The primary antibody solution was removed and the slides were rinsed (3 x 5 mins) in PBS.

Secondary antibody Incubation

The horseradish peroxidase (HRP) – conjugated secondary antibody was diluted in 1% BSA diluent. Excess fluid was removed from the slide. The secondary antibody solution was added to each slide and incubated for 1 hour at room temperature in the dark. The slides were rinsed (3 x 5 mins) in PBS and the excess fluid was removed.

Color development

The chromogen, 3, 3'-Diaminobenzidine (DAB) solution was added to each slide and kept for 15 minutes. The slides were washed in de-ionized water for 5 minutes.

Counter-stain

a. The slides were dipped into a staining plate of hematoxylin for 30 seconds.

b. The slides were rinsed with distilled water.

c. The slides were removed and placed inside an acid bath for 15 seconds for de-staining (200ml distilled water and 1-3 drops of acetic acid).

d. The slides were rinsed with distilled water.

Cover Slips

Cover slips were placed and mounted with DPX over the slides for examination under the microscope.

IMMUNOCYTOCHEMISTRY PROCEDURE FLOW CHART

Growing cells on APES coated slide

⇩

Wash in PBS (3 x 5 minutes each)

⇩

Fix cells with methanol (10 – 15 minutes)

⇩

Wash in PBS (3 x 5 minutes each)

⇩

Blocking with 1% Bovine Serum Albumin (BSA) (30 minutes)

⇩

Blot excess serum

⇩

Primary antibody added and incubated at 4°C (Overnight)

⇩

Wash in PBS (3 x 5 minutes each)

⇩

Secondary antibody added and incubated in dark (30 minutes)

⇩

Wash in PBS (3 x 5 minutes each)

⇩

Incubate with 1-3 drops of 3, 3'-Diaminobenzidine (DAB) (15 minutes)

⇩

Wash in de-ionized water (5 minutes)

⇩

Counter stain with hematoxylin (30 seconds)

⇩

Rinse in de-ionized water

⇩

De-stain in acid alcohol (15 seconds)

Rinse in de-ionized water

⇩

Mount the slides using DPX

⇩

Observe the slides under the Microscope

IMMUNOHISTOCHEMISTRY (IHC) PROCEDURE

POSITIVE CONTROL

Oral squamous cell carcinoma paraffin embedded tissue sections to which primary antibody was added were used as positive control.

NEGATIVE CONTROL

Oral squamous cell carcinoma paraffin embedded tissue sections to which primary antibody was not added were used as negative control.

STATISTICAL ANALYSIS

Data analysis was done using SPSS^{TM} (Statistical Package for Social Science) version 20.0.

Linear regression analysis was used to derive the slope from growth curves of each cell populations for determination of the population doubling time.

Armamentarium

EQUIPMENTS FOR CELL CULTURE

Figure 1: **CO_2 Incubator**

Figure 2: **Inverted Phase contrast microscope**

Figure 3: **Weighing Balance**

Figure 4: **Cyclomixer**

Figure 5: **Incubator**

Figure 6: **pH meter**

Figure 7: **Laminar Flow**

ARMAMENTARIUM AND REAGENTS FOR CELL CULTURE AND IMMUNOCYTOCHEMISTRY

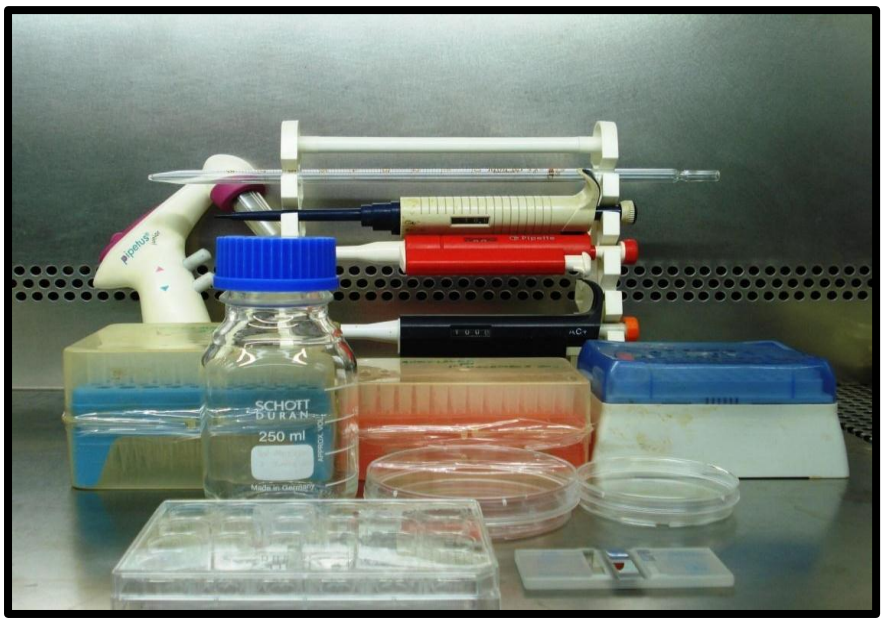


Figure 8: **Cell Culture Armamentarium**

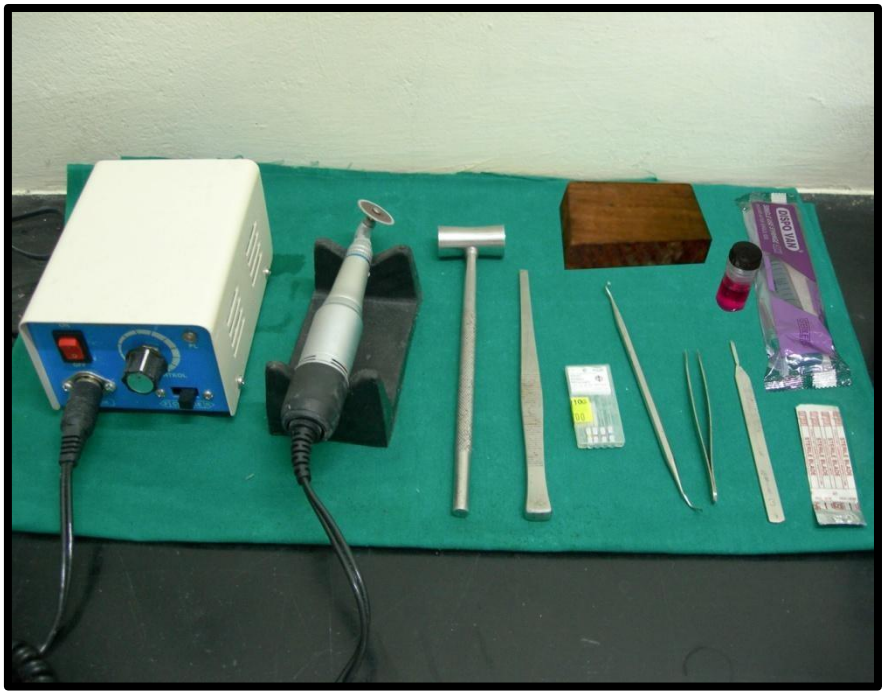


Figure 9: **Pulp Tissue Isolation Armamentarium**

Figure 10: **Reagents for isolation of dental pulp tissue**

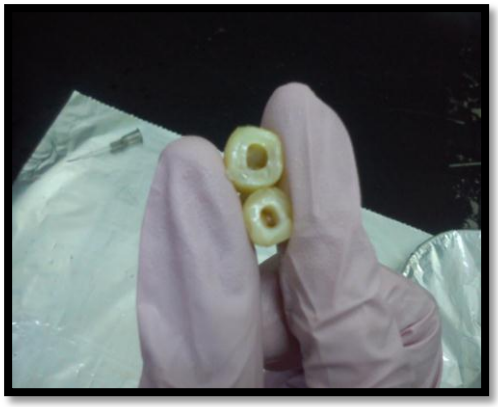


Figure 11: **Tooth sectioned at the cemento-enamel junction**

Figure 12: **Tissue taken for enzyme disaggregation**

Figure 13: **Primary and Secondary antibodies**

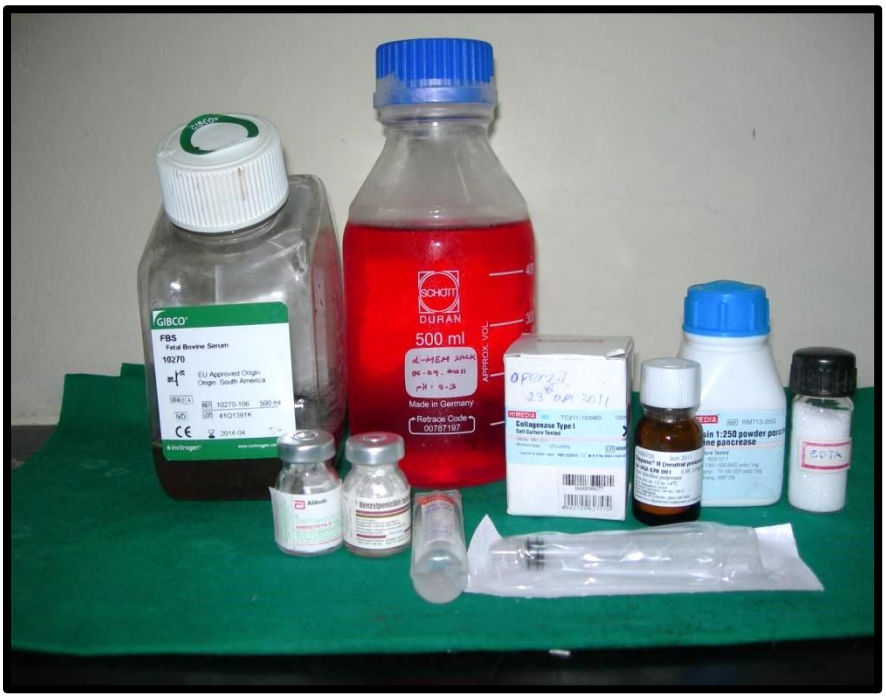


Figure 14: **Reagents used for cell culture**

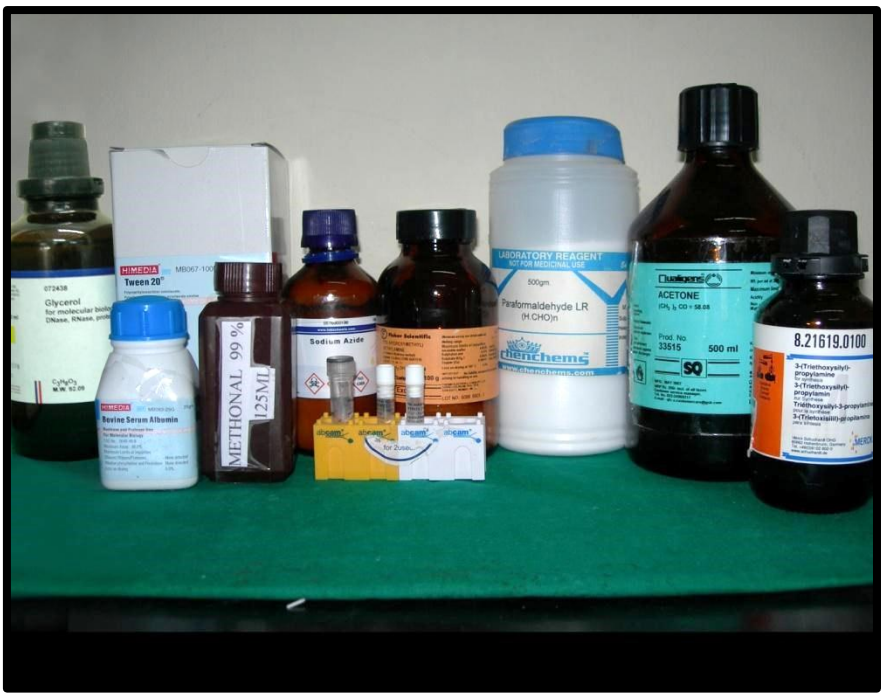


Figure 15: **Reagents used for Immunocytochemistry**

Reviewof Literature

A ‘‘stem cell’’ is defined as a “clonogenic, undifferentiated cell that is capable of self-renewal and multi-lineage differentiation”[1]. Self-renewal is one of the properties of the stem cells by which the cells self-replicate through multiple generations by maintaining its undifferentiated state. There are two types of replication: One is the obligatory asymmetric self-replication and the second type is stochastic differentiation. Obligatory asymmetric self-replication results in two daughter cells, in which one is undifferentiated, identical to the mother cell and the other is the daughter cell that is differentiated. In stochastic differentiation, two daughter cells were produced from lineage of the same cell[15].

Potency refers to the ability of the cell to differentiate into different cell lineages. Based on their potential to differentiate, the stem cells are classified as totipotent, pluripotent and multipotent stem cells. Totipotent cells are true stem cells with the capacity to differentiate into embryonic and extra – embryonic cells which can replicate into a distinct viable organism. Pluripotent cells are capable of differentiating into cells of all the three germ layers. Multipotent cells have the ability to differentiate into different cell types of similar tissue type[13, 16]. Multipotent stem cells are of two types, multipotent fetal stem cell and multipotent adult stem cell[8].

Based on their origin, stem cells are of two types. They are embryonic and adult stem cells. Embryonic stem (ES) cells are totipotent cells, derived from inner cell mass of the mammalian blastocysts that can virtually

differentiate into any cell type, as well as being propagated indefinitely in an undifferentiated state[17].

Adult stem cells are undifferentiated (unspecialized) cell that are found in a differentiated (specialized) tissue. These cells have the ability to proliferate and self-renew for long term. The two common examples of adult stem cells are hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). The HSCs are derived from bone marrow whereas MSCs are derived from epidermis, retina, neural tissue, adipose tissue, dental pulp and the periodontal ligament[18].

MESENCHYMAL STEM CELLS (MSCs):

Mesenchymal stem cells (MSCs) are non-haematopoietic, plastic-adherent and fibroblast-like cells, which are conventionally able to differentiate and self-renew into tissues of the mesodermic lineage, such as bone, adipose tissue and cartilage[19]. **Friedenstein *et al*** was the first to isolate and identify the mesenchymal stem cells from bone marrow[20].

The heterogenous culture contain cells of different size, proliferative rate and differentiation capacity. Human mesenchymal stromal cells express a panel of cell surface markers such as CD73, CD90 and CD105 and lack the expression of endothelial or haematopoietic cell markers such as CD11b, CD14, CD31, CD34 and CD45. These markers are used for identifying MSCs[21].

INDUCED PLURIPOTENT STEM CELLS (iPS):

In 2006, **Takahashi** and **Yamanaka** were the first to generate the induced pluripotent stem cells (iPS)[22]. The generation of human iPS cells are done by transducing Oct4, Sox2, Nanog, and Lin28 in human fibroblasts. Oct4, Sox2, Nanog, and Lin28 are the four important transcription factors that are responsible for induced pluripotent stem cells[23].

Thomson *et al* reprogrammed human somatic cells with the same four transcription factors. Reprogramming pluripotent stem cells from human somatic cells are done in the presence of these transcription factors. Thus reprogrammed pluripotent stem cells exhibits the essential characteristics of embryonic stem (ES) cells[24]. **Yamanaka** in 2007, reprogrammed human somatic cells with the combination of transcription factors like Oct4, Sox2, Klf-4 and c-Myc[25]. Both the group of transcriptional factors shows that the generated human iPS cells resemble human ES cells. These iPS cells have similar phenotype, proliferation capacity, expression of pluripotency markers, gene expression profiles and epigenetic status and also has the ability to differentiate into derivatives of all the three primary germ layers[26]. Differentiated somatic cells can be reprogrammed into iPS cells by forceful expression of Oct4, Sox2, Klf-4 and c–Myc. The achievement of human iPS cells holds great promise for regenerative medicine. Such induced pluripotent human cell lines are useful in the production of new disease models and in drug development, and also in the field of transplantation medicine. Human

iPS cells produced either by expression of Oct4, Sox2, c-Myc, and Klf-4 or by Oct4, Sox2, Nanog and Lin28 are similar to each other and resembles human ES cells[27]. There are many efficient sources for iPS cell generation, such as peripheral blood, human keratinocytes from hair follicles or epidermal biopsies, mesenchymal stem cells of dental origin from dental pulp and impacted third molars[28].

SOMATIC CELL NUCLEAR TRANSFER:

Somatic cell nuclear transfer (SCNT) is defined as "a process in which a somatic cell nucleus is fused with a mature enucleated oocyte" [29]. This process is also called as **'therapeutic cloning'**. The undifferentiated state of reprogrammed somatic cell nuclei is achieved in somatic cell nuclear transfer by transferring the transacting proteins present in the mammalian oocyte[30]. Cytoplasm of human oocytes reprograms transplanted somatic cell nuclei to pluripotency. Thus obtained stem cells are called "nuclear transfer embryonic stem cells (NT-ESCs)". These cells are efficiently derived from high-quality human oocytes. Human NT-ESCs are similar to ES cells derived from fertilized embryos. The difference of SCNT-based reprogramming is that NT-ESCs contain mtDNA which is an advantage over iPSC derivation. Thus, mtDNA mutations and diseases can be treated and corrected by SCNT[31].

The NT-ESCs obtained by somatic cell nuclear transfer (SCNT) are genetically identical to ES cells. These cells have the potential to cure or

alleviate the symptoms of many degenerative diseases where rejection by the host immune system is present[32].

STEM CELL NICHE:

The stem cells can differentiate and self-renew into multiple lineages, which contributes to tissue maintenance and regeneration after injury. These stem cells reside in a specialized microenvironment called Stem cell niche. The stem cells niche is a highly regulated microenvironments which maintains a balance of self – renewal and differentiation. The stem cell niche varies from location and nature for each type of tissue[33].

The niche protects the stem cells from differentiation stimuli, apoptotic stimuli and other stimuli that disturbs their stores. The niche also protects the stem cells from overproduction[34].

In 1978, **Schofield** was the first who proposed the stem cell niche concept. Niches are of two types.

- **Lineage niches** are based on asymmetric differentiation of the stem cells. One daughter cell moves away and undergoes differentiation and the other daughter cell retains its stem cell property.

- **Population niches** are based on the symmetric division of the stem cells. Either the daughter cells can remain within the

microenvironment and become stem cells or both daughters can undergo differentiation[35].

The primary function of niche is to provide signals that regulates stem cells self-renewal, survival and maintenance. The niche also provides spatial relationship between stem cells and other cells which promote asymmetric stem cell divisions, adhesion between stem cells and supporting stroma. E-cadherin and N- cadherin are the anchoring molecules which helps in the adhesion of stem cells. Thus, the stem cell niche regulates stem cell function, provides structural support, trophic support and topographical information[36,37].

There are various types of stem cell niches. They are present in:

- Bone Marrow
- Skin
- Intestine
- Neuron
- Cornea
- Tooth

Bone Marrow:

Bone marrow (BM) tissue is composed of network of mesenchymal stromal cells and vascular endothelial cells. There are more than eight different hematopoietic cell lineages. The bone marrow micro-environment is a major source of stem cell niche in the body. Hematopoietic stem cells (HSCs) and Mesenchymal stem cells (MSCs) niches are found in adult BM tissue. These cells help in maintaining normal hematopoietic homeostasis and re-establishing hematopoiesis after injury[38, 39].

The Epithelial Stem Cell Niche in Skin:

The epithelial stem cells are situated and maintained in the bulge area of the hair follicle and functions as a stem cell niche. These stem cells have characters similar to other keratinocytes. Epithelial stem cells are multipotent, giving rise to daughter cells that migrate downwards and converts into hair – matrix progenitors. Later these migrated cells gives rise to the hair shaft. The daughter cells can also migrate to upward and serves as progenitors for generating epidermal cells during wound repair[40].

Each hair follicle is composed of an inner root sheath, cortex, cuticle and medulla, which includes sebaceous glands and the underlying bulge area and a dynamic renewing portion. These bulge area undergoes a renewing process in a cycle of three phases. The three phases are anagen phase (active

growth phase), catagen phase (apoptosis-driven retraction) and telogen phase (a short resting phase) [41].

The Intestinal Stem Cell Niche (ISCs):

The intestinal architecture is composed of a sequential array of compartments along with the villus-crypt axis. Intestinal regeneration begins with the migration of intestinal stem cells. These cells migrate up the walls of the intestinal crypt. ISCs are located at the fourth or fifth position from the crypt bottom, above the Paneth cells. The intestinal stem cell progeny differentiate into four main cell types - Paneth cells, enteroendocrine cells, goblet cells and columnar cells[42].

The Neural Stem Cell Niche (NSC):

NSCs resides and can be isolated from various regions in the adult brain and peripheral nervous system. The sub ventricular zone (SVZ) and the sub granular zone (SGZ) of the hippocampus region are the primary and well-characterized germinal regions in which NSCs reside and support neurogenesis in the adult brain. The SVZ and SGZ structures have endothelial cells that form blood vessels and the specialized basal lamina, an essential component of the NSC niche[39].

The Stem Cell Niche in Eye:

At the cornea-scleral junction in an area known as the limbus. There is a population of limbal epithelial stem cells (LESCs). The characteristics of LESCs are similar to that of adult somatic stem cells including small size. The limbal region of the cornea, the LESC niche is thought to be located within the palisades of Vogt – an undulating region of increased surface area. The palisades are highly pigmented with melanocytes and are infiltrated with Langerhans's cells and T-lymphocytes[43].

The Stem Cell Niches in Tooth:

Human stem cells can also be isolated from the teeth. To date, six different human dental stem cells have been isolated and characterized[44].

- **D**ental **P**ulp **S**tem **C**ells (DPSCs) by **Gronthos *et al* 2000**[4]
- **S**tem **C**ells from **H**uman **E**xfoliated **D**eciduous teeth (SHED) by **Miura M *et al* 2003**[5]
- **P**eriodontal **L**igament **S**tem **C**ells (PDLSCs) by **Seo *et al*** in **2004**[51]
- The **D**ental **F**ollicle **P**rogenitor **C**ells (DFPCs) by **Morsczeck *et al*** in **2005**[54]
- **S**tem **C**ells from the **A**pical **P**apilla (SCAP) by **Sonoyama *et al*** in **2006**[56]

- **G**ingival **S**tem **C**ells (GSCs) by **Zhang *et al*** in **2009**[57]

Adult dental stem cells can differentiate into many dental components, such as dentin, periodontal ligament, cement and dental pulp tissue, but not into enamel[45].

DENTAL PULP STEM CELLS:

Dental pulp tissue is derived from migration of the neural crest cells during development and harbours various populations of multipotent stem/progenitor cells. The multipotent human dental stem/progenitor cells have been isolated, characterised and classified as 'dental pulp stem cells' (DPSCs). These include stem cells from exfoliated deciduous teeth (SHEDs), periodontal ligament stem cells (PDLSCs), dental follicle progenitor cells (DFPCs) and stem cells from apical papilla (SCAP) [46]. These cells have mesenchymal stem cell (MSC)-properties such as the capacity for self-renewal, the potential to differentiate into multiple lineages, including osteoblasts and chondroblasts and a potential for *in vitro* differentiation. The DPSCs are capable of differentiating into cell types from various embryonic layers, including adipose, bone, endothelial and neural-like tissues. DPSCs are one of the new source of adult stem cell for the repair and regeneration of a variety of mesenchymal tissues, such as bone, cartilage and muscle[47]. These cells was first discovered by **Gronthos *et al*** in 2000[3]. The DPSCs can be reprogrammed into induced pluripotent stem cells by transcriptional factors

like Oct-4, Nanog and Sox-2. Human dental pulp from the third molar is one of the potential sources for adult dental pulp stem cells [47, 48].

Stem cells from human exfoliated deciduous teeth (SHEDs):

Human exfoliated deciduous teeth are a relatively easily accessible source of adult stem cells. The remnants of the dental pulp derived from exfoliated deciduous teeth contains a multipotent stem-cell population. SHEDs can be isolated from the coronal pulp of exfoliated deciduous teeth. Primary incisors are the major source of SHEDs[4]. These cells are identified to be a population of highly proliferative, clonogenic cells capable of differentiating into a variety of cell types including neural cells, adipocytes, chondrocytes, osteocytes and odontoblasts. Deciduous teeth are significantly different from permanent teeth with regard to their developmental processes, tissue structure and function[49]. The STRO-1-positive region in the pulp of deciduous teeth is similar to that of permanent teeth, also in the perivascular regions. These cells show positive expression for CD73, CD90, CD105, CD146, CD166, and SSEA-4 and show negative expression for CD45, CD34, CD14, CD19, and HLA-DR[50].

Periodontal ligament stem cells (PDLSCs):

The periodontal ligament (PDL) is a ligament that holds the tooth in its alveolus, connects the alveolar bone to the root cementum. The PDL contains stem cells which have the potential to form periodontal structures such as

cementum and ligament. It can be obtained from the roots of extracted teeth[51,52].

In vitro, PDLSCs can differentiate into osteoblasts, cementoblasts and adipocytes[53]. STRO-1/CD146/CD44 staining of the PDL has shown that it is located mainly in the perivascular region, with small clusters of cells in the extravascular region suggesting that these are the niches of PDLSCs[17].

Dental follicle stem cells (DFSCs):

The dental follicle is a loose connective tissue that surrounds the developing tooth. It plays a major role in the genesis of cementum, periodontal ligament, and alveolar bone. DFSCs can be isolated from the follicles of impacted third molars. DFSCs cultivated *in vitro* exhibit characteristics of cementoblasts and osteoblasts, and can differentiate neurally[54, 55].

Stem Cells from the Apical Papilla (SCAP):

Dental stem cells can also be extracted from the apical papilla of shed primary teeth (SCAP). Stem cells from the dental apical papilla are stem cells from the apical part of the papilla, a precursor tissue of the dental pulp. Impacted third molars serve as a suitable source. *In vitro*, SCAP can differentiate osteogenically, odontogenically and adipogenically. *In vivo*, SCAP have been found to differentiate into odontonblasts and osteoblasts. STRO-1 staining of apical papilla has shown positivity in perivascular region[49, 56].

Stem Cells derived from Gingiva (GSCs):

The stem cell population can also be isolated from gingiva (GSCs). The stem cells reside in the spinous layer of human gingiva and these cells are referred as gingival stem cells (GSCs). GSCs show similar characteristics of mesenchymal stem cells like multipotency with high proliferation rate. In terms of markers, it has been shown that GSCs are negative for CD45/CD34, but positive for CD29, CD44, CD73, CD90, CD105, CD146, STRO-1 and SSEA-4[57,58]

Biological Characteristics:

In 2003, **Gregory CA, Singh H, Perry AS *et al*** conducted a study on Human Adult Stem Cells from Bone Marrow, in which they observed three phases of growth[59].

3 phases of growth are:

(1) An early lag phase

(2) A Rapid proliferation phase

(3) A Late stationary phase.

Phenotypic Characterization:

Studies on rat skin and lung fibroblasts revealed the presence of three subpopulations of cells[60]

i. F I – Spindle shaped cells with high proliferation potential

ii. F II – Epitheloid cells with comparatively lower proliferation rate

iii. F III - Large stellate cells with slow proliferation potential

Bayreuther K, Rodemann HP, Hommel R *et al* 1988[61] performed studies on human skin fibroblasts of cell lines isolated from the lower abdominal region. They stated that the fibroblasts *in vitro* can spontaneously differentiate into a seven stages, where the seventh stage is the terminal stage. Among seven stages, they can be broadly divided into **Mitotic fibroblast subtypes** and **Post mitotic fibroblast subtypes.** The seven stages of cell lineage are as follows.

Mitotic fibroblast subtypes

i. F1 – small spindle shaped cells

ii. F2 – small epitheloid cells

iii. F3 – larger pleomorphic epitheloid cells

Post mitotic subtypes

i. F4 – large spindle shaped cells

ii. F5- larger epitheloid cells

iii. F6- largest epitheloid cells

iv. F7-degenerating fibroblasts

DPSCs and SHED populations are morphologically distinct. Cells from permanent tooth pulp showed a higher proportion of fibroblastoid cells, whereas deciduous pulp culture showed a higher proportion of epitheloid cells. Epithelioid cells are considered as contaminating cells during the isolation and propagation of mesenchymal stem cells[62].

Though DPSCs and BMMSCs share many common properties, there are differences. The ability to form dentin and differentiate into odontoblasts is a unique property of DPSCs. While both osteogenic and odontogenic medium are identical, the mineralized deposits of DPSCs are nodular in nature. This is similar to in vitro dentin formation, but markedly different than that of osteogenesis by BMMSCs[3]. Elevated expression of basic fibroblast growth factor (bFGF) and matrix metalloproteinase-9 (MMP-9) is found with the formation of hematopoietic marrow by BMSSC, but not in the connective tissue formed by DPSCs transplants[63].

STEM CELL MARKERS:

Stem cells are best defined functionally by a number of cell surface markers and generic molecular markers. These markers are being used to characterize various stem cell populations. Proteins involved in signal pathways are known to have an important functions in cell fate decision. Thus,

the unique expression pattern of markers is used as an essential tool to identify and isolate stem cells[9].

A systematic review on cell surface characterization of adult mesenchymal stem cells was done in which it was concluded that there are various cell surface markers for mesenchymal stem cells like CD105, CD90, CD44, CD73, CD29, CD13, CD34, CD146, CD106, CD54 and CD166 had a positive expression for the cell surface markers. Whereas the adult mesenchymal stem cells reported negative expression for CD14, CD11b, CD49d, CD34, CD106, CD10 and CD31. It was also observed that the markers like CD10, CD34, CD45 and CD106 did not have a uniform expression in the cell types and it varied. The variability of the expression of the markers can be attributed to the heterogenicity of the cell types or to the different cell passages that was used to access the expression of markers[64].

The international society for cellular therapy has proposed the minimal criteria for identifying the MSC[65]:

- MSC must be plastic-adherent in standard culture conditions.
- MSC must express CD105, CD73 and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface markers.

- MSC must be capable of differentiation into osteoblasts, adipocytes and chondroblasts in vitro.

The pluripotency of human stem cells can be assessed in two different ways: teratoma formation by cells and the aggregation and generation of embryoid bodies (EBs). These cells are positive for SSEA-4, Oct3/4, Nanog, Sox2, Lin28, CD13, CD105, CD90, CD29, CD73 and STRO-1. These cells are negative for CD34, CD45 and CD146 markers[48, 66]. The following table shows various stem cell markers, their properties and their expression in stem cells.

No.	Stem cell markers	Common Synonyms	Characteristics	Classification
1	CD 13	Alanyl membrane aminopeptidase	BMMSCs	Haematopoietic stem cell marker
2	CD34	gp 105 -120	Human ES cells, HSCs	Haematopoietic stem cell marker
3	CD 29	Integrin β1	Human ES cells	Cell Surface marker
4	CD 44	Extracellular matrix receptor III	Human ES cells, HSCs	Cell Surface marker
5	CD 73	Ecto 5' nucleotidase	Human ES cells, HSCs	Cell Surface marker
6	CD 90	Thy - 1 membrane glycoprotein	Human ES cells, HSCs	Haematopoietic stem cell surface marker
7	CD 105	Endoglin	MSCs, ESCs	Cell Surface marker

8	CD 106	Vascular cell adhesion molecule (VCAM)	Haematopoietic Stem cells (HSCs)	Cell Adhesion marker
9	CD 146	Melanoma cell adhesion molecule (MCAM)	Human ES cells, MSCs	Cell Adhesion marker
10	CD 54	Intercellular adhesion molecule (ICAM)	Human ES cells, MSCs	Cell Adhesion marker
11	CD 166	Activated leukocyte cell adhesion molecule	Human ES cells	Cell Adhesion marker
12	CD 10	Common acute lymphocytic leukemia antigen	Human ES cells, HSCs	Cell Surface marker
13	CD 11b	Integrin α M	Human ES cells	Cell Surface marker
14	CD 31	Platelet endothelial cell adhesion molecule (PECAM)	Human ES cells, HSCs	Cell Adhesion marker
15	CD 19	B4 - B lymphocyte antigen	Human ES cells	Cell surface marker

Bone marrow MSCs express many embryonic stem cell markers like Oct4, Nanog, alkaline phosphatase and SSEA-4. The adipose tissue and dermis MSCs express Oct4, Nanog, Sox2, alkaline phosphatase and SSEA-4, whereas cardiac MSCs express Oct4, Nanog, Sox2 and SSEA-4. Thus SSEA-4

is one embryonic stem marker which is expressed in all the MSCs and associated with the property of multilineage differentiation[67].

Limbal stem cells (LSCs) are epithelial in nature. The LSCs show strong positive expression for cytokeratin (CK) -14 by which their epithelial nature is confirmed. ABCG2, ABCB5, vimentin, connexin and cytokeratin 19 shows weak positive staining in LSCs. CD34 and CD45 shows negative expression in LSCs[43].

Dental pulp stem cells (DPSCs) have similar properties as that of bone marrow stem cells (BMSCs). Both DPSCs and BMSCs exhibit expression of similar surface markers and matrix proteins associated with formation of mineralized tissue. DPSCs have high proliferation rate when compared to that of BMSCs[69].

Immunocytochemically DPSCs, show positive expression of numerous stem cell markers, including Nanog, Sox2, SSEA-4, Nestin, Musashi-1 and Nucleostemin and negative expression of differentiated neural, vascular, and hepatic cells. Immunoblotting analyses also revealed similar results. These cells showed slight expression of smooth muscle actin and variable expression of CD 146[68].

Dental pulp stem cells (DPSCs) expressed embryonic stem cell markers (Oct-4, Nanog and Stage Specific Embryonic Antigen (SSEA) 3,

SSEA-4) as well as mesenchymal stem cell markers during experiments by at least 25 passages[70].

One of the sources for pluripotent stem cells are the human third molars. The stem cells derived from the third molars are also called as Dental Pluripotent Pulp Stem Cells (DPPSCs). DPPSCs differentiate into tissues that have similar characteristics to embryonic mesoderm, endoderm and ectoderm layers. They also generate embryoid bodies (EB)-like structures and develop into teratoma like structures. DPPSCs are derived from an easily accessible source and are used in regeneration of tissues from the three embryonic layers[66].

Stage Specific Embryonic Antigens (SSEAs): SSEAs were identified by three monoclonal antibodies (Abs) recognizing defined carbohydrate epitopes associated with lacto and globo - series glycolipids. SSEA -1, -3 and -4. SSEA-1 is expressed on the surface of preimplantation - stage of murine embryos (i.e. at the eight cell stage) and has been found on the surface of teratocarcinoma stem cells, but not on their differentiated derivatives. SSEA-3 and SSEA-4 are synthesized during oogenesis and are present in the membranes of oocytes, zygotes and early cleavage-stage embryos. SSEA-3 and SSEA-4 are expressed in undifferentiated primate ES cells, human embryonic germ (EG) cells, human teratocarcinoma stem cells and ES cells[9]. SSEA-4 expression is absent in murine ES cells, but appears following differentiation. SSEA-4, a globo-series ganglioside is used as a marker for

distinguishing primitive human embryonic carcinoma cells and embryonic stem cells[11].

The extracellular markers that have been used to characterize hESCs are primarily carbohydrate epitopes on proteoglycans or sphingolipids, such as stage-specific embryonic antigen-4 (SSEA-4).

Stage-specific embryonic antigen-4 (SSEA-4) is a globo-series ganglioside. Ganglioside is a molecule which is composed of glycosphingolipid and sialic acids linked to a sugar chain. The glycosphingolipids play an important role in cell proliferation and differentiation during embryogenesis. They also have a major role in cell membrane events such as cellular interactions, cell signaling and trafficking[12].

In a study on human ES cells, SSEA-4 showed negative expression and showed properties for pluripotency. Thus, the authors stated that the negative expression of SSEA-4 do not play any critical role in function and maintaining the pluripotency of hESCs. They also stated that SSEA-4 has role in cellular differentiation[75].

SSEA-4, a marker helps to identify and isolate mesenchymal stem cell population from bone marrow. SSEA-4 positive cells also expressed Oct-4 and failed to express CD34 and CD45. Thus, the authors showed SSEA-4 marks an adult mesenchymal stem cell population and also can be used to isolate MSCs[71].

The identified and isolated the pluripotential stem cell subpopulations in human dental pulp from the third molar was known as the DPPSCs. These cells have the phenotype similar to embryonic stem cells and have the capacity to differentiate *in vitro* into tissues that are similar to embryonic mesoderm and endoderm layers. In this study, the dental pulp tissue was cultured in media with the presence of growth factors such as LIF, EGF and PDGF. The isolated cells from dental pulp (DPPSCs) expressed positivity for SSEA-4, Oct4, Nanog, FLK-1, HNF3beta, Nestin, Sox2, Lin28, c-Myc, CD13, CD29 and CD105. The cells expressed negativity for CD3, CD45 and CD146. These cells also had low positivity for CD90, CD73 and STRO-1[47].

SSEA-4 is a marker to isolate and to identify multipotent DPSCs analogous in identifying PDL stem cells and bone marrow MSCs. MC813-70, a monoclonal antibody against SSEA-4. This reacts with the gangliosides GL-7, GM1b, and GD1a. The gangliosides GM3, GM2, and GD1a are present in dental pulp cells, whereas GM1, GD3, GD1b, GT1b and GQ1b are absent. Although DPSCs express SSEA-4, there is no evidence to support the fact that DPSCs have the pluripotentiality analogous to embryonic stem cells. It is also noted that DPSCs can differentiate into chondrogenic, osteogenic, neural lineages but lacked adipogenic potential[13].

The stem cell properties of deciduous PDL stem cells are similar to those of permanent PDL stem cells. Both cell types have plastic-adherent properties and displays MSC immunophenotype. In addition, both of them

display multipotential lineage towards adipocytes, osteoblasts and chondrocytes. The difference between deciduous PDL cells and permanent PDL cells is that the deciduous PDL cells were negative for CD120a and CD318, while the permanent PDL cells expresses these antigens. The deciduous PDL stem cells can be isolated with SSEA-4 as a specific marker of multipotent stem cells[72].

Later it was proved that, DPPSCs have the ability to form embryoid bodies (EB) like or teratoma-like structures which is one source for ES or iPS cells. He also stated that, the induction of iPS cells are easier from stem cells than from differentiated cells that is reprogramming process occurs in DPPSCs, but not in differentiated dermal fibroblasts[48].

DPSCs express SSEA-4 and other embryonic stem cell-associated antigens. As STRO-1 expression in MSCs is controversial, the use of SSEA-4 appears to be advantageous as an alternative marker for identifying DPSCs. The majority of the SSEA-4 positive DPSCs have the potential for multilineage differentiation toward osteoblasts and chondrocytes, while some also had the ability to differentiate into adipocytes, suggesting that they appear to be a promising source of stem cells for regenerative therapy[73].

SSEA-4 shows positive expression in human corneal stem cells but failed to express in human limbal stem cells (LSCs). This is because the anti-SSEA-4 antibody recognizes only the globo-series carbohydrate core and not the protein. It is also noted that SSEA-4 plays role only in cell differentiation and not in cell pluripotency. Thus, the negative expression of SSEA-4 can be used as marker to isolate limbal stem/ progenitor cell population[74].

Results

We successfully isolated dental pulp cells from four permanent teeth samples 1 to sample 30. The tooth was collected in the transport medium after extraction or surgery. The transport media was α-MEM with twice the concentration of antibiotics added to prepare the working media and without serum. The demographics of all the samples are listed in *Table 1*. *Graph 1* summarizes the total number of tooth samples cultured and the days taken to reach its confluency. Based on the yield obtained from each culture we concluded that processing the tooth immediately after the surgery or extraction was more important than processing the tooth collected in the transport media. After 48 hours of plating, single cells were scattered *(Figure 16)* and cluster of cells were seen arising from each foci of disaggregated pulp tissue *(Figure 17)* in the plate. Thus, the plates were observed daily until 70% to 80% confluency *(Figure 18)* was reached. Meanwhile, media change was done every alternate day from plating. The primary culture reached 70% to 80% confluency in about 27 to 35 days. The first subculture derived cells took about 7 to 10 days to reach confluency. The second subculture derived cells took about 4 to 8 days. The third subculture derived cells took about 5 to 8 days to reach confluency. Cells from the 3^{rd} passage to 4^{th} passage were used to study the growth characteristics and morphology. The plates considered for subpopulation analysis took about 30 days to reach confluency as only minimal cells, 5×10^3 cells were plated.

In the cultured DPSCs, growth characteristics and morphology was assessed for two adult pulp (AP) samples, 27th and 28th respectively (27AP, 28AP). Subpopulation analysis was done for both the samples. Immunocytochemistry was done in 1st, 2nd and 3rd passage of cells in both 27th and 28th sample in duplicate (27AP and 28AP).

CULTURE OF DENTAL PULP STEM CELLS FROM PERMANENT TEETH

In this study, the permanent teeth were collected immediately after extraction and transported in the transport medium to the laboratory and used as a source of pulp.

Permanent sample 1(1AP) was obtained from a 34 - year old male with pericoronitis in relation to 18. On splitting the tooth to extirpate the pulp; all the three roots were completely calcified. No pulp tissue was obtained. Sample was discarded.

Permanent sample 2(2AP) was obtained from an 18 - year old male with pericoronitis in relation to 18.The pulp tissue obtained was <1mm^3. The media showed cell debris. Following the media change on the third day after primary plating the media remained clear. The plate was checked daily. But no cells were found growing.

Media was changed every alternate day and the pH of the media with FBS was 7.5. No cells were seen till 20 days after plating. So the plate was discarded.

Permanent sample 3(3AP) were obtained from a 20 - year old female with pericoronitis in 28. The pulp tissue obtained were <1mm^3. The media remained clear. There were no signs of contamination. The pH was 7.5 with FBS. No cells were seen in the plate till 23 days. The plate was discarded.

Permanent sample 4(4AP) and 5(5AP) were obtained from an 18 year old male patient and a 20 year old female with pericoronitis in 38 and 48 respectively. The pulp tissue obtained were ~1mm^3 in both the teeth. The plating was done separately in 2 plates. The media had debris but then remained clear on subsequent media change in both the plates. A slow growth of cells was seen but the growth cessated after 23 days in 4AP and 5AP. Later, no growth was seen in both the plates and the cells started degenerating. Change in the colour (pale yellow) of the media was noted. The pH of the media was checked and the pH was acidic (6.35).The plates were discarded. Because of the change in the pH of the media there was increased level of carbon dioxide in the incubator which led to degeneration of the cells.

Permanent sample 6(6AP) and 7(7AP) was obtained from a 21 year old male patient (18, 48). The cells in 7AP reached confluency in 18 days (48). Sufficient cells were not there for analysis of growth curve and

phenotype. There was no growth seen till 20 days in 6AP. The media was clear. There were no signs of contamination. 6AP plate was discarded.

Permanent sample 8(8AP) was obtained from a 29 year - old female with pericoronitis in 18. The pulp tissue obtained was ~1mm^3. On the third day of plating, the media appeared turbid. When observed under a phase contrast microscope, filamentous structures was seen suggestive of fungal contamination. The plate was immediately discarded *(Figure 19).*

Permanent sample 9(9AP) was obtained from a 21 year old female (28). The pulp tissue obtained was ~1mm^3. On the third day, the media appeared turbid. When observed under a phase contrast microscope, bacterial colonies were seen throughout the plates. The plate was immediately discarded *(Figure 20).*

Permanent sample 10(10AP) and 11(11AP) were obtained from a 21 years old female (28 and 38). The pulp tissue obtained were <1mm^3 and >1mm^3 respectively. On the third day, the media appeared turbid in 11AP. When observed under a phase contrast microscope, bacterial colonies were seen throughout the plates. The plates were immediately discarded. The media remained clear. There were no signs of contamination. The pH was 7.5 with FBS. No cells were seen in the plate till 23 days in 10AP. The plate was discarded.

Permanent sample 12(12AP) were obtained from a 28 year male (28). The pulp tissue obtained was ~1mm^3. On the 3rd day when observed under the phase contrast microscope filamentous structures was seen suggestive of fungal contamination. The plate was immediately discarded.

Permanent sample 13(13AP) was obtained from a 26 year male with pericoronitis in 18. The pulp tissue obtained were <1mm^3. The media remained clear. There were no signs of contamination. The pH was 7.5 with FBS. No cells were seen in the plate till 23 days. The plate was discarded.

Permanent sample 14(14AP) and 15(15AP) were obtained from a 21 year female (28 and 38). Pulp tissue was very less than 1mm^3 so both tissues were combined and plated in the same plate. On the 23rd day when observed under the phase contrast microscope filamentous structures was seen suggestive of fungal contamination. The plate was immediately discarded.

Permanent sample 16(16AP) were obtained from a 27 year old female (48). Pulp tissue < 1mm^3 was obtained. On the 13th day when observed under the phase contrast microscope filamentous structures was seen suggestive of fungal contamination. The plate was immediately discarded.

Permanent sample 17(17AP) and 18(18AP) were obtained from a 25 year female with pericoronitis in 18 and 48. The pulp tissue obtained were >1mm^3 in both the teeth. The plating was done separately in 2 plates. On the 10th day of plating, the media appeared turbid in both the plates. When

observed under a phase contrast microscope, bacterial colonies were seen throughout the plates. The plates were immediately discarded.

Permanent sample 19(19AP) and 20(20AP) were obtained from a 23 year old male (28 and 38). Pulp tissue was very less than $1mm^3$ so both tissues were combined and plated in the same plate. On the 18^{th} day when observed under the phase contrast microscope filamentous structures was seen suggestive of fungal contamination. The plate was immediately discarded.

Permanent sample 21(21AP) and 22(22AP) were obtained from 29 year - old female patient and 25 year old male with pericoronitis in 28 and 38 respectively. The pulp tissue obtained were ~$1mm^3$ in both the teeth. The plating was done separately in 2 plates. The media had debris but then remained clear on subsequent media change in both the plates.

Permanent sample 23(23AP) obtained from a 20 year old female (18 open apex) during orthodontic prophylaxis treatment. The pulp tissue obtained was ~$2mm^3$ processed and plated. On the third day, the media was found turbid. When viewed under phase contrast microscope filamentous structures was seen suggestive of fungal contamination. Immediately the plate was discarded.

Permanent sample 24(24AP) obtained from a 29 year old male (38). The pulp tissue was less than $1mm^3$ and plating was done. There was no

growth seen till 30 days. The media was clear. There were no signs of contamination. The plate was discarded.

Permanent sample 25(25AP) and 26(26AP) were obtained from a 25 year old female patient with pericoronitis in 18 and 48. The pulp tissue obtained were ~1mm^3 in both the teeth. The plating was done separately in 2 plates. The media had debris but then remained clear on subsequent media change in both the plates. The cells reached confluency in 29 days (18). Sufficient cells were not there for growth curve and phenotype in 25AP. There was slow growth in the cells in 26AP. Then, the growth cessated after 33 days in 26AP. Later, the cells started degenerating. Change in colour (pale yellow) of the media was also noted. The pH of the media was checked and the pH was acidic (6.5).The plate was discarded. Because of the change in the pH of the media there was increased level of carbon dioxide in the incubator which led to degeneration of the cells. The pH was corrected and brought to the ideal pH (7.35).

Permanent sample 27(27AP) and 28(28AP) were obtained from a 22 year old male (18 and 48). The pulp tissue obtained was ~1mm^3. On the fourth day, the cells seen were more of a spindle shaped and few epitheliod cells were also observed (*Figure 29*). On the eighth day of primary culture, clusters of cells were seen (*Figure 30)*. On fifteenth day, the cells reached 50% confluency (*Figure 31*). The cells obtained from 48 reached confluency in 23 days (*Figure 32)* while cells from 18 reached confluency in 30 days.

After reaching 70 – 80% confluency, trypsinisation procedure was done (*Figure 33).* Growth characteristics, phenotype, immunostaining for Stage Specific Embryonic Antigen-4 (SSEA-4) was done for the samples 27AP and 28AP.

Characteristics of sample 27(27AP):

- Growth characteristics 27AP *(Graph 2, Table 2)*: The cells were seeded in the concentration of 12 $x10^3$ cells/ well/ ml in a 24 well plate. On the first two days, there was a decrease in the cell count, following seeding. Then a gradual increase was seen in the log phase at day 3 and the cell count thereafter increased steadily. The seeding efficiency was calculated to be 70.08% and the population doubling time calculated from the slope of the curve was 3.39 days.

- Morphological phenotype *(Graph 3, 4 and Table 3, 4)*: Mitotic and Post-Mitotic phenotype of the cells were seen from day 1 to day 8 (*Figure 22 – 28*). Crystal violet staining (*Figure 34)* and Giemsa staining were done to assess the morphology of cells. There was a notable decrease in mitotic phenotype from day 1 to day 3 and a gradual increase in the post-mitotic phenotype from day 4 to day 8 of culture. The F III subpopulation was the highest in the first few days of culture. At day 8, the F V subpopulation was the highest. The population of cells that exhibited epitheloid morphology was more

than the population that had spindle cell morphology from day 1 to day8.

- Immunocytochemistry: Immunocytochemistry was done for the cells grown in APES coated slide in 1st, 2nd and 3rd passages. The cells did not express Stage Specific Embryonic Antigen-4 (SSEA-4) (*Figure 37*).

Characteristics of sample 28(28AP):

- Growth characteristics 28AP *(Graph 5, Table 5)*: The cells were seeded in the concentration of 12 $x10^3$ cells/ well/ ml in a 24 well plate. On the first two days, there was a decrease in cell count, following seeding. Then gradual increase was seen in the log phase at day 3 and the cell count thereafter increased steadily. The seeding efficiency was calculated to be 69.5% and the population doubling time calculated from the slope of the curve was 3.15 days.

- Morphological phenotype *(Graph 6, 7 and Table 6, 7)*: There was a decrease in mitotic phenotype and increase in the post-mitotic phenotype from day 1 to day 8 of culture. The F III subpopulation was the highest population from day 1 to day 8 The population of cells that exhibited epitheloid morphology was more than the population that had spindle cell morphology from day 1 to day 8.

- Immunocytochemistry: Immunocytochemistry was done for the cells grown in APES coated slide in 1st, 2nd and 3rd passages. The cells did not express Stage Specific Embryonic Antigen-4 (SSEA-4) (*Figure 38*).

Permanent sample 29(29AP) and 30(30AP) obtained from 28 and 29 year old male respectively (48 and 38). The pulp tissue was ~1mm^3 and plating was done. The growth was seen in the corners of the plate. Re - plating was done on 30th day of the primary culture in 29AP. The media showed cell debris. Following the media change on every alternate day after re - plating the media remained clear. The plate was checked daily. But no cells were found growing till 39th day. The media was clear. There were no signs of contamination. The plate was discarded. In 30AP, there was slow growth in the cells. Then, the growth cessated after 29 days in 30AP. Later, the cells started degenerating. So the plate was discarded.

The stem cells isolated and cultured from the dental pulp of permanent teeth did not express SSEA-4. Oral squamous cell carcinoma (OSCC) paraffin embedded tissue section were used as positive controls. Imunohistochemically, the oral squamous cell carcinoma tissue sections showed positive staining for Stage Specific Embryonic Antigen-4 (SSEA-4) (*Figure 35* and *36)*.

Tables and Graphs

TABLE 1: DEMOGRAPHIC DATA

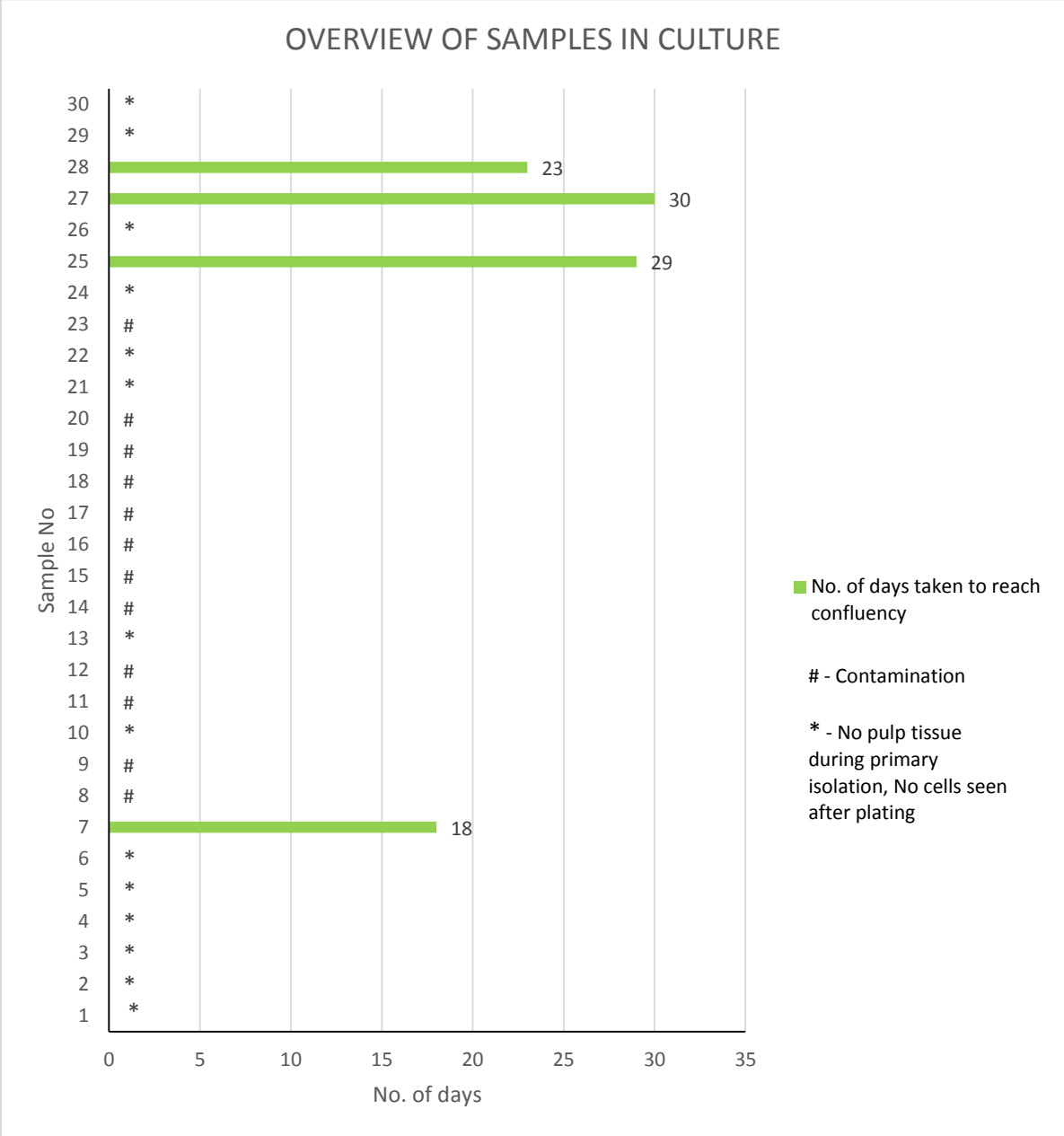
Sample No.	Sample Type	Age (yrs) / Sex	Source	Disease	Time to reach confluence (Days)	Final passage number	Outcome	Growth / phenotype characteristics	ICC
1	Permanent	34/M	18	Pericoronitis	-	-	Calcified	-	-
2	Permanent	18/M	18	Pericoronitis	-	-	No cells	-	-
3	Permanent	20/F	28	Pericoronitis	-	-	No cells	-	-
4	Permanent	18/M	38	Pericoronitis	-	-	Slow growth, pH-6.35-plate discarded.	-	-
5	Permanent	20/ F	48	Pericoronitis	-	-	Slow growth, pH-6.35-plate discarded.	-	-
6	Permanent	21/ M	18	Impacted	-	-	No cells	-	-
7	Permanent	21/ M	48	Impacted	18	3	Successful	Sufficient cells were not present	
8	Permanent	29/ F	18	Pericoronitis	-	-	Fungal contamination	-	-

9	Permanent	21/F	28	Impacted	-	-	Bacterial contamination	-	-
10	Permanent	21/ F	28	Impacted	-	-	No cells	-	-
11	Permanent	21/ F	38	Impacted	-	-	Bacterial contamination	-	-
12	Permanent	28/ M	28	Impacted	-		Fungal contamination		
13	Permanent	26/ M	18	Pericoronitis	-		No cells		
14	Permanent	21/ F	28	Impacted	-		Fungal contamination		
15	Permanent	21/ F	38	Impacted	-		Fungal contamination		
16	Permanent	27/F	48	Impacted	-		Fungal contamination		
17	Permanent	25/F	18	Pericoronitis	-		Bacterial contamination		
18	Permanent	25/F	48	Pericoronitis	-		Bacterial contamination		
19	Permanent	23/M	28	Impacted	-		Fungal contamination		
20	Permanent	23/M	38	Impacted	-		Fungal contamination		

21	Permanent	29/F	28	Pericoronitis	-		No cells		
22	Permanent	25/M	38	Pericoronitis	-		No cells		
23	Permanent	20/F	18	Impacted	-		Fungal contamination		
24	Permanent	29/M	38	Impacted	-		No cells		
25	Permanent	25/F	18	Pericoronitis	29 days	1	Successful	Sufficient cells were not present	
26	Permanent	25/F	48	Pericoronitis			Slow growth, pH-6.5-plate discarded.		
27	Permanent	22/M	18	Pericoronitis	30 days	3	Successful	Yes	Yes
28	Permanent	22/M	48	Pericoronitis	23 days	3	Successful	Yes	Yes
29	Permanent	28/M	48	Impacted			No cells		
30	Permanent	29/M	38	Impacted			No cells		

- Successful cultures

GRAPH 1: SAMPLES IN CULTURE



GROWTH CHARACTERISTICS FOR PERMANENT SAMPLE 27 ADULT PULP

TABLE 2: GROWTH CURVE DERIVATIVES FOR PERMANENT SAMPLE 27 ADULT PULP

Days	Cell count/well/ml x 10^3	Slope	Standard Error (SE)	Population doubling time (days)	Seeding efficiency (%)
0	12	6.82	6.43	3.39	70.8
1	8.5				
2	13.56				
3	20				
4	26.5				
5	31.75				
6	42.54				
7	55.95				
8	61.3				

GRAPH 2: GROWTH CURVE FOR PERMANENT SAMPLE 27 ADULT PULP

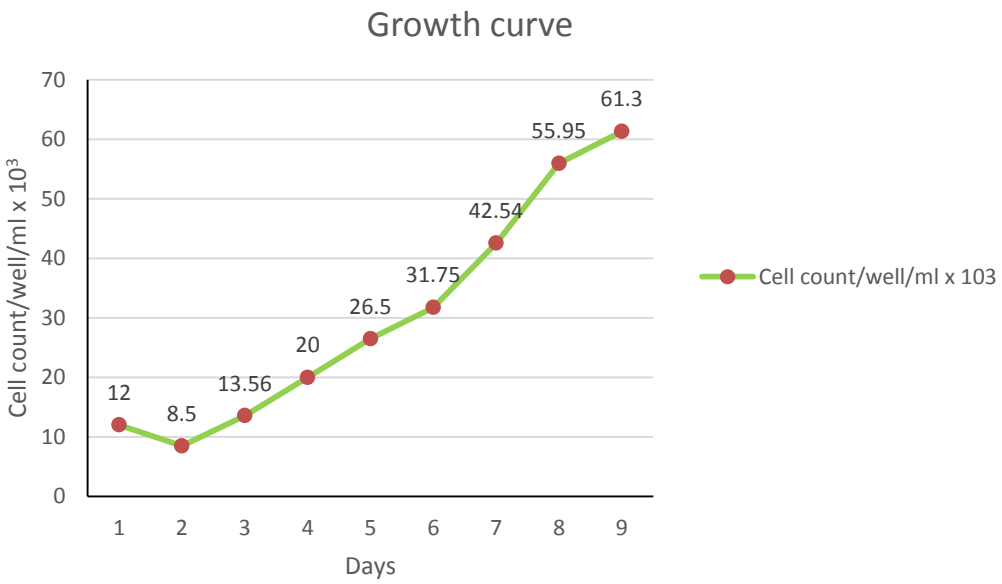


TABLE 3: F I-F VII SUBPOPULATION OF PERMANENT SAMPLE 27 ADULT PULP

Day	F I	F II	F III	F IV	F V	F VI	F VII
1	20	22	40	7	1	0	0
2	17	20	39	9	3	1	1
3	20	22	30	10	5	2	1
4	23	21	24	11	6	4	1
5	20	19	20	15	7	6	3
6	14	18	18	19	11	6	4
7	11	15	11	20	16	10	7
8	7	13	13	18	22	10	7

GRAPH 3: F I-F VII SUBPOPULATION OF PERMANENT SAMPLE 27 ADULT PULP

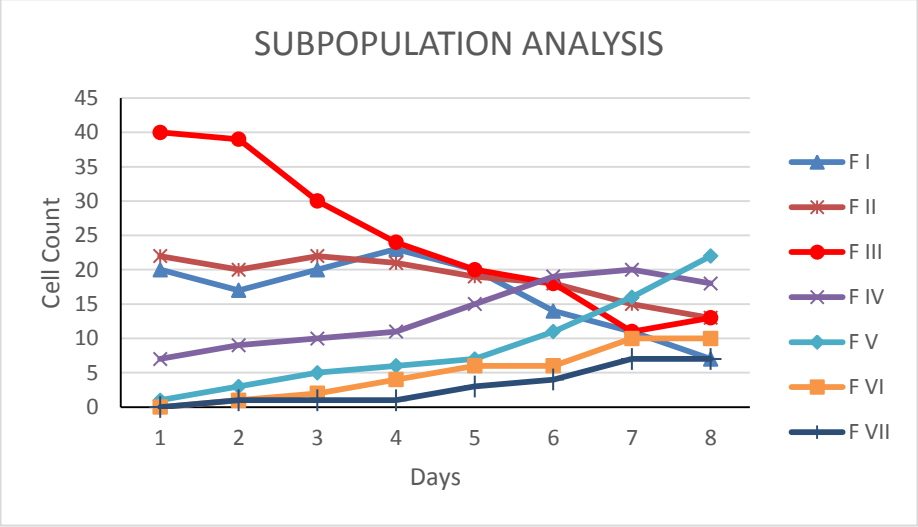
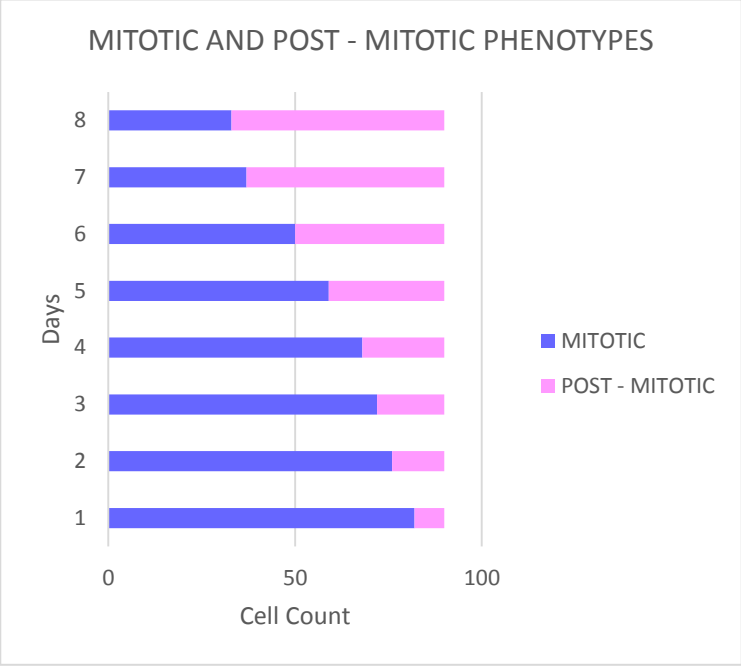


TABLE 4: MITOTIC AND POST-MITOTIC PHENOTYPES IN PERMANENT SAMPLE 27 ADULT PULP

Days	MITOTIC	POST - MITOTIC
1	82	8
2	76	14
3	72	18
4	68	22
5	59	31
6	50	40
7	37	53
8	33	57

GRAPH 4: MITOTIC AND POST-MITOTIC PHENOTYPES IN PERMANENT SAMPLE 27 ADULT PULP



GROWTH CHARACTERISTICS FOR PERMANENT SAMPLE 28 ADULT PULP

TABLE 5: GROWTH CURVE DERIVATIVES FOR PERMANENT SAMPLE 28 ADULT PULP

Days	Cell count/well/ml x 10^3	Slope	Standard Error (SE)	Population doubling time (days)	Seeding efficiency (%)
0	12	7.67	7.49	3.15	69.5
1	8.344				
2	11.976				
3	14.449				
4	19.302				
5	31.473				
6	42.555				
7	59.2				
8	69.435				

GRAPH 5: GROWTH CURVE FOR PERMANENT SAMPLE 28 ADULT PULP

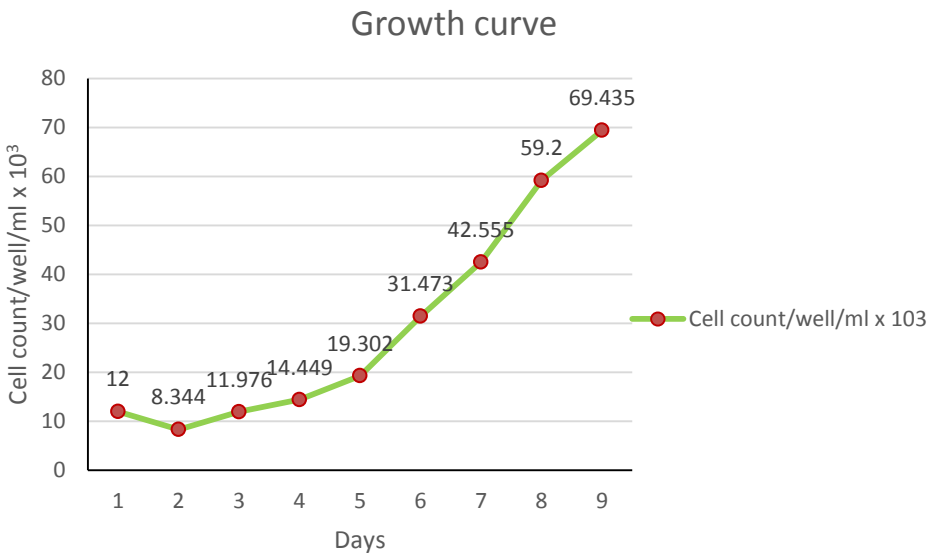


TABLE 6: F I-F VII SUBPOPULATION OF PERMANENT SAMPLE 28 ADULT PULP

Day	F I	F II	F III	F IV	F V	F VI	F VII
1	18	26	38	3	5	0	0
2	12	30	34	5	7	1	1
3	10	28	33	8	5	3	3
4	15	26	27	11	7	3	1
5	18	21	24	15	7	4	1
6	14	16	26	17	9	5	3
7	10	13	25	20	11	7	4
8	7	13	22	15	18	9	6

GRAPH 6: F I-F VII SUBPOPULATION OF PERMANENT SAMPLE 28 ADULT PULP

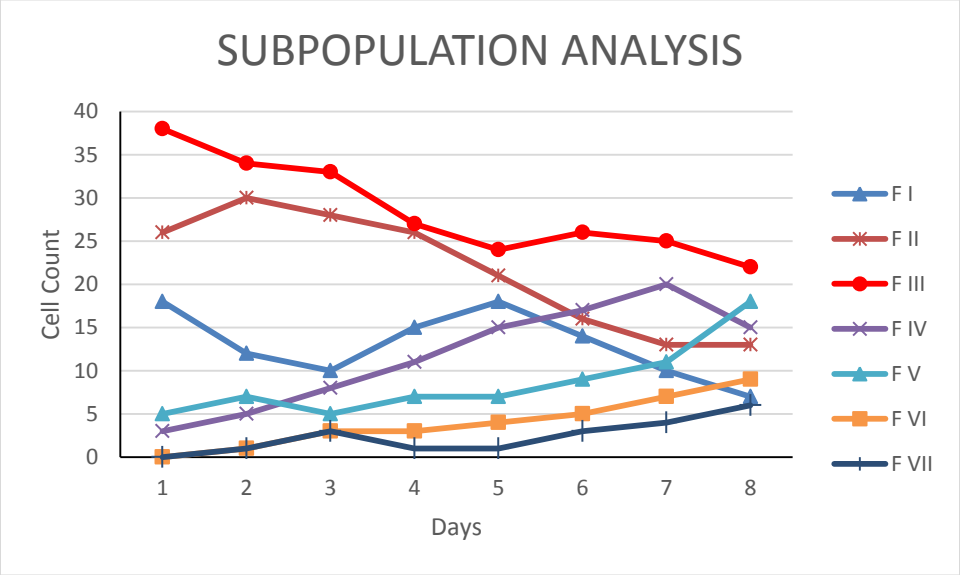


TABLE 7: MITOTIC AND POST-MITOTIC PHENOTYPES IN PERMANENT SAMPLE 28 ADULT PULP

Days	MITOTIC	POST - MITOTIC
1	82	8
2	76	14
3	71	19
4	68	22
5	63	27
6	56	34
7	48	42
8	42	48

GRAPH 7: MITOTIC AND POST-MITOTIC PHENOTYPES IN PERMANENT SAMPLE 28 ADULT PULP

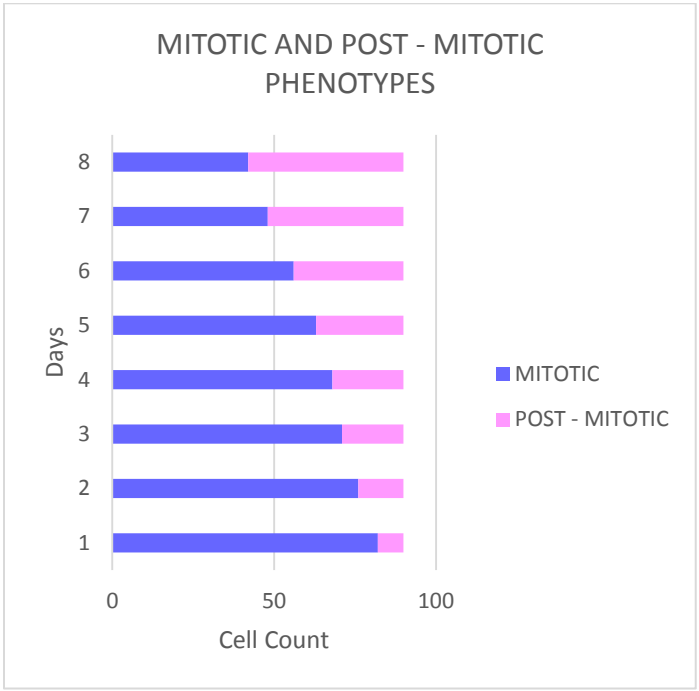


TABLE 8: NO OF SAMPLES CONTAMINATED

Permanent sample	Type of contamination	
	Bacterial	Fungal
No of samples contaminated (n)	4	8

GRAPH 8: TYPE OF CONTAMINATION

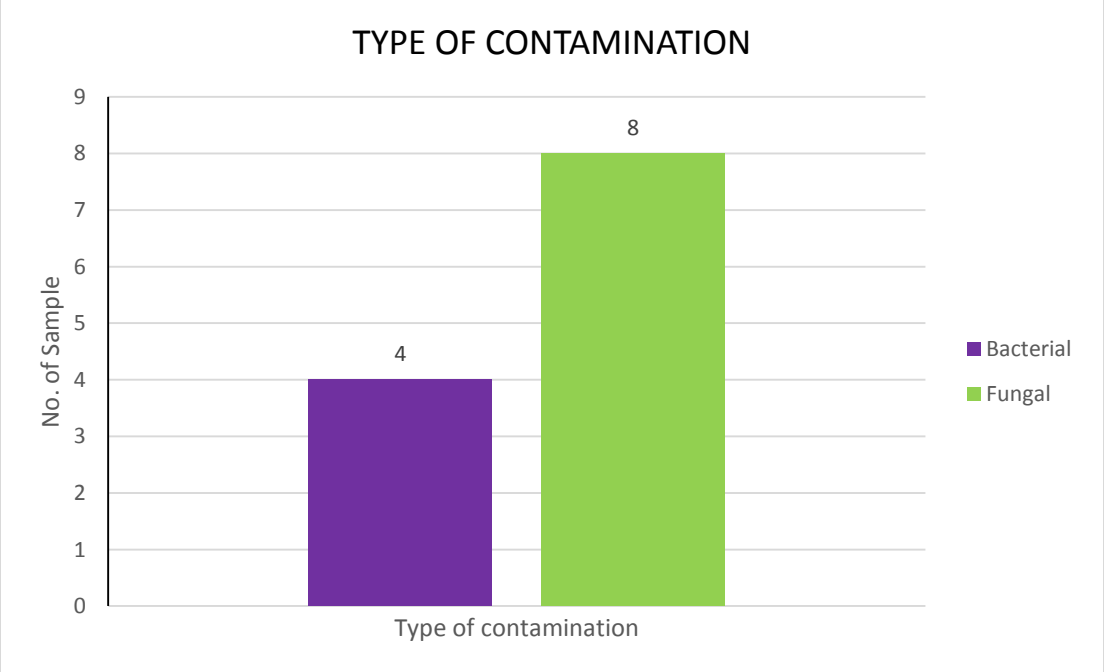


TABLE 9: SUMMARY OF IMMUNOCYTOCHEMICAL ANALYSIS OF STAGE SPECIFIC EMBRYONIC ANTIGEN - 4 (SSEA – 4) STAINING IN DPSCs

No	Sample	Fixation	PBS(pH)	Primary dilution	Primary incubation	Secondary dilution	Secondary incubation	Result	Positive Control
1	27	Methanol	PBST 7.4	1:100	Overnight	1:1000	1hour	Negative	Positive
2	28	Methanol	PBST 7.4	1:100	1 hour	1:1000	1hour	Negative	Positive
3	27	Methanol	PBST 7.6	1:100	Overnight	1:1000	1hour	Negative	Positive
4	28	Methanol	PBST 7.6	1:100	1 hour	1:1000	1hour	Negative	Positive
5	27	Methanol	PBST 7.4	1:50	Overnight	1:1000	1hour	Negative	Positive
6	28	Methanol	PBST 7.4	1:50	Overnight	1:1000	1hour	Negative	Positive
7	27	Methanol	7.5	1:50	Overnight	1:1000	1hour	Negative	Positive
8	28	Methanol	7.5	1:50	Overnight	1:1000	1hour	Negative	Positive
9	27	Methanol	7.4	1:50	Overnight	1:1000	1hour	Negative	Positive
10	28	Methanol	7.4	1:50	Overnight	1:1000	1hour	Negative	Positive
11	27	Methanol	7.6	1:50	Overnight	1:1000	1hour	Negative	Positive
12	28	Methanol	7.6	1:50	Overnight	1:1000	1hour	Negative	Positive

PRIMARY CULTURE OF DENTAL PULP STEM CELLS (DPSCs)

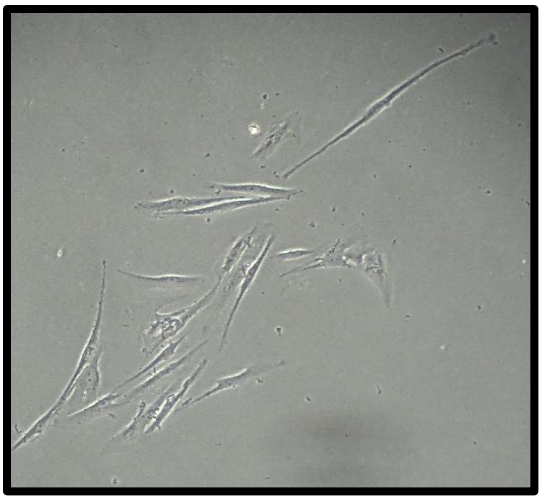


Figure 16: **Individual Cells – 10x**

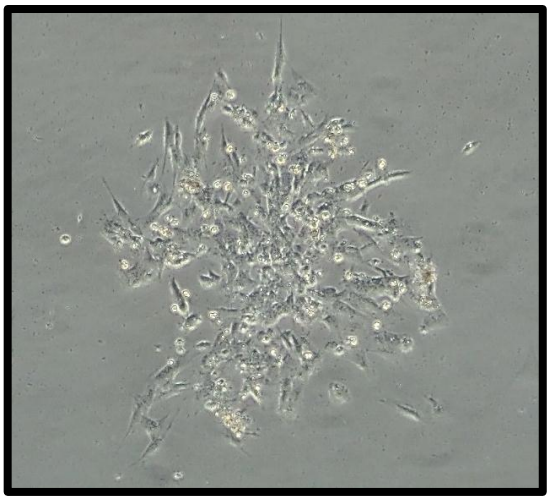


Figure 17: **Cluster of Cells – 10x**

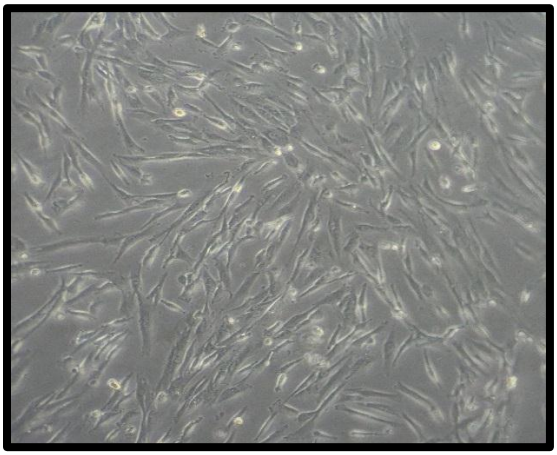


Figure 18: **Confluent primary culture – 10x**

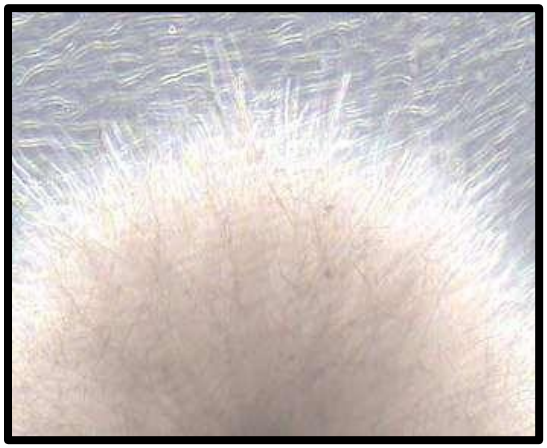


Figure 19: **Fungal contamination – 20x**

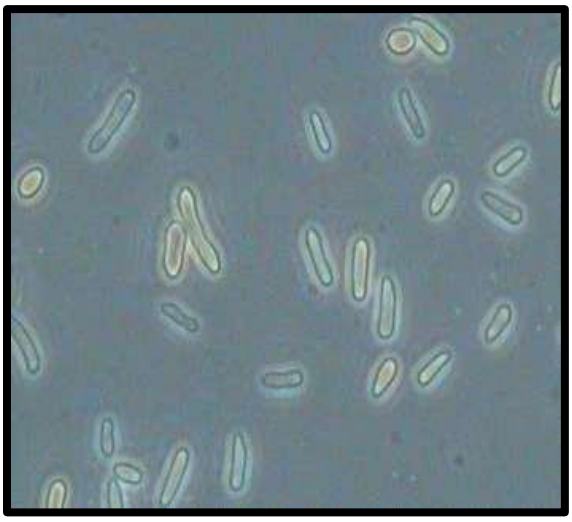


Figure 20: **Bacterial contamination – 20x**

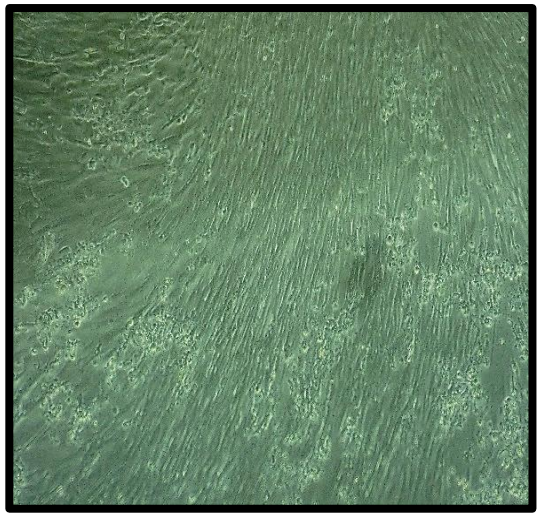


Figure 21: **Contact Inhibition – 10x**

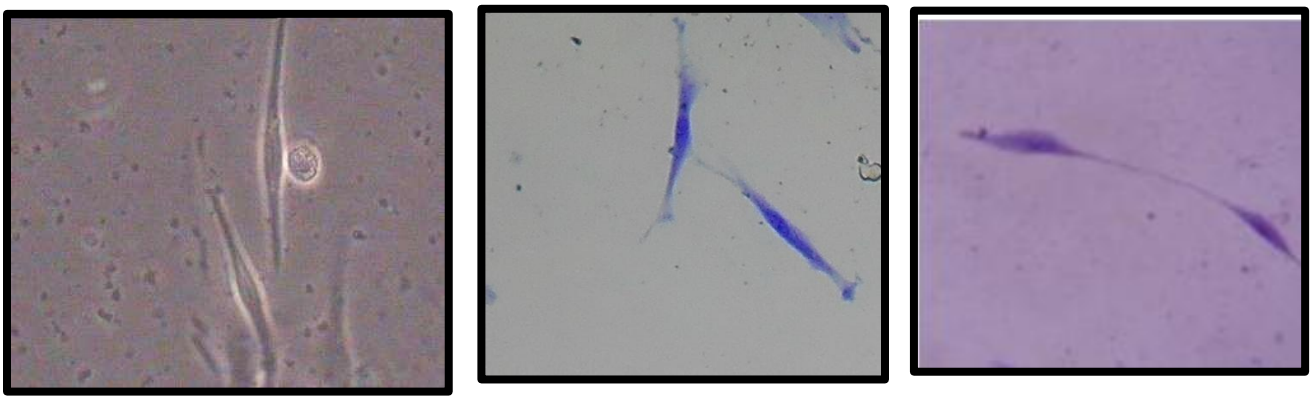


Figure 22: **F I Post-Mitotic phenotype [i) Phase contrast, ii) Crystal Violet, iii) Giemsa staining]**

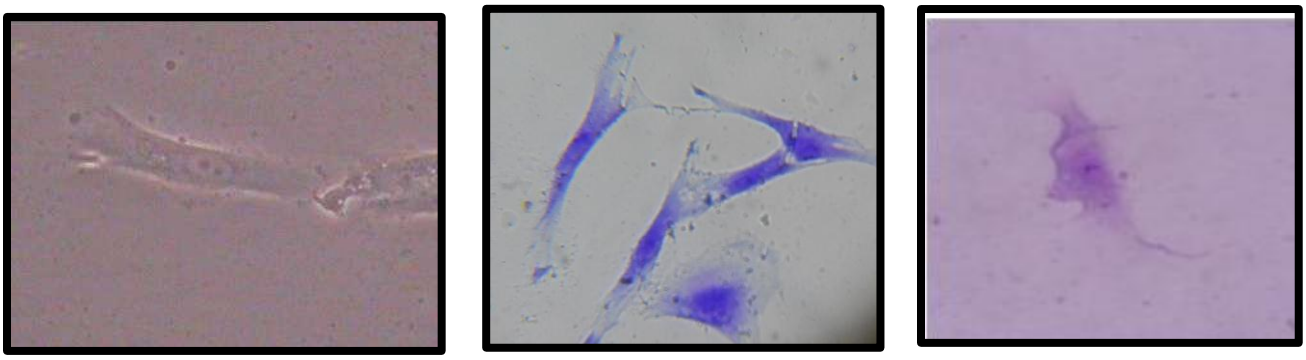


Figure 23: **F II Post-Mitotic phenotype [i) Phase contrast, ii) Crystal Violet, iii) Giemsa staining]**

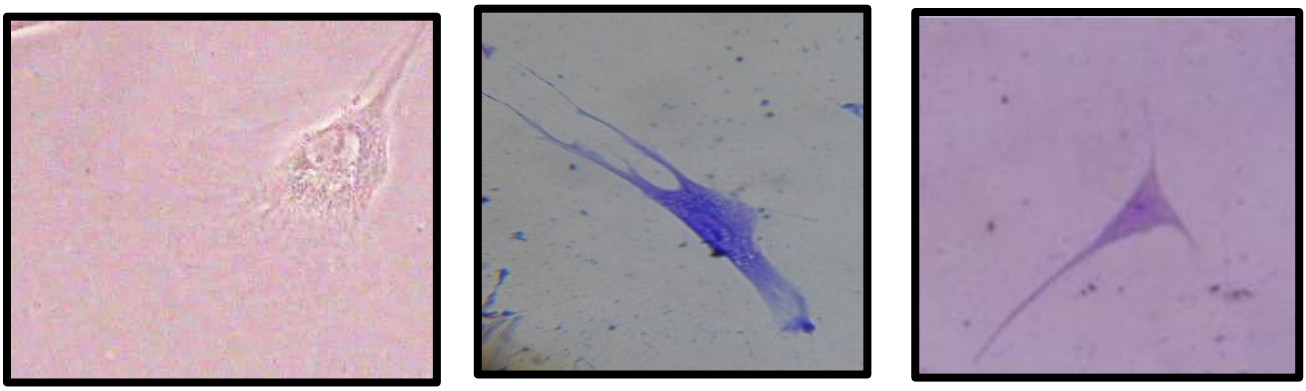


Figure 24: **F III Post-Mitotic phenotype [i) Phase contrast, ii) Crystal Violet, iii) Giemsa staining]**

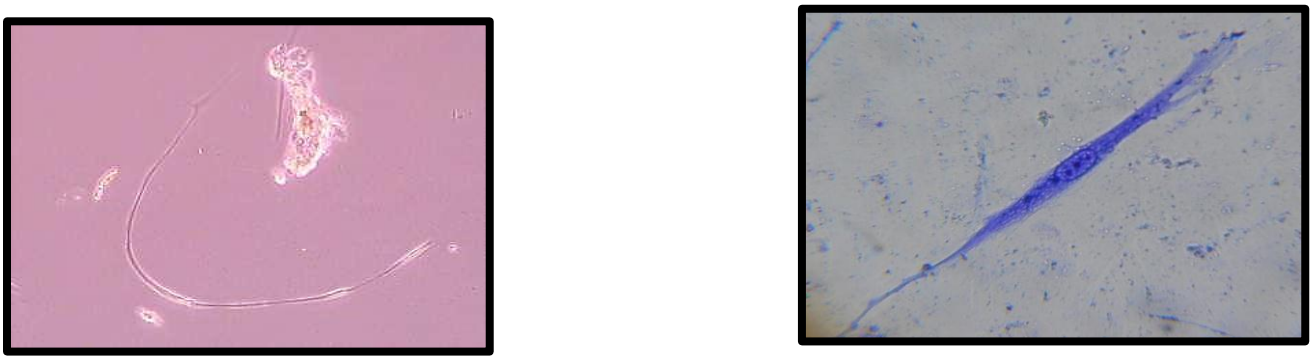


Figure 25: **F IV Post-Mitotic phenotype [i) Phase contrast, ii) Crystal Violet]**

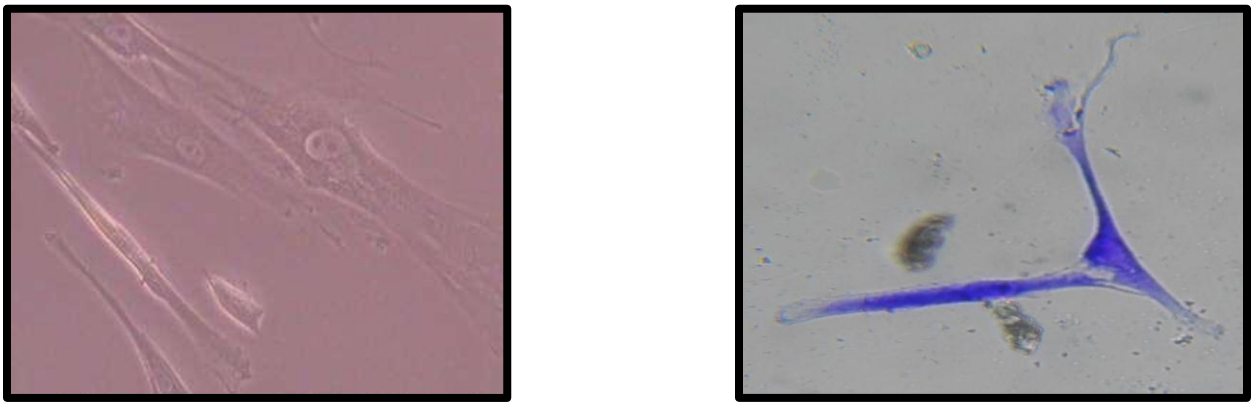


Figure 26: **F V Post-Mitotic phenotype [i) Phase contrast, ii) Crystal Violet]**

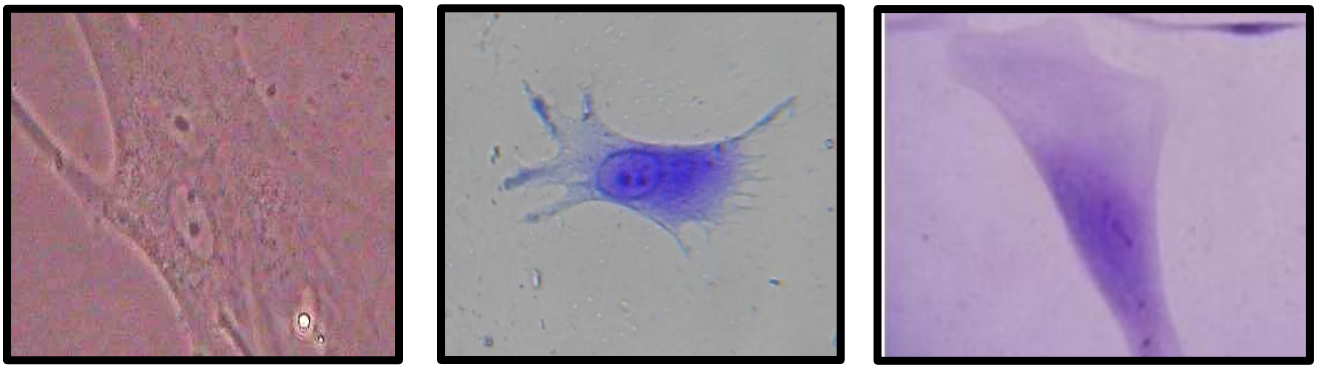


Figure 27: **F VI Post-Mitotic phenotype [i) Phase contrast, ii) Crystal Violet, iii) Giemsa staining]**

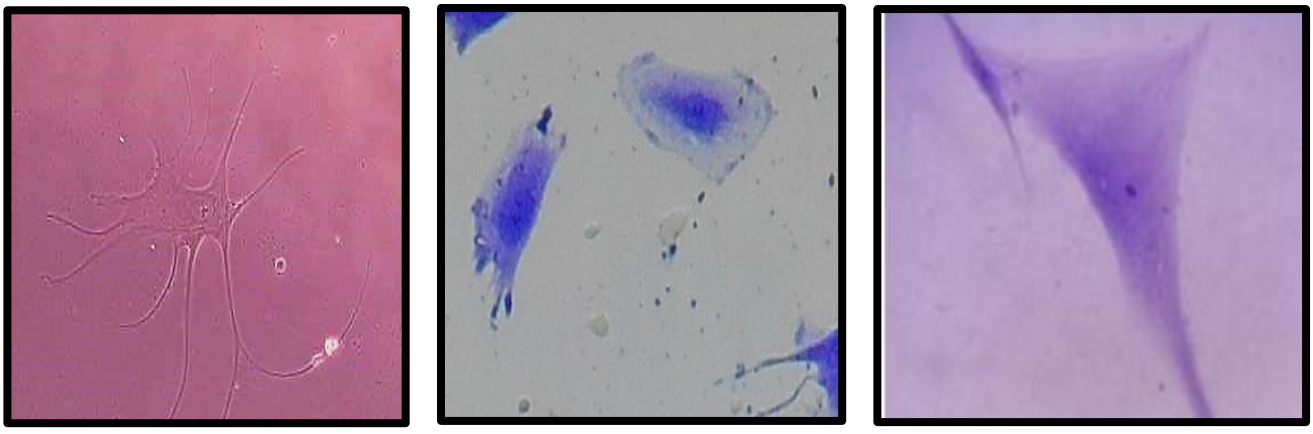


Figure 28: **F VII Post-Mitotic phenotype [i) Phase contrast, ii) Crystal Violet, iii) Giemsa staining]**

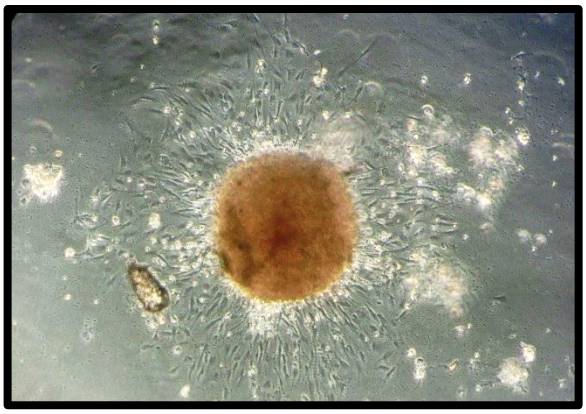


Figure 29: **Day 4 – 10x**

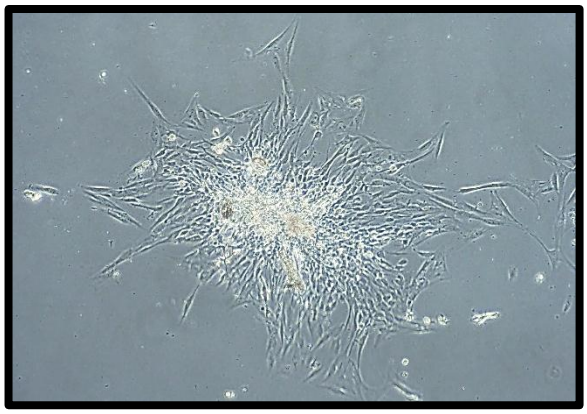


Figure 30: **Day 8 – 10x**

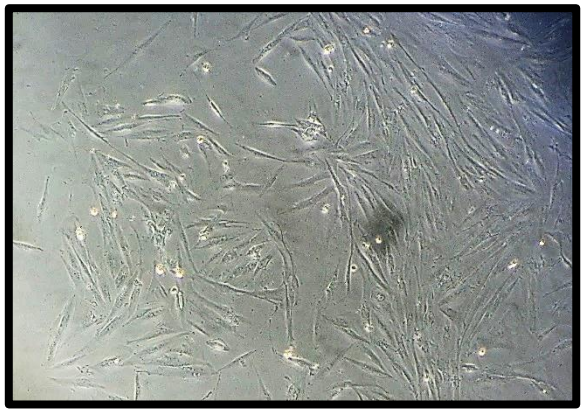


Figure 31: **Day 15 - 10x**

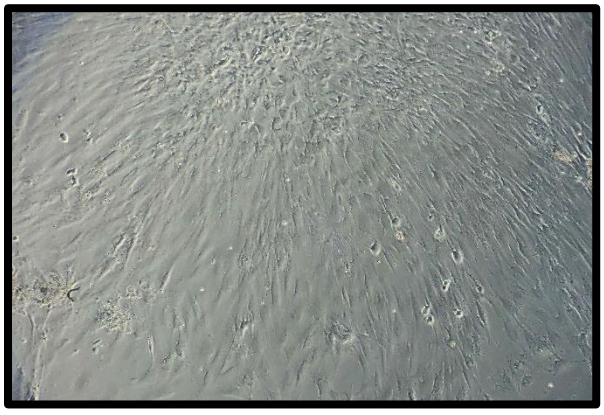


Figure 32: **Day 23 – 10x**

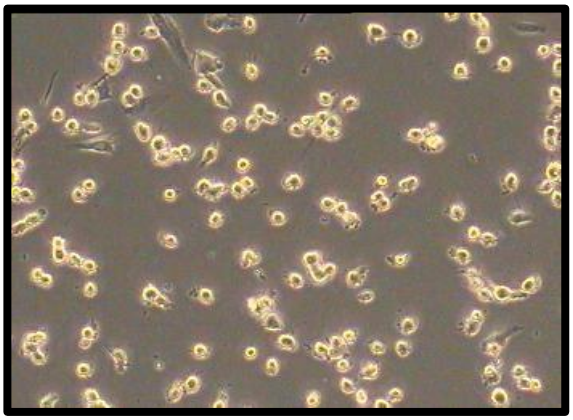


Figure 33: **Trypsinisation – 10x**

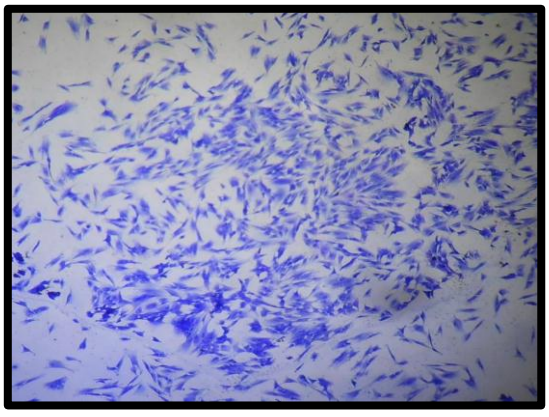


Figure 34: **Crystal Violet staining – 10x**

IMMUNOHISTOCHEMISTRY STAINING WITH SSEA-4 IN OSCC

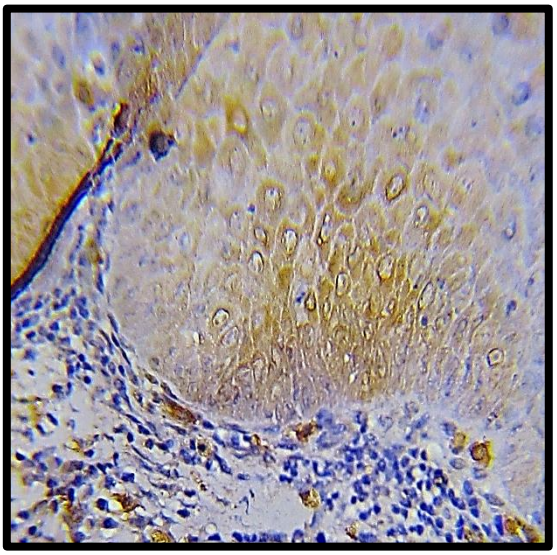


Figure 35: **Positive control - 40x**

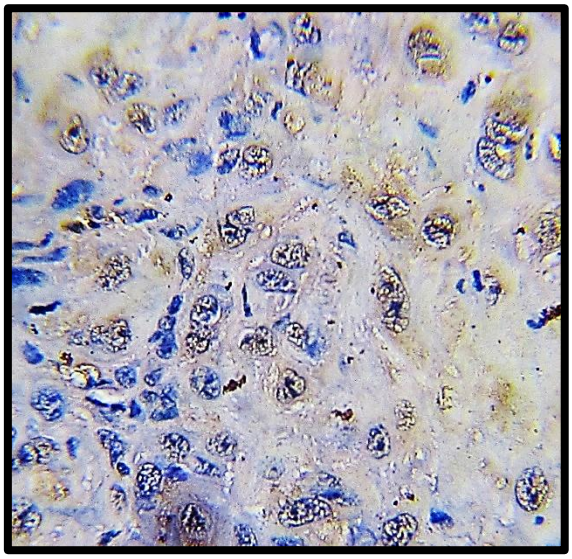


Figure 36: **Positive control - 40x**

IMMUNOCYTOCHEMISTRY STAINING WITH SSEA-4

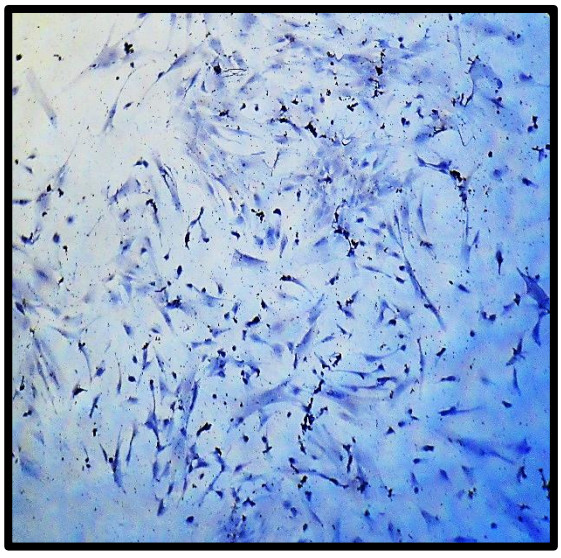


Figure 37: **Negative staining 27AP 1st passage - 10x**

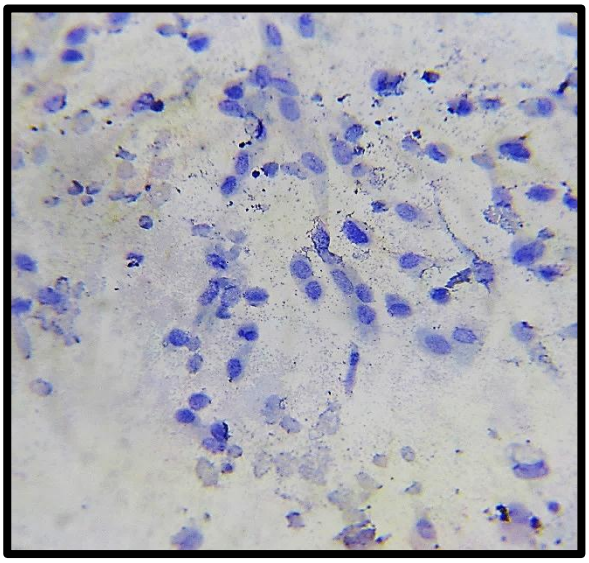


Figure 38: **Negative staining 28AP 3rd passage - 40x**

Discussion

This study was done to isolate and characterize the stem cells from dental pulp of permanent teeth in α-MEM and to ascertain that the dental pulp stem cells have embryonic property by studying the expression of SSEA-4.

Kawanabe N *et al.,* used premolars and molars as the source of stem cells[14]. In this study, we chose to extract the pulp from permanent third molars. This decision was based on our previous experience in which we tried extraction of pulp from premolars as the source of stem cells. But we did not yield sufficient cells[77]. 30 permanent third molar teeth were collected in which 4 yielded successful cultures after some trouble shooting in culture technique. The protocol of this study was similar to that of **Gronthos *et al***[4] and **Ian Freshney's Textbook of human cell culture**[76].

Tissue disaggregation is the foremost step to produce the suspension of cells. The tissue disaggregation for primary culture can be done in 3 types. They are fine dissection, mechanical disaggregation and enzyme disaggregation. In our study, we chose the enzyme disaggregation technique as it gives better yield. Collagenase and dispase were the enzymes we used for tissue disaggregation because the extracellular matrix of the pulpal tissues contains collagen, so collagenase was the obvious choice. The perivascular niche is made up of type IV collagen where the stem cells in pulp reside. Hence dispase, was used to disaggregate type IV collagen and to get a better yield of cells. According to the study done by **Shekar R and Ranganathan K**

in characterization of DPSCs, the authors used similar enzyme disaggregation technique and enzymes in ratio of 2:1 which gave a good yield of cells[62].

The enzymes we used were 3mg of collagenase and 1mg of dispase. Previous studies in our department also used the same method of enzyme disaggregation techniques for tissue disaggregation in primary culture[77, 78].

Out of 30, 10 third molar teeth did not yield any cells. Among 10 samples, 5 samples were stored in the transport medium containing the double strength of antibiotics and without the serum and two samples were transported in normal saline. We collected teeth samples from Department of Oral and Maxillofacial Surgery, Ragas Dental College, Chennai and also from private clinical centres in and around our institution. By maintaining the temperature at 4˚C the specimen was transported. On reaching the laboratory (15 mins to less than 24 hours), the specimen was completely rinsed in Phosphate buffered saline (PBS) to remove blood clot or debris that might contaminate the pulp tissue while extirpation. Yield could not be obtained in 10 samples. One of the reason could be that, the time lag between extraction and transport of the sample to the lab for processing. Alternatively, this could be due to excess washing of the tissue sample in PBS with 5x antibiotics[78]. According to **Perry *et al,*** DPSCs from human third molars processed immediately after extraction yielded more cells than from the molars stored in the transport medium at 4˚C[80].

Despite the use of antibiotics and following aseptic conditions 12 samples could not be used further in this study due to contamination. (*Table 8*). Similar findings were reported by **Takeda *et al.*,** in which 9% of their samples reported bacterial and fungal contamination[79]. In our study the resultant contamination would be attributed to either inadequate sterilization procedure or could be due to the oral hygiene status of the patients from whom the samples were collected.

In our study, the inability to standardize the sterilization procedure and aseptic technique from the clinics where the samples have been collected could also have contributed to contamination. During our study, we perpetually had continuous, fungal and bacterial contamination in the culture plates of primary culture (*Figure 4 & 5*). To overcome this, we did the following precautions: i) Complete washing and sterilization of the instruments was done cautiously, ii) Complete fumigation of the culture laboratory was done with an interval period of 12 hours, 24 hours and 48 hours, iii) Complete flaming of the instruments was done before using it for the procedures. New α-MEM was prepared to avoid and eliminate preexisting microorganisms in the old medium.

The pH of culture medium is critical to the growth of stem cells. The pH is determined by carbon dioxide levels, constitution of the culture medium, and cell metabolism[62,76]. In our study, slow growth of cells and cellular degeneration was seen in sample 4, 5 and 26 because of the uncontrolled CO_2

level in the incubator which led to altered pH (less than 6.5) of the media. So, the CO_2 level in the incubator and the pH of the stock media was adjusted.

Media was changed every alternate day till the primary culture reached its confluency. Growth characteristic and phenotype was done for 2 samples (27AP, 28AP). Cells took 23 days (lower third molars) to reach confluency. Seeding efficiency is the percentage of the inoculums that attached to the substrate after 12 hours or the first day count. It implies only the cell viability or survival, but not the proliferation capacity. In our study, the seeding efficiency was 70.8% and 69.5% in the samples 27 and 28 respectively (*Table 2 and 5*). In the studies previously conducted in our department, the seeding efficiency was 72.2 ± 5.5 SD[77], and in another study it ranged from 37 to 112%[78]. The seeding efficiency was 88.9% and 91.7% for DPSCs in a study by **Shekar R and Ranganathan K**[62] and the seeding efficiency was 42.8% ± 1.65 SD in another study by **Sivashankar V and Ranganathan K**[81].

The mitotic population was higher than the post mitotic population in 1 to 3 days. There was a significant decrease in the mitotic population and an increase in post-mitotic population from day 5 to day 8 of culture in both the samples (*Table 4 and 7*).

Population doubling time (PDT) is the interval period required by a cell population to double at the middle of the logarithmic phase of growth. The PDT in our study was 3.37 and 3.15 days in 27AP and 28AP respectively. In

one of the previously conducted study in our department, the population doubling time was 3.1 days (72 hours)[77] and in other study, the PDT ranged from 37 hours to 144 hours[78]. In another study by **Shekar R and Ranganathan K,** the average PDT of DPSCs was 26 hours[62]. Sufficient cells were not present in subsequent subcultures for growth characteristic and phenotype of the samples 7AP and 25AP.

Trouble shooting reasons encountered in cell culture were due to:

- Storing samples in the transport medium
- Contamination due to aseptic procedures
- pH alteration due to uncontrolled CO_2 level
- Tooth with open apex.

The problems could be overcome by ensuring adequate sterilization procedure, washing the tooth with povidone - iodine, fumigating the lab, monitoring CO_2 levels and avoiding tooth samples with open apex. Thus, we were able to yield successful cultures.

Sivashankar V and Ranganathan K used CD73 and CD146 as markers to characterize DPSCs (Dental Pulp Stem Cells) as mesenchymal stem cells[81]. Studies have shown that 90% of DPCs positively express markers that are similar to the expression in BMSCs such as CD29, CD44, and endothelial cell marker CD146 which is shown by flowcytometry.

Gronthos *et al.,* found that DPSC's express a higher positivity of CD146 similar to that of BMSCs[82]. Another study by **Shi and Gronthos** used magnetic and fluorescence activated cell sorting for STRO-1 positive cells in an attempt to identify the stem cell niche of DPSCs[83]. Thus it is shown from various studies that the DPSCs have mesenchymal stem cell properties.

The cells from DPSCs and SHED are positive for both embryonic stem cell markers and mesenchymal stem cell markers. Embryonic markers like CD73, CD90, CD105, CD146, CD166, and SSEA-4 show strong positive expression[5, 10]. These cells also show positive expression for mesenchymal markers like Sox2, Lin28, CD13, CD105, CD90, CD29, CD73 and STRO-1 and show negative expression for CD45, CD34, CD14, CD19, and HLA-DR[11,50, 81].

Gang *et al* (2007)[71] stated that SSEA-4, a marker helps to identify and isolate mesenchymal stem cell population from bone marrow. **Kerkis I *et al*** (2006)[70], **Atari *et al*** (2011)[47], **Atari *et al*** (2012)[48] successfully isolated dental pulp stem cells that expressed the various embryonic stem cell markers Oct-4, Nanog, Stage Specific Embryonic Antigen (SSEA)-3, SSEA-4. Thus, Stage Specific Embryonic Antigen-4 (SSEA-4) is also a marker for isolating pluripotent dental pulp stem cells.

Kawanabe N *et al* [14] stated, the DPSCs have the embryonic property and also the capacity to differentiate into all the lineages from the germ layers.

Cell surface antigen profiles reflect the phenotype of cells and expression of embryonic stem cell marker.

In a recent study of stem cells from human corneal epithelium, it was found that SSEA-4 positive cells constituted 40% of limbal epithelial cells. Interestingly, the SSEA-4 negative cell population had 5 times more progenitor cells than the SSEA-4 positive cells. Also, stem cell markers ABCG2, ΔNp63α and Cytokeratin-14 expression was less in SSEA-4 positive compared to SSEA-4 negative cells. In addition to this, differentiation markers were less in SSEA-4 negative cells and the colony forming efficiency was higher which had led them to conclude that SSEA-4 could be a "negative marker to enrich limbal stem cells" [74].

Brimble *et al*[75] stated, pluripotent stem cells isolated from human ES cells show negative expression for SSEA-4. The authors stated that SSEA-4 plays a role in cellular proliferation rather than cell differentiation. Thus, the pluripotential nature of the stem cells do not depend on SSEA-4 expression.

In our study, SSEA-4 was used to study the embryonic property in DPSCs. Immunocytochemistry was done using antibodies to SSEA-4 to evaluate the expression on the surface of the cells cultured from permanent tooth pulp. The expression of SSEA-4 marker was evaluated to assess the embryonic property in the 1st, 2nd and 3rd passage of culture. The presence or absence of the marker on the cell surface can be elicited by

immunocytochemistry method. The cells in 1st, 2nd and 3rd passage showed negative expression for SSEA-4. As recommended by the manufacturer Abcam™, oral squamous cell carcinoma tissue section were used as positive control. The DPSCs from 1st, 2nd and 3rd passages showed negative expression to SSEA-4 (*Table 9*).

Thus in our study, the stem cells were isolated from dental pulp by enzyme disaggregation method and cultured. The cultured cells showed phenotype similar to that of mesenchymal stem cells. The cells also showed negative staining for SSEA-4. Interestingly, there is one study in the ophthalmological literature which says that SSEA-4 has a role in cell differentiation and not in pluripotency. And the investigators of this study state that negative expression of SSEA-4 is a marker of progenitor cell population. However, further studies are needed to ascertain as this finding can be extrapolated to cells of the dental pulp.

The DPSCs from third molars have adult stem cell population. Along with the adult stem cells, the DPSCs also have embryonic like stem cell population and pluripotent like stem cell population[47, 48].

Our results showed that DPSCs and SSEA-4 negativity would indicate that cells in pulp are not embryonic/ pluripotential. Previous studies from our lab have shown their mesenchymal nature[78, 81]. Thus, combined results proved to a multipotential nature of stem cells rather than pluripotentiality.

Summary and Conclusion

- In this study, we successfully isolated and cultured four Dental Pulp Stem Cells (DPSCs) from thirty samples of permanent teeth. We also evaluated the immunocytochemical expression of Stage Specific Embryonic Antigen-4 (SSEA-4) on cultured DPSCs.
- The average population doubling time of DPSCs was revealed by plotting the growth curve and the population doubling time (PDT) was estimated as 3.26 days (78.24 hours). The average seeding efficiency of DPSCs were 70.15%.
- The morphologically characterized DPSCs did not express the embryonic stem cell marker, SSEA–4.
- Further characterization with combination of markers for Mesenchymal Stem Cells (MSCs) and Embryonic Stem Cells (ESCs) are needed to define their differentiation potential of DPSCs.

Bibliography

1. **Smith A**

   A glossary for stem-cell biology

   Nature 2006; 441: 1060

2. **Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM**

   Embryonic stem cell lines derived from human blastocysts

   Science 1998; 282(5391): 1145 – 7

3. **Gearhart J**

   New potential for human embryonic stem cells

   Science 1998; 282(5391): 1061 - 2

4. **Gronthos S, Mankani M, Brahim J, Robey PG, Shi S**

   Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo

   Proceedings of the National Academy of Sciences USA 2000; 97: 13625-13630

5. **Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S**

   SHED - Stem cells from human exfoliated deciduous teeth

   Proceedings of the National Academy of Sciences USA 2003; 100: 5807- 5812

6. **Ulloa-Montoya F, Verfaillie CM, Hu WS**

   Culture systems for pluripotent stem cells

   Journal of bioscience and bioengineering 2005; 100(1): 12 – 27

7. **Nedel F, André Dde A, de Oliveira IO, Cordeiro MM, Casagrande L,**

   Tarquinio SB, Nor JE, Demarco FF

   Stem cells: therapeutic potential in dentistry

   Journal of Contemplary Dental Practice 2009; 10: 90 - 6

8. **Rastegar F, Shenaq D, Huang J, Zhang W, Zhang BQ, He BC, Chen L, Zuo GW, Luo Q, Shi Q, Wagner ER**

   Mesenchymal stem cells: Molecular characteristics and clinical applications

   World Journal of Stem Cells 2010; 26: 67 - 80

9. **Karamzadeh R, Eslaminejad M B**

   Dental Related Stem Cells and Their Potential in Regenerative Medicine

   Regenerative Medicine and Tissue Engineering, Intech, 2013; 95 - 115

10. **Zhao W, Ji X, Zhang F, Li L, Ma L**

    Embryonic Stem Cell Markers

    Molecules 2012; 17: 6196 - 6236

11. **Lv FJ, Tuan RS, Cheung K, Leung VYL**

    Concise Review: The Surface Markers and Identity of Human Mesenchymal Stem Cells

    Stem Cells 2014; 32: 1408 – 1419

12. **Kannagi R, Cochran NA, Ishigami F, Hakomori SI, Andrews P, Knowles BB, Solter D**

Stage-specific embryonic antigens (SSEA - 3 and - 4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells

The EMBO journal 1983; 2(12): 2355

13. **Yamashita T, Wada R, Sasaki T, Deng C, Bierfreund U, Sandhoff K, Proia RL**

A vital role for glycosphingolipid synthesis during development and differentiation

Proceedings of the National Academy of Sciences USA 1999; 96: 9142 – 9147

14. **Kawanabe N, Murata S, Fukushima H, Ishihara Y, Yanagita T, Yanagita E, Ono M, Kurosaka H, Kamioka H, Itoh T, Kuboki T**

Stage-specific embryonic antigen-4 identifies human dental pulp stem cells

Experimental Cell Research 2012; 318(5): 453 - 63

15. **Kumar V, Abbas AK, Fausto N, Aster J**

Robbins and Cotran's Pathologic Basis of Diseases

8th Edition, Elsevier 2010; 82 - 86

16. **Suzuki T, Lee CH, Chen M, Zhao W, Fu SY, Qi JJ, Chotkowski G, Eisig SB, Wong A, Mao JJ**

Induced migration of dental pulp stem cells for in vivo pulp regeneration

Journal of Dental Research 2011; 90: 1013 - 8

17. Ivanovski S, Gronthos S, Shi S, Bartold PM

Stem cells in the periodontal ligament

Oral diseases 2006; 12: 358 - 363

18. Koc ON, Lazarus HM

Mesenchymal stem cells: heading into the clinic

Bone Marrow Transplantation 2001; 27: 235 – 239

19. Neirinckx V, Coste C, Rogister B, Wislet-Gendebien S

Neural Fate of Mesenchymal Stem Cells and Neural Crest Stem Cells: Which Ways to Get Neurons for Cell Therapy Purpose?

INTECH Open Access Publisher, 2013

20. Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV

Osteogenesis in transplants of bone marrow cells

Journal of embryology and experimental morphology 1966; 16(3): 381 - 390

21. Kolf CM, Cho E, Tuan RS

Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation

Arthritis Research & Therapy 2007; 9(1): 204

22. Takahashi K, Yamanaka S

Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors

Cell 2006; 126: 663 – 676

23. Zaehres H, Kim JB, Scholer HR

Induced Pluripotent Stem Cells

Methods in enzymology 2010; 476: 309 – 325

24. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS and Jones JM

Embryonic stem cell lines derived from human blastocysts

Science 1998; 282: 1145 – 1147

25. Yamanaka S

Strategies and new developments in the generation of patient-specific pluripotent stem cells

Cell Stem Cell 2007; 1: 39 – 49

26. Zhang X, De Los Angeles A, Zhang J

The Art of Human Induced Pluripotent Stem Cells: The Past, the Present and the Future

The Open Stem Cell Journal 2010; 2: 2 - 7

27. Yu J, Thomson JA

Pluripotent stem cell lines

Genes and Devlopment 2008; 22(15): 1987 - 1997

28. Egusa H, Okita K, Kayashima H, Yu G, Fukuyasu S, Saeki M, Matsumoto T, Yamanaka S, Yatani H

Gingival Fibroblasts as a Promising Source of Induced Pluripotent Stem Cells

PLoS ONE 2010; 5(9): e12743

29. **Telpalo-Carpio SA, Aguilar-Yañez JM, Gonzalez-Garza MT, Cruz-Vega DE, Moreno-Cuevas JE**

iPS cells generation: an overview of techniques and methods

Journal of stem cells & regenerative medicine 2013; 9(1): 2

30. **Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Thomson JA**

Induced pluripotent stem cell lines derived from human somatic cells

Science 2007; 318(5858): 1917 - 1920

31. **Tachibana M, Amato P, Sparman M, Gutierrez NM, Tippner-Hedges R, Ma H, Mitalipov S**

Human embryonic stem cells derived by somatic cell nuclear transfer

Cell 2013; 153(6): 1228 - 1238

32. **Byrne J, Pedersen D, Clepper L, Nelson M, Sanger W, Gokhale S, Mitalipov S**

Producing primate embryonic stem cells by somatic cell nuclear transfer

Nature 2007; 450(7168)

33. **Harada H, Ohshima H**

New perspectives on tooth development and the dental stem cell niche

Arch Histology and Cytology 2004; 67(1): 1-11

34. **Mitsiadis TA, Barrondon O, Rochat A, Barrandon Y, De Bari C**

Stem cell niches in mammals

Experimental Cell Research 2007; 313: 3377 – 3385

35. Schofield R

The relationship between the spleen colony-forming cell and the haemopoietic stem cell

Blood Cells 1978; 4: 7 – 25

36. Sprading A, Drummond-Barbosa D, Kai T

Stem cells find their niche

Nature 2001; 414: 98 - 104

37. Jones DL, Wagers AJ

No place like home: anatomy and function of the stem cell niche

Nature Reviews Molecular Cell Biology 2008; 9(1): 11 - 21

38. Bianco P, Riminucci M, Gronthos S, Robey PG

Bone Marrow Stromal Stem Cells: Nature, Biology and Potential Applications

Stem cells 2001; 19(3): 180 - 192

39. Li L, Xie T

Stem cell niche: structure and function

Annual Review Cell Developmental Biology 2005; 21: 605 - 31

40. Sun TT, Cotsarelis G, Lavker RM

Hair follicular stem cells: the bulge-activation hypothesis

Journal of Investigative Dermatology 1991; 96: 77 - 78

41. Niemann C, Owens DM, Hulsken J, Birchmeier W, Watt FM

Expression of DeltaNLef1 in mouse epidermis results in differentiation of hair follicles into squamous epidermal cysts and formation of skin tumors

Development 2002; 129: 95 – 109

42. Sancho E, Batlle E, Clevers, H

Signaling pathways in intestinal development and cancer

Annual Review Cell Developmental Biology 2004; 20: 695 - 723

43. Secker GA, Daniels JT

Limbal epithelial stem cells of the cornea

The Stem Cell Research Community, StemBook, June 30, 2009

44. Rodríguez-Lozano FJ, Bueno C, Insausti CL, Meseguer L, Ramirez MC, Blanquer M, Moraleda JM

Mesenchymal stem cells derived from dental tissues

International endodontic journal 2011; 44(9): 800 - 806

45. Ulmer FL, Winkel A, Kohorst P, Stiesch M

Stem cells--prospects in dentistry

Schweiz Monatsschr Zahnmed 2010; 120: 860 - 83

46. Karaöz E, Doğan BN, Aksoy A, Gacar G, Akyüz S, Ayhan S, Sarıboyacı AE

Isolation and in vitro characterisation of dental pulp stem cells from natal teeth

Histochemistry and cell biology 2010; 133(1): 95 - 112

47. **Atari M, Barajas M, Hernández-Alfaro F, Gil C, Fabregat M, Ferres Padro E, Giner L, Casals N**

Isolation of pluripotent stem cells from human third molar dental pulp

Histology and histopathology 2011; 26(7): 1057

48. **Atari M, Gil-Recio C, Fabregat M, García-Fernández D, Barajas M, Carrasco MA, Giner L**

Dental pulp of the third molar: a new source of pluripotent-like stem cells

Journal of cell science 2012; 125(14): 3343 - 3356

49. **Bluteau G, Luder HU, De Bari C, Mitsiadis TA**

Stem cells for tooth engineering

European Cells and Materials 2008; 16: 1 - 9

50. **Yamaza T, Kentaro A, Chen C, Liu Y, Shi Y, Gronthos S, Shi S**

Immunomodulatory properties of stem cells from human exfoliated deciduous teeth

Stem cell research & therapy 2010; 1(1): 5

51. **Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, Young M, Robey PG, Wang CY, Shi S**

Investigation of multipotent postnatal stem cells from human periodontal ligament

The Lancet 2004; 364(9429): 149 - 55

52. **Liu Y, Zheng Y, Ding G, Fang D, Zhang C, Bartold PM, Gronthos S, Shi S, Wang S**

Periodontal ligament stem cell-mediated treatment for periodontitis in miniature swine

Stem Cells 2008; 26: 1065 – 1073

53. **Yalvac ME, Ramazanoglu M, Rizvanov AA, Sahin F, Bayrak OF, Salli U, Palota´s A, Kose GT**

Isolation and characterization of stem cells derived from human third molar tooth germs of young adults: implications in neo-vascularization, osteo-, adipo- and neurogenesis

The Pharmacogenomics Journal 2009; 1 – 9

54. **Morsczeck C, Götz W, Schierholz J, Zeilhofer F, Kühn U, Möhl C, Sippel C, Hoffmann KH**

Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth

Matrix Biology 2005; 24(2): 155 - 65

55. **Honda MJ, Imaizumi M, Tsuchiya S, Morsczeck C**

Dental follicle stem cells and tissue engineering

Journal of Oral Science 2010; 52(4): 541 - 52

56. **Sonoyama W, Liu Y, Fang D, Yamaza T, Seo BM, Zhang C, Liu H, Gronthos S, Wang CY, Shi S, Wang S**

Mesenchymal Stem Cell-Mediated Functional Tooth Regeneration in Swine

PLoS ONE 2006; 1(1): e79

57. Zhang Q, Shi S, Liu Y, Uyanne J, Shi Y, Shi S, Le AD

Mesenchymal stem cells derived from human gingiva are capable of immunomodulatory functions and ameliorate inflammation-related tissue destruction in experimental colitis

The Journal of Immunology 2009; 183(12): 7787 - 98

58. Jin SH, Lee JE, Yun J-H, Kim I, Ko Y, Park JB

Isolation and characterization of human mesenchymal stem cells from gingival connective tissue

Journal of Periodontal Research 2015; 50: 461 – 467

59. Gregory CA, Singh H, Perry AS, Prockop DJ

The Wnt signaling inhibitor dickkopf-1 is required for reentry into the cell cycle of human adult stem cells from bone marrow

Journal of Biological Chemistry 2003; 278: 28067 - 78

60. Mollenhauer J, Bayreuther K

Donor age related changes in the morphology, growth potential and collagen biosynthesis in rat fibroblasts subpopulations in vitro

Differentiation 1986; 32: 165 - 72

61. Bayreuther K, Rodemann HP, Hommel R, Dittmann K, Albiez M, Francz PI

Human skin fibroblasts in vitro differentiate along a terminal cell lineage

Proceedings of the National Academy of Sciences USA 1988; 85: 5112 - 16

62. Shekar R, Ranganathan K

Phenotypic and growth characterization of human mesenchymal stem cells cultured from permanent and deciduous teeth

Indian Journal of Dental Research; 2012: 23(6)

63. Batouli S, Miura M, Brahim J, Tsutsui TW, Fisher LW, Gronthos S, Shi S

Comparison of stem cell mediated osteogenesis and dentinogenesis

Journal of Dental Research 2003; 82(12): 976 - 981

64. Mafi P, Hindocha S, Mafi R, Griffin M, Khan WS

Adult mesenchymal stem cells and cell surface characterization – a systematic review of the literature

The open orthopaedics journal 2011; 5: 253 - 60

65. Hynes K, Menicanin D, Han J, Marino V, Mrozik K, Gronthos S, Bartold PM

Mesenchymal stem cells from iPS cells facilitate periodontal regeneration

Journal of Dental Research 2013; 92(9): 833 - 839

66. Neeraj Malhotra, Kundabala Mala

Regenerative endodontics as a tissue engineering approach: Past, current and future

Australian Endodontic Journal 2012; 38: 137–148

67. Riekstina U, Cakstina I, Parfejevs V, Hoogduijn M, Jankovskis G, Muiznieks I, Ancans J

Embryonic stem cell marker expression pattern in human mesenchymal stem cells derived from bone marrow, adipose tissue, heart and dermis

Stem Cell Reviews and Reports 2009; 5(4): 378 - 386

68. **Karbanová J, Soukup T, Suchánek J, Pytlík R, Corbeil D, Mokrý J**

Characterization of dental pulp stem cells from impacted third molars cultured in low serum-containing medium

Cells organs tissues 2011; 193: 344 – 365

69. **Vidya L, Ranganthan K**

Stem cells of dental pulp

Indian Journal of Dental Research 2012; 23(4)

70. **Kerkis I, Kerkis A, Dozortsev D, Stukart-Parsons GC, Gomes Massironi SM, Pereira LV, Caplan AI, Cerruti HF**

Isolation and characterization of a population of immature dental pulp stem cells expressing OCT-4 and other embryonic stem cell markers

Cells Tissues Organs 2006; 184: 105 - 16

71. **Gang EJ, Bosnakovski D, Figueiredo CA, Visser JW, Perlingeiro RC**

SSEA - 4 identifies mesenchymal stem cells from bone marrow

Blood 2007; 109(4): 1743 - 1751

72. **Fukushima H, Kawanabe N, Murata S, Ishihara Y, Yanagita T, Balam TA, Yamashiro T**

SSEA-4 is a marker of human deciduous periodontal ligament stem cells

Journal of Dental Research 2012; 91(10): 955 – 960

73. Kawanabe N, Fukushima H, Ishihara Y, Yanagita T, Kurosaka H, Yamashiro T

Isolation and characterization of SSEA-4-positive subpopulation of human deciduous dental pulp cells

Clinical oral investigations 2015; 19(2): 363 - 371

74. Truong TT, Huynh K, Nakatsu MN, Deng SX

SSEA4 is a potential negative marker for the enrichment of human corneal epithelial stem/progenitor cells

Investigative ophthalmology & visual science 2011; 52(9): 6315

75. Brimble SN, Sherrer ES, Uhl EW, Wang E, Kelly S, Merrill AH, Robins AJ, Schulz TC

The cell surface glycosphingolipids SSEA-3 and SSEA-4 are not essential for human ESC pluripotency

Stem Cells 2007; 25: 54 – 62

76. Freshney I

Characterization, Culture of Animal Cells, A manual of basic technique

5th edition, John Wiley & Sons publication 2005; 247 – 80

77. Vasaki A

Immunocytochemical expression of Dentin Sialoprotein and von Kossa Staining Pattern of Mesenchymal Pulp Stem Cells from Permanent Teeth

Dissertation submitted to Tamil Nadu Dr. M. G. R Medical University, 2014

78. Soundarya S

Analysis of growth, phenotypic characteristics and expression of STRO1 and CD106, in dental pulp stem cells and stem cells from human exfoliated deciduous teeth – a cell culture study

Dissertation submitted to Tamil Nadu Dr. M. G. R Medical University, 2012

79. Takeda T, Tezuka Y, Horiuchi M, Honosa K, Tezuka K

Characterization of Dental Pulp Stem Cells of Human Tooth Germs

Journal of Dental Research 2008, 87(7): 676 - 68

80. Perry BC, Zhou D, Wu X, Yang FC, Byers MA, Chu TM, Hockema JJ, Woods EJ, Goebel WS

Collection, cryopreservation, and characterization of human dental pulp derived mesenchymal stem cells for banking and clinical use

Tissue Engineering Part C: Methods, 2008, 14: 149 – 56

81. Sivashankar V, Ranganathan K

Growth characteristics and expression of CD73 and CD146 in cells cultured from dental pulp

Journal of Investigative and Clinical Dentistry 2015; 0: 1 – 8

82. Gronthos S, Brahim J, li W, Fisher LW, Cherman N, Boyed A, DenBesten B, Robey PG, Shi S

Stem cell properties of human dental pulp stem cells

Journal of Dental Research 2002; 81: 531 - 535

83. Shi S, Gronthos S

Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp

Journal of Bone and Mineral Research 2003; 18: 696 - 704

Annexures

ANNEXURE I

PRIMARY ANTIBODY

abcam®
discover more

Anti-SSEA4 antibody [MC813] ab16287

★★★★ 12 Abreviews | 41 References | 6 Images

Overview

Product name	Anti-SSEA4 antibody [MC813]
Description	Mouse monoclonal [MC813] to SSEA4
Specificity	This antibody reacts with the Stage-specific embryonic antigen-4 (SSEA-4, a glycolipid carbohydrate epitope) that is expressed upon the surface of human teratocarcinoma stem cells (EC), human embryonic germ cells (EG) and human embryonic stem cells (ES). No immunoreactivity is evident with undifferentiated murine EC, ES and EG cells.Expression of SSEA-4 is down regulated following differentiation of human EC cells. In contrast, the differentiation of murine EC and ES cells may be accompanied by an increase in SSEA-4 expression.
Tested applications	ICC/IF, WB, Flow Cyt, IHC-FoFr
Species reactivity	**Reacts with:** Mouse, Rat, Human
Immunogen	Human embryonal carcinoma cell line 2102Ep.
General notes	The mouse myeloma was fused with a spleen from a Balb/c mouse.

Properties

Concentration	1.000 mg/ml
Form	Liquid
Storage instructions	Shipped at 4°C. Store at +4°C short term (1-2 weeks). Upon delivery aliquot. Store at -20°C or -80°C. Avoid freeze / thaw cycle.
Storage buffer	pH: 7.50 Preservative: 0.02% Sodium azide Constituents: 5.88% Sodium citrate, Tris HCl, 2.9% Sodium chloride Some batches of this product contain 0.4M Arginine as a stabilising agent. If you would like information about the formulation of a specific lot, please contact our scientific support team who will be happy to help.
Purity	IgG fraction
Clonality	Monoclonal
Clone number	MC813
Myeloma	Sp2/0
Isotype	IgG3
Light chain type	kappa

Applications

Our Abpromise guarantee covers the use of **ab16287** in the following tested applications.
The application notes include recommended starting dilutions; optimal dilutions/concentrations should be determined by the end user.

Application	Abreviews	Notes
ICC/IF	★★★★	Use a concentration of 15 µg/ml.
WB		Use at an assay dependent concentration.

ANNEXURE II

SECONDARY ANTIBODY

abcam®
discover more

Rabbit Anti-Mouse IgG H&L (HRP) ab6728

★★★★★ 3 Abreviews | 34 References | 4 Images

Overview

Product name	Rabbit Anti-Mouse IgG H&L (HRP)
Description	Rabbit polyclonal Secondary Antibody to Mouse IgG - H&L (HRP)
Target species	Mouse
Tested applications	Dot Blot, ELISA, IHC-P, IHC-Fr, Immunomicroscopy, ICC/IF, WB
Immunogen	Mouse IgG whole molecule
Conjugation	HRP

Properties

Concentration	2.000 mg/ml
Form	Liquid
Storage instructions	Shipped at 4°C. Store at +4°C.
Storage buffer	pH: 7.20 Preservative: 0.01% Gentamicin sulphate Constituents: 0.42% Potassium phosphate, 0.87% Sodium chloride, 1% BSA
Purity	IgG fraction
Purification notes	This product was prepared from monospecific antiserum by immunoaffinity chromatography using Mouse IgG coupled to agarose beads.
Conjugation notes	Horseradish Peroxidase (HRP)
Clonality	Polyclonal
Isotype	IgG
General notes	Many of our customers have reported seeing brown precipitates in the vials. The brown precipitates are very common with HRP conjugated antibodies; we suggest vortexing the vial and using this antibody as normal. Our customer's feedback says the antibody worked great. If in case the antibody fails to give results then please contact our Scientific Support team for assistance.

Applications

Our Abpromise guarantee covers the use of **ab6728** in the following tested applications.
The application notes include recommended starting dilutions; optimal dilutions/concentrations should be determined by the end user.

Application	Abreviews	Notes
Dot Blot		Use at an assay dependent dilution.
ELISA		1/1000.
IHC-P		Use at an assay dependent dilution.
IHC-Fr		Use at an assay dependent dilution.
Immunomicroscopy		Use at an assay dependent dilution.
ICC/IF		1/1000 - 1/5000.
WB	★★★★★	1/2000 - 1/10000. PubMed: 17200442

ANNEXURE III

RAGAS DENTAL COLLEGE HOSPITAL
(JAYA GROUP)
(Department of Oral & Maxillofacial Pathology)
2/102, East Coast Road, Uthandi, Chennai - 600 119, INDIA.
Tele : (044) 24530002-6-Exten. 33 Principal (Dir) 24530001 Fax : (044) 24530009

29.12.2015

TO WHOMSOEVER IT MAY CONCERN

From

Institutional Review Board,

Ragas Dental College and Hospital,

Uthandi, Chennai

The dissertation topic titled **'Expression of Stage Specific Embryonic Antigen - 4 (SSEA - 4), in dental pulp isolated from human permanent teeth – an Immunocytochemical study'** submitted by **Dr. V. Saranya** has been approved by the Institutional Review Board of Ragas Dental College and Hospital on 5th May 2014.

RAGAS DENTAL COLLEGE Chennai

Dr. S. Ramachandran, M.D.S.,
IRB, Secretary,
Head of the Institution
Ragas Dental College& Hospital
Chennai.

PRINCIPAL
RAGAS DENTAL COLLEGE AND HOSPITAL
UTHANDI, CHENNAI - 600 119.

ANNEXURE IV

DISSERTATION PROTOCOL

Title of the proposed research project

Expression of Stage Specific Embryonic Antigen-4 (SSEA-4), in dental pulp isolated from human permanent teeth – An Immunocytochemistry study

Name and designation of the principal investigator

V. SARANYA

I Year Post graduate student

Department of Oral and Maxillofacial Pathology

Name of HOD & staff in charge

Dr. Ranganathan. K (Professor and Head)

Dr. Uma Devi. K (Professor)

Dr. Elizabeth Joshua (Professor)

Dr. Rooban. T (Professor)

Department where project is to be carried out

Department of Oral and Maxillofacial Pathology,

Ragas Dental College,

Chennai

Duration of the project-

I year

Signature of principal investigator _____

Signature of Head of Department _____

Remarks of committee _____

Permission Granted YES / NO

Modifications / Comments _____

AIM

To isolate and characterize Mesenchymal Stem Cells (MSCs) from dental pulp in α- modified minimum essential medium (α-MEM) and to study the expression of Stage Specific Embryonic Antigen-4 (SSEA-4) in their 1st, 2nd and 3rd passages.

OBJECTIVE

1. To isolate and culture MSCs from permanent teeth using enzyme disaggregation technique in α-MEM growth medium.

2. To study the phenotypic and growth characteristics of cells isolated from the dental pulp of permanent teeth in the 2nd and 3rd passage of the culture respectively.

3. To study the population doubling time of cells isolated from the dental pulp of permanent teeth in the 3rd passage of the culture.

4. To study the expression of SSEA-4 in duplicate in the cells of 1st, 2nd and 3rd passages of the culture.

METHODOLOGY

PATIENT SELECTION

Healthy human third molars are to be obtained from the patients in the Department of Oral and Maxillofacial surgery, Ragas Dental College after obtaining informed consent from each subject.

SAMPLE SIZE

Pulp tissue specimens for culture is taken from freshly extracted third molar teeth following informed consent.

INCLUSION CRITERIA:

- Extracted third molars teeth extracted for orthodontic treatment, trauma.
- Freshly extracted third molars/premolars teeth immediately transferred to transport medium until pulp extirpation.
- Teeth with vital pulpal tissue

EXCLUSION CRITERIA:

- Teeth with evidence of decay or pulpal necrosis.
- Extracted third molars/premolars that have not been transferred to transport media immediately.

SPECIMEN COLLECTION

Permanent third molar with no evidence of decay or pulpal necrosis are collected immediately following extraction.

TRANSPORTATION OF THE SPECIMEN

α-MEM without serum at a pH of 7.2 to 7.4 with 2 x Antibiotics (Penicillin-100 IU, Streptomycin-100µg/ml) maintained at 4°C with ice pack is used to transport tissue specimens. They are transported in leak proof sterilized culture vials and the pulpal tissue is extirpated within 1 hour following collection.

PULP EXTIRPATION

- Tooth surface is cleaned well by washing three times with Phosphate Buffer Saline (PBS).

- Grooves are placed around the cemento-enamel junction with a tungsten carbide bur. The tooth is soaked intermittently in cold PBS to avoid heating while cutting.
- Tooth is split with chisel and mallet to reveal the pulp chamber.
- Separation of pulp tissue from pulp chamber is done with small fine forceps and spoon excavator.
- The pulp tissue is put into a on a Petri dish containing 2ml of Mesenchymal Stem Cell (MSC) medium (α-modified minimal essential medium (α-MEM) with 2 mM glutamine and supplemented with 15% fetal bovine serum (FBS), 0.1 mM l-ascorbic acid phosphate, 100 U/ml penicillin, and 100 µg/ml streptomycin) to avoid drying.

PRIMARY CULTURE OF DENTAL PULP CELLS:

- The dental pulp tissue is minced into tiny pieces with a surgical blade.
- The tissue is immersed into a mixed collagenase /dispase solution (3:1)
- It is incubated at 37^0C for up to 30-60 minutes and mixed well intermittently.
- After the digestion, the enzymes are inactivated by dilution in sufficient MSC medium.
- Cells centrifuged at 2500 rpm for 5 minutes.
- The supernatant is removed and the pellet re-suspended with MSC medium.
- The cells are counted (using a counting chamber) and then seeded into dishes at 1-10 X $10^3/cm^2$.(6-well plate)
- The cells are cultured in MSC medium at 37^0C and 5% CO_2 in the incubator.

- Attachment of the cells can be observed after 48 hours. Media change is to be done on every alternate day. Mono layer of cells can be observed within 2-3 week time.

SUBCULTURE

Usually around one week after the cell isolation, colonies are identified in the culture plates, where the cells have a typical fibroblast – like spindle shape. Before the cells become 100% confluent (usually after about 2-3 weeks), they are sub-cultured.

Immunocytochemistry:

Cells are fixed in APES coated slide and immunologically stained and evaluated for SSEA-4 expression.

ANNEXURE V

Consent Form

I……..…s/o, w/o, d/o……..……, aged about…….years, Hindu/Christian/Muslim …….……. residing at…….do hereby solemnly and state as follows.

I am the deponent herein; as such I am aware of the facts stated here under.

I state that I came to Ragas Dental College and Hospital, Chennai for my treatment for…….I was examined by Dr……..and I was requested to do the following tests.

1.
2.
3.

I was also informed and explained about the pros and cons of the treatment/test in the …… language known to me.

I was also informed and explained that the results of the individual test will not be revealed to the public. I give my consent after knowing full consequences of the dissertation/thesis/study and I undertake to cooperate with the doctor for the study.

I also assure that I shall come for each and every sitting without fail.

I also authorize the doctor to proceed with further treatment or any other/suitable/alternative method for the study.

I have given voluntary consent to undergo the treatment without any individual pressure or duress.

I am also aware that I am free to withdraw the consent given at any time during the study in writing

Signature of the Patient/Attendant

The patient was explained the procedure by me and he has understood the same and signed in
(English/Tamil/Hindi/Telugu/…….) before me.

Signature of the Doctor

ANNEXURE V

ஒப்புதல் படிவம்

நான் ……. வயது ….. இந்த ஒப்புதல் கடித்தை, இதில் அடங்கியிருக்கும் அனைத்து விஷயங்களையும் தெரிந்து, புரிந்துக்கொண்டு என் முழுசம்மதத்துடன் ஒப்புதல் அளிக்கிறோன். நான் சென்னை ராகாஸ் பல் மருத்துவக் கல்லூரி மற்றும் மருத்துவமனையில் பல் சிகிச்சை எடுத்துக் கொள்ளும் பொருட்டு வந்துள்ளேன்.

என்னை மருத்துவர் ……. பரிசோதனை செய்து பார்த்தார். மேலும் கீழ்க்கண்ட சோதனைகளை செய்து வருமாறு அறிவுறித்தினர். அவை,

1.
2.
3.

என்னிடம் மேற்கண்ட சிகிச்சை மற்றும் சோதனை செய்யும் போது ஏற்படும் பின் விளைவுகள் பற்றி எனக்கு …….. மொழியில் விளக்கம் அளித்தனர். என்னை தவிர வேறு யாரிடமும் இந்த சிகிச்சை மற்றும் சோதனைகள்/அதன் முடிவுகள் பற்றி சொல்ல மாட்டோம் என்று விளக்கினார்.

நான் இந்த ஆராய்ச்சி/படிப்பு/விளக்கவுரைக்கு மருத்துவருக்கு முழு ஒத்துழைப்பு அளிக்கிறேன். இந்த சோதனை மற்றும் சிகிச்சை பற்றிய விளைவுகளை நான் நன்கு அறிந்துகொண்டு என் முழுசம்மதத்தையும் அளிக்கிறேன். நான் சோதனை/சிகிச்சையின் தொடர் அமர்வுகளுக்கு தொடர்ந்து தவறாமல் வருவேன் என்று உறுதி சொல்கிறேன்.

என் சிகிச்சை காரணமாக செய்ய வேண்டிய மாற்று வழிமுறைகள் தொடர்ந்து அளிக்கும்படி மருத்துவமனைக்கு முழு அதிகாரம் அளிக்கிறேன். நான் இந்த சிகிச்சைக்கு என்னை உட்படுத்தி கொள்ள யாரும் என்னை கட்டாயப்படுத்தவோ அல்லது வேறு வகையில் வற்புறுத்தவோ இல்லை, நானே எனது சுய விருப்பத்தின் அடிப்படையில் சம்மதம் தெரிவிக்கிறேன்.

நான் சிகிச்சையின் பொருட்டு கொடுத்திருக்கும் சம்மதத்தை எந்த நேரத்திலும் எழுத்து மூலமாக தெரிவித்து திரும்பப் பெற்றுக் கொள்ளலாம் என்று எனக்கு தெரியும்.

நோயாளி/உடனிருப்பவரின் கையொப்பம்

நோயாளிக்கு நன்கு தெரிந்த …….. மொழியில் சிகிச்சையின் செய்முறைகள் பற்றி விவரித்த பின் அவர் நன்கு புரிந்து கொண்டு கையொப்பம் செய்தார்.

மருத்துவரின் கையொப்பம்

ANNEXURE VI

α-MEM	Alpha Modification Minimal Essential Medium
ABCG2	ATP – binding cassette sub family G member 2
ALP	Alkaline Phosphatase
APES	3-aminopropyl-triethoxy-silane
bFGF	Basic epidermal growth factor
BM	Bone Marrow
BMP	Bone Morphogenetic Protein
BMSC	Bone Marrow Stromal/Stem Cell
BSA	Bovine Serum Albumin
BSP	Bone Sialoprotein
BSP	Bone SialoProtein
CD	Cluster of Differentiation
CO_2	Carbon Dioxide
Col-I	Collagen type-I
DAB	3, 3’ -Diaminobenzidine
DEJ	Dentin Enamel Junction
DFSC	Dental Follicle Stem Cell
DPSC	Dental Pulp Stem Cell
EB	Embryoid bodies

ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetate
EGF	Epidermal growth factor
ELISA	Enzyme Linked Immunosorbent Assay
ESC	Embryonic Stem Cell
FBS	Fetal Bovine Serum
GMSC	Gingival mesenchymal stem cell
GSC	Gingival stem cell
hDPSC	human Dental Pulp Stem Cell
HEPA	High Efficiency Particulate Air filter
HRP	Horseradish Peroxidase
HSC	Haematopoietic Stem Cell
ICC	Immunocytochemistry
iPS	Induced Pluripotent Stem Cell
IRB	Institutional Review Board
ISC's	Intestinal Stem Cells
LESC	Limbal epithelial stem cell
LIF	Leukemia inhibitory factor
MMP	Matrix Metalloproteinase
MSC	Mesenchymal Stem/Stromal Cell
NCP	Non-Collagenous Protein
NSC's	Neural Stem Cells
NT – ESC	Nuclear transfer embryonic stem cell

Oct4	Octamer-Binding Transcription Factor 4
OSCC	Oral Squamous Cell Carcinoma
PBS	Phosphate Buffered Saline
PDGF	Platelet derived growth factor
PDL	Periodontal Ligament
PDLSC	Periodontal Ligament Stem Cell
PDT	Population Doubling Time
PFA	Paraformaldehyde
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SC	Stem cell(s)
SCAP	Stem Cell of the Apical Papillae
SCNT	Somatic cell nuclear transfer
SGZ	Sub granular zone
SHED	Stem Cells from the Human Exfoliated Deciduous Teeth
SPSS	Statistical Package for Social Science
SSEA	Stage-Specific Embryonic Antigen
SVZ	Sub ventricular zone
UV	Ultra Violet

ANNEXURE VII

DECLARATION OF PLAGIARISM CHECK

FROM

V. Saranya
III - Postgraduate student
Department of Oral and Maxillofacial Pathology
Ragas dental college and hospital
Chennai.

TO

The Head of the Department
Department of Oral and Maxillofacial Pathology
Ragas dental college and hospital
Chennai.

SUB: Declaration of plagiarism check of my dissertation to be submitted to "The Tamil Nadu Dr. M.G.R Medical University" – April 2016

I hereby declare that I have checked my dissertation for plagiarism using "writecheck"- plagiarism checker software on 29.12.2015 date for this dissertation. The unique content was 70% and the plagiarism content was 30%. The plagiarism content corresponds to definitions and terminologies that have to be quoted.

Yours sincerely,

V. Saranya

ANNEXURE VIII

DEPARTMENT DECLARATION FORM

The study title "**EXPRESSION OF STAGE SPECIFIC EMBRYONIC ANTIGEN-4 (SSEA-4), IN DENTAL PULP ISOLATED FROM HUMAN PERMANENT TEETH – AN IMMUNOCYTOCHEMICAL STUDY**" has been done under the guidance of the staffs of the Department of Oral Pathology and Microbiology during my post-graduation during 2013- 2016. The same has been submitted as a part of the syllabus MDS degree programme in Oral Pathology and Microbiology of the Tamil Nadu Dr. M.G.R. Medical University. I shall publish in full or part of this work in any media only with the prior written approval of the head of the department.

V. Saranya

V. Saranya

Post-graduation 2013-2016

Department of Oral and Maxillofacial Pathology