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DEMONSTRATION AND CHARACTERIZATION OF STEM CELLS IN PULP TISSUE OF HUMAN THIRD MOLAR TEETH – AN EX - VIVO STUDY

Dental Science

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ABSTRACT

Aims and Objective- Aim of the study was isolation, demonstration and characterization of stem cells from the pulp tissue derived from human third molar teeth that are extracted for therapeutic purposes.

Materials and methods- Study was carried out from December 2013 to August 2015 in the Department of Oral and Maxillofacial surgery, MGM Dental College and Hospital. 10 Samples were selected as per the inclusion and exclusion criteria and patients were explained about the study. Pulp tissue was retrieved from the samples and processed to achieve single cell suspension. Smears prepared from the single cell suspensions were processed for H and E stating and Immunocytochemistry (using Primary antibodies against CD44, CD133, Oct-4 and SSEA-4).

Results- Significant number of samples demonstrated stem cells by H and E staining. Significant numbers of cells were positive for immunocytochemistry (ICC) by CD44, CD133 and Oct-4; while the expression of SSEA-4 was not clinically significant. All the P values of <0.05 were considered clinically significant.

Conclusion- Human pulp tissue is a viable source of stem cells, which is easily accessible and from which these stem cells can be isolated with minimum infrastructure and minimum morbidity. Immunocytochemical studies confirm the presence and coexistence of Dental Pulp Mesenchymal Stem cells (DPMSC's) and Dental Pulp Pluripotent stem cells (DPPSC's) in the pulp tissue from human third molar teeth.

KEYWORDS

Third Molars, Stem cells, Dental Pulp, Immunocytochemistry

Introduction

Stem cells have the ability to self-renew and to generate mature differentiated cells.[1] Stem cells are the key subset of cells in the body, functioning as ancestor cells to produce a variety of types of functionally specialized mature cells (differentiation) in a given tissue, while at the same time maintaining the capacity to continuously divide and reproduce themselves (self renewal). Stem cell research has been hailed for the potential to revolutionize the future of medicine with the ability to regenerate damaged and diseased organ.[2] In addition to offering unprecedented hope in regenerative medicine, the research on stem cell may advance the understanding of basic normal biological processes like development, repair and regeneration and explain the abnormal phenomenon such as cancer.[3]

Amongst the myriad of human tissues in which these immortal cells have been demonstrated, the dental pulp is one of the most accessible, thus opening a range of new possibilities for regenerative medicine.[4] Stem cell therapy involves manipulation of the cells in vitro and using for therapeutic purposes. [5] The possible applications of stem cells are replacement and repair of damaged or lost tissues and organs. Isolation of stem cells is a basic key step for any research in the field of regenerative medicine. The aim of the study was isolation, demonstrat ion and characterization of stem cells from the pulp tissue derived from human third molar teeth extracted for therapeutic purposes.

Materials and methods

Study was carried out from December 2013 to August 2015 in the Department of Oral and Maxillofacial surgery, MGM Dental College and Hospital, India. This study was approved by local institutional review board of MGM dental college and hospital. 10 Samples were selected as per the inclusion and exclusion criteria and patients were explained about the study. Patients consent to participate in the study was obtained and they were subjected to investigations as deemed necessary for the selection criteria. The selected third molar teeth were extracted under local anaesthesia under strict aseptic conditions.

Samples were from patients ranging in the age of 18 - 50 years with completely formed maxillary and mandibular third molars which are indicated for extraction and can be removed in toto with minimal manipulation. Third molars were removed because they are impacted or not likely to erupt fully or they are non-functional or removed for orthodontic purposes and prosthetic purposes were included in this study. Medically compromised patients were excluded from the study. Teeth with pulpal and periapical pathology, pericoronitis and requiring sectioning for removal were also excluded.

Study was carried out under following objectives-

1. Isolation of stem cells from the human third molar pulp tissue. 2. Identification of stem cells based on morphological characteristics on Haematoxylin & Eosin (H and E) stained smears. 3. Characterization of types of stem cells derived from pulp tissue of human third molar teeth based on presence of cell surface markers by Immunoc ytochemistry (ICC).

Methodology involves following steps-

1. Retrieval of pulp tissue 6 - Immediately after extraction, the third molars were washed using 70% ethanol, followed by a wash with sterile distilled water and dried with gauze. A cut was made at cement-enamel junction of teeth with diamond disc (Fig.1) and teeth were fractured manually along the same line and pulp tissue was retrieved from the pulp chamber using a sterile endodontic K file no.15 (Fig.2) in a laminar flow hood. Tissue retrieved was stored and transported in a sterile container containing 2ml Phosphate buffer solution (PBS).

Figure1 Sectioning of a tooth using sterile diamond disc

Figure 2 Retrieval of pulp tissue using K file

2. Pulp tissue processing 6 - pulp tissue was transferred in fresh PBS solution in 1.5 ml of Eppendoff tube. Tissue was then placed in the centre of petri dish and was minced using surgical blade of 200 microns. Cellular separation was completed by digesting the divided pulp tissue with 1 ml of collagenase type I (3 mg/ml) for 30 minutes at 37°C in a water bath. Collagenase activity was inactivated by adding media with PBS upto 10 ml. The suspension was centrifuged for 10 minutes at 1000 rpm. The filtrate was collected in separate tube and again centrifuged at 3000 rpm for 5minutes.The precipitate was washed with PBS twice.
3. Preparation of smears- The precipitate was filtered through 40 micron strainer, and precipitate was loaded onto the slide to make

smears, each smear being of 5 microns. Smear was air dried and treated with 4% Para formaldehyde (PFA) for 15 minutes, followed by PBS wash. 2 washes of PBS each for 5 min were carried out. Slide was air dried and stored at 40C in a freezer. Prepared slides were then processed for Haematoxylin & Eosin staining and Immuno cytochemistry staining.

4. Haematoxylin and Eosin staining[7] (H &E) and immunocytoc hemistry staining using CD44, CD133, Oct-4 and SSEA-4 primary antibodies.
5. Immunocytochemistry staining (ICC)[7] - Heat- induced antigen retrieval was carried out using commercial microwave antigen retrieval system for 10 minutes at 800 watt, 10 minutes at 400 watt and 5 minutes at 320 watt and then washed in PBS saline. The smears were then treated with peroxidase consisting of 3% H_2O_2 in water for 5 minutes to block the endogenous peroxide activity. This was followed by a power block for 15 minutes to block any non- specific antigenic sites. Incubation for 45 minutes at room temperature with primary antibody, (CD-44, CD 133, OCT- 4, SSEA-4) was then carried out and washed with PBS. Smears were then subjected to a super enhancer for 10 minutes. Smears were then incubated in secondary antibody polymer – (Horse radish Peroxidase) HRP reagent for 20-25 minutes. Visualization was enhanced using freshly prepared DAB for about 7 minutes (depending upon acquisition of color intensity) and washed in PBS saline. The smears were then counterstained with Harris haematoxylin stain. Smears were then dehydrated in different grades of alcohol, cleared in xylene and mounted using DPX (mountant). Smears were then observed under a light microscope at 40 X magnification.

Results

The Data was entered into a MS-Excel worksheet and analysed using Statistical Software IBM SPSS 21.0. The data was presented using frequency and percentage followed by graphical presentation (pie chart). Further analysis was done using z-test for proportion. The level of significance was set at 5%. All p-values less than 0.05 were treated as significant.

Out of 10 selected samples derived from the pulp tissues of human third molars 8 samples showed presence of stem cells based on the morphological characteristics in H & E stained smears. P value is < 0.005, which is considered clinically significant. Immunocyto chemistry staining was carried out using CD44, CD133, Oct-4 and SSEA-4 markers. 7 samples were positive for the expression of cell surface marker CD44, 7 samples were positive for the expression of cell surface marker CD133, 7 samples were positive for nuclear and cytoplasmic expression of Oct-4. P value for results of CD44, CD133 and Oct-4 was <0.005, which was considered as clinically significant. Out of 10 samples 4 samples were positive for expression of SSEA-4. P value was >0.005; thus results for SSEA-4 expression were insignificant clinically.

Disccussion

The third molars are the most common source of dental stem cells, because wisdom tooth extraction is widely performed and the teeth are usually considered to be medical waste. Studies have shown that extracted non decayed impacted third molars are capable of producing an optimum quantity of dental pulp tissue for the isolation of DPSCs. Another positive point is that harvesting stem cells from this unavailing tissue after extraction causes no ethical controversy. 8 In this study we have isolated stem cells from the pulp of 10 third molar teeth from patients with age group of 24- 33 years.

Many methods have been tried to isolate pulp tissue from the extracted teeth, some studies used carbide burs [9]while some have used forceps to break the tooth, 10 others have used mini hammer to crack the teeth and have got access to the pulp. [11] Heating of pulp tissue and contamination are some of the disadvantages of rotary instrument. In this study diamond disc was used to create a furrow without reaching the pulp in order to reduce pulp heating. The tooth was fractured manually and an endodontic K-file no.15 was used to extirpate the pulp in the laminar flow hood.

Traditionally two methods have been employed for isolation of dental pulp stem cells; the enzyme digestion method, the explant outgrowth method. The explant method is based on outgrowth of cells from tissue fragments and subsequent adherence on a plastic surface. The enzyme digestion consists of sterile surgical removal of dental pulp, digestion in collagenase/dispase, characterization, and screening through the use of specific markers. In this study collagenase type I was used in a concentration of 3mg/ml of type I for the treatment of pulp tissue. The key to the success of the widely used explant method is the culture medium used and the culture conditions which are not yet standardised and may lead to genetic instability. Hence the enzymatic digestion method which provides single cell suspension with unaltered characterisation was chosen in this study. Phosphate buffer solution (PBS) was found to be an effective transport medium in this study. It is isotonic and non-toxic to most cells and was thus used for substance dilution.

In our study H&E staining revealed the presence of cells with dark stained nuclei surrounded by a thin rim of cytoplasm and were easily distinguished from the surrounding epithelial cells with pale nuclear staining which fulfilled the criteria of the presence of stem cells. The DPMSC's were larger in size and flatter while the DPPSC's were spherical and smaller in size (Figure.3) Out of 10 samples studied 8 samples showed presence of stem cells, the value of which is statistically significant ($P < 0.05$).

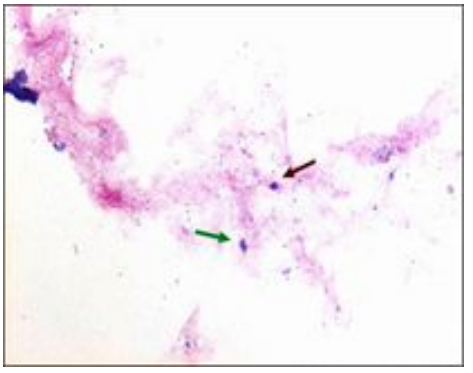

Figure 3 Photomicrograph of H and E Stained pulp smear from sample no. 1 showing DPPSCs (Red arrow), Somatic cell (Green arrow), [Haematoxylin and Eosin 400x]

and characterisation of stem cells. Cells need to be demonstrated by some definitive confirmatory techniques, such as Immunofluorescence (IF), Immunohistochemistry (IHC) /Immunocytochemistry (ICC), flow cytometry or RT- PCR.

This study demonstrated two different populations of stem cells by immunocytochemistry. CD44, CD105, CD90, CD73, CD271 and STRO-1 are typical markers for mesenchymal stem cells. In this study antibodies against CD44 and CD133 was used, which are specific for mesenchymal stem cells. Out of 10 samples, 7 samples were positive against CD44 and CD133 antibodies and suggestive of presence of mesenchymal stem cells in dental pulp (DPMSC's). It is found that there are two populations of cells one showing cytoplasmic expression and other showing nuclear expression of Oct-4. It is thought MSCs shows cytoplasmic expression of Oct-4 and Embryonic cells shows nuclear expression (Fig.4). 7 samples showed cytoplasmic (Fig.5) and nuclear expression of Oct-4, thus demonstrating MSCs as well as embryonic stem cells.

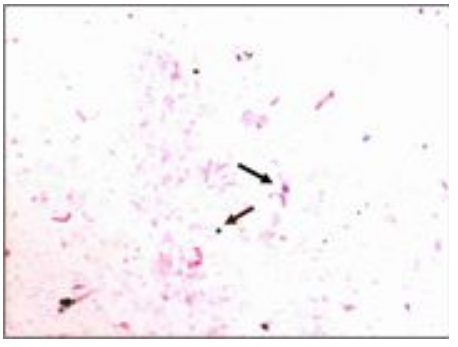

Figure 4 Photomicrograph of Oct-4 positive stained smear obtained from sample no.5 of pulp tissue showing nuclear expression of Oct-4 by Immunocytochemistry, suggestive of DPPSC's (400x)

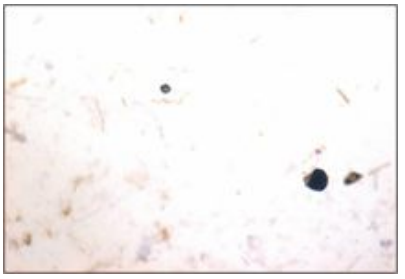

Figure 5 Photomicrograph of Oct-4 positive stained smear obtained from sample no.5 of pulp tissue showing cytoplasmic expression of Oct-4 by Immunocytochemistry, suggestive of DPMSC's (400x).

We have used primary antibodies against SSEA-4, 4 samples were positive (Fig.6).

The p value was not clinically significant thus further studies are required for the same. Expression of Oct-4 and SSEA-4 suggest pluripotent differential potential of stem cells. It is found that expression of Oct-4 decreases as the age advances and expression of SSEA-4 increases as the age advances. 7 Results of this study for expression of SSEA-4 are insignificant clinically; this can be attributed to the narrow age range of selected samples which was 24-33 years. Results of this study prove that DPPSC's and DPMSC's are coexistent as same pulp sample demonstrate presence of markers for DPPSC's and DPMSC's.

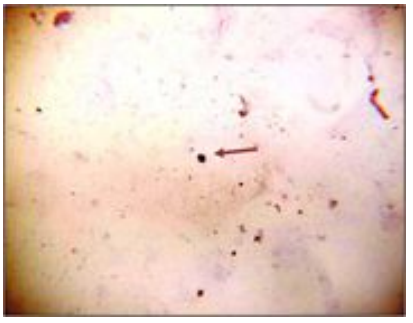

Figure 6 Photomicrograph of SSEA-4 positive stained smear obtained from sample no.5 of pulp tissue by Immunocytochemistry, suggestive of DPPSC's (400x).

Conclusion

Human pulp tissue is a viable source of stem cells, which is easily accessible and from which these stem cells can be isolated with minimum infrastructure and minimum morbidity. Morphological study showed two different populations of stem cells which are DPMSC's and DPPSC's. These cells characteristically differ from somatic cells, where in they have larger nucleo-cytoplasmic ratio with a darkly stained nucleus and a well defined shape. Amongst the two populations of stem cells DPPSC's are smaller in size than DPMSC's. DPPSC's are spherical in shape with minimal cytoplasm whereas DPMSC's are flatter.

On immunocytochemical studies dental pulp stem cells were positive for CD44, CD133, OCT-4 and SSEA-4. DPMSC's are positive for expression of CD44, CD133 and cytoplasmic expression of Oct-4 whereas DPPSC's shows positive expression of SSEA-4 and nuclear expression of Oct-4. Immunocytochemical studies confirm the presence and coexistence of DPMSC's and DPPSC's in the pulp tissue from human third molar teeth.

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