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Interaction of dental pulp stem cells in bone regeneration on titanium implant. An in vitro study

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ABSTRACT

Aim To investigate *in vitro* interaction of dental pulp stem cells (DPSCs) in bone regeneration on titanium implants.
Materials and methods Dental pulp tissue collected from extracted teeth without infection was used to obtain DPSCs. The biocompatibility of titanium implant was studied by culturing the DPSCs in conditioned media obtained by incubating the titanium implants for 72 hours. Following which the interaction of DPSCs on titanium implants was checked by wrapping the DPSCs cell sheet on SLA (sandblasted large grit acid etched) treated and smooth implants.
Results Cytotoxicity test revealed that the SLA treated implants were biocompatible and did not affect the proliferation rate of DPSCs. Osteogenic study with titanium implant revealed DPSCs have the capacity to undergo osteogenic differentiation in the presences of titanium implants.
Conclusions This study postulates that it is possible to construct a biologically modified implant by wrapping the dental stem cell sheet around commercially available implants in order to improve the process of osseointegration as the implant- stem cell complex contains all the factors required for osseointegration.

KEYWORDS Dental pulp stem cells, Dental follicular stem cells, Titanium implants.

INTRODUCTION

William Gregor discovered Titanium in the year 1791, one and half century after the discovery, the metal was found to be biocompatible in the biological environment. This phenomenon was termed as osseointegration coined by Per-Ingvar Brånemark in the year 1970 (1). Through the years several implant design, implant materials and several surface modification have been introduced to hasten the process of osseointegration (2). Implant material, type of cells, type of bone, cytokines and growth factors act in a coordinated manner to bring about healing of the osteotomy site. This clearly states that, apart from the type of implant material, the inherent regenerative potential of the surrounding bone is also responsible for the process of osseointegration (3). Bone is a dynamic tissue that experiences constant remodelling. When a dental implant is placed, it causes injury to the bone and requires a cascade of events to complete regeneration. Immediately after implant placement, local blood vessel growth allows the recruitment of migratory MSCs to the surgical site and the implant surface. These cells then proliferate and differentiate into mature osteoblasts responsible for bone matrix formation, which is essential for implant integration (4).
The most important factor in healing at bone-implant interface is the type of cell that has been attracted to the implant site, which is determined by the proteins that bound to the implant surface when it comes in contact with the body fluids. Such proteins in many cases involve cell binding components that will selectively bind to distinct receptors at the surface of the invading cells. Hence it should be feasible to modulate the responses with regards to cell infiltration by changing surface properties that only particular proteins will bind more selectively to the implant surface. Recently, biological molecules like "extracellular matrix components, designed peptides, and growth factors" were introduced onto implant surfaces to stimulate osteogenic cells in the early stages of implantation, to accelerate bone formation around the implants (6).
The extracellular matrix (ECM) not only serves as a

structural scaffold to house the cells, and support their adhesion but also plays a dynamic role in modulating cellular function such as differentiation, proliferation and migration1. Growth factors are bioactive proteins which control the process of wound healing. Growth factors have a critical role in cell migration, cell proliferation, and angiogenesis for tissue regeneration. These growth factors are present in blood, within platelet and in plasma. "Platelet concentrates such as platelet-rich plasma (PRP), platelet-rich fibrin (PRF), and concentrated growth factor (CGF) have been used for reconstruction of bony defects. They contain many growth factors including: platelet-derived growth factor (PDGF), transforming growth factor b (TGF-b), insulin-like growth factor (IGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and bone morphogenic proteins (BMP)" (7). Recently these platelet aggregates are being used at implant site owing to the fact that they accelerate wound healing and improve the process of osseointegration. The process of osseointegration cannot be improved by improving the surface property of the implant or by providing growth factor when the inherent regenerative capacity of the bone surrounding the implant is defective, particularly in case of osteoporotic and irradiated bone. When the bone quality is poor, then the number of mesenchymal stem cells (MSC) that will migrate to the osteotomy site will be limited and thereby delaying the healing time. In such situations stem cell therapy will be a feasible treatment option. Wei Zhou et al., conducted a study wherein they have used MSC-implant complex *in vitro* using partially mineralized and strong stem cell sheets under appropriate cell culture conditions. And they demonstrated that "MSC-implants possessing osteogenic and vascularization abilities can be produced using cell sheet engineering techniques in conjunction with routine implant materials", which provide a novel technique to modify the implant surface (5). MSCs are also found in dental tissues like dental pulp, dental follicle, apical papilla, gingiva and periodontal ligament. Several studies are being conducted to improve the process of osseointegration, of which most of the studies were carried out to improve the surface properties of the implant. Taking into consideration the importance of stem cells, ECM and growth factors in the process of osseointegration, it would be advantageous to develop a biologically modified implant surface by incorporating it with all these three components. In our present study we are developing a three-dimensional tissue scaffold for inducing bone formation at the implant surface using DPSCscell sheets, as it contains stem cells, growth factors and cellular derived ECM. ECM will also act as a niche for attracting more stem cells to the surgical site. This will potentiate the use of allogenic dental stem cells at the implant site in the near future.

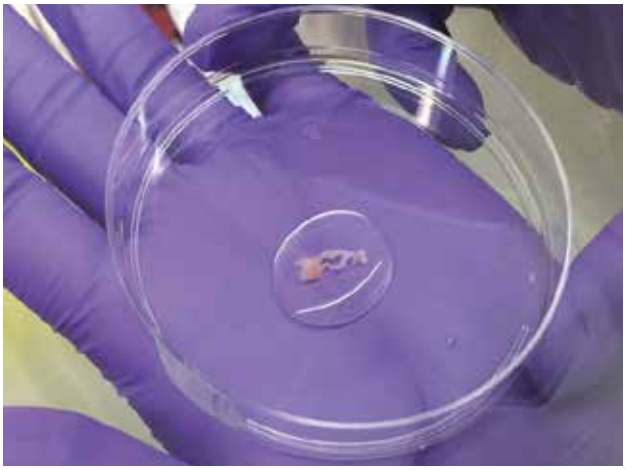
MATERIALS AND METHODS

Sample collection, storage and processing

Five freshly extracted teeth were collected and washed thrice with Dulbecco's phosphate buffered saline solution (DPBS). A standard endodontic access cavity was prepared on each tooth with Endo Access Bur (Dentsply) in high speed aerotor hand-piece with water coolant. Pulp was gently separated using a small size broach and transported to stem cell laboratory (NUCSReM) within 4 hours after the extraction in DPBS solution mixed with antibiotic penicillin and streptomycin (Pen-Strep, 1% solution, Gibco, Life) at 4°C in a sterile tube.

Establishment of primary culture

Under a sterile laminar flow cabinet (Thermo Fisher scientific class II), the pulp tissues were minced with a sterile scalpel (Fig. 1), and then digested using 0.1% collagenase Type I (Gibco, Invitrogen) for 1 hour at



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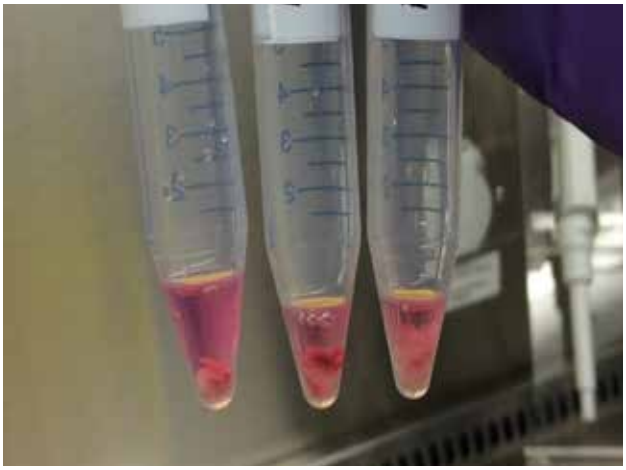

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FIG. 1 A Pulp tissue after removal from the freshly extracted teeth B Enzymatic digestion of pulp tissue.

37°C centrifugation at 1000 rpm for 5 min; the cells released from the digested tissue were passed through a 70 µM filter (Merck Millipore) and cell suspensions were seeded on plastic tissue culture dishes containing complete medium (Minimum Essential Medium-Alpha) supplemented with 20% fetal bovine serum (FBS, Gibco), 100 U/ml of penicillin and 100 µg/ml of streptomycin and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. Fresh MEM-Alpha with 20% FBS and 1.0% penicillin-streptomycin was changed once in three days of culture. When the culture reached 80% confluence, approximately after 14 days of incubation, cells were washed twice with DPBS, and harvested using 0.25% trypsin-ethylenediamine tetra acetic acid (EDTA) (Gibco). Complete medium was added to the solution containing cell suspension to neutralize the trypsin activity and were replated in 6-well plate. All the analyses for the basic characterization of cell cultures were initially carried out up to the fifth passage

Cell morphology analysis

Cell morphology was assessed at all initial passages using phase contrast microscope (Olympus, Japan). Cells were gently washed with DPBS to remove the cellular debris in culture suspension before photomicrography.

Cell viability

The cell viability was assessed for the successfully expanded cell line in triplicate using trypan blue dye exclusion (0.4%) to determine viability. Cell number was assessed by diluting in 0.4% trypan blue (Gibco, Invitrogen). The stained cells were viewed in a Neubauer haemocytometer with an inverted phase-contrast light microscope (Olympus), before cell counting and seeding into a tissue culture plate/flask. Cell viability was assessed at every passage (P1-P4).

Cell proliferation and population doubling time analysis

Expanded cells were analysed for cell proliferation and population doubling time (PDT). 10,000 cells were seeded in a 12-well culture plate (BD Falcon USA). The cells from each well were harvested on day three (3), six (6), nine (9) and twelve (12) (three), and counted with Neubauer hemocytometer under phase contrast microscope. The population doubling time (PDT) at that particular passage was determined using the formula PDT = t (log 2)/ (log Nt – log No), where t represent culture time, No and Nt represents the cell number before and after seeding, respectively. Proliferation rate and population doubling time were expressed in Mean± SD.

Colony forming unit (CFU) assay

After 14 days of culture, cells were fixed with 4% paraformaldehyde (Sigma, USA) and then stained with crystal violet (Sigma). Aggregates that contained 50 or more cells were counted as colonies under the microscope.

Flow cytometry analysis

Flow cytometry was performed on cultures from dental pulp tissue and in the 3-5 passages. A total number of 0.5×10^6 cells was incubated with antibodies against human clusters of differentiation (CDs) makers. DPSCs were analysed for the expression of MSC markers (CD 44, CD 90, STRO-1, and CD73) and absence of CD34 and CD45 using flow cytometry BD FACS (Calibur, Becton Dickinson, USA). At 80% confluence DPSCs were harvested and incubated with unconjugated CD73 (Bio legend, 1:100), Stro-1 (E-bioscience, 1:100), CD44 (Bio legend, 1:100), CD90 (Bio legend, 1:100) and CD34 (Biolegend, 1:100), CD45 (Bio legend, 1:100) were incubated for 1 hrs at 37°C. Following washes with cell staining buffer (Biolegend, USA), fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (E-bioscience, 1:100) was used as a secondary antibody and was incubated for 1 hour. Cell sorting was performed with a BD FACS calibur Flow Cytometer using CELLQuest Software (BD Biosciences, San Jose, CA, USA). IsotypeIgG was used as control to assess background fluorescence.

Osteogenic and adipogenic differentiation

For osteogenic differentiation, DPSCs were seeded in a 12-well plate at a density of 1.5×10^4 cells/well and cultured in maintenance medium consisting of MEM-Alpha with 10% FBS. When the cell monolayer reached 70% confluence, the medium was replaced with osteogenic differentiation medium. Osteogenic induction media consisted of MEM-Alpha, 10% FBS, 0.1 µM dexamethasone (Sigma, USA), 10 mM sodium β-glycerophosphate (Sigma) and 100 µM ascorbic acid (Sigma). The differentiation was performed for 21 days. Control cultures were maintained with basal medium. Both osteogenic induction medium and basal culture medium were changed 3 to 4 days in a week. Alizarin red S staining was carried out once calcified mineralised nodules were observed after three weeks of induction. The medium was removed and the cells were fixed with 70% ethanol (Sigma, v/v). The plates were rinsed three times with DPBS to remove non-adherent cells and stained with 40 mM. Alizarin Red S (Sigma, pH 4.2) for 10 to 30 min at room temperature. Excess dye was removed in case of over-staining by washing three times with DPBS. Cells were observed and images were captured using an inverted phase-contrast microscope (Olympus, Japan).

For adipogenic differentiation, an adipogenic differentiation medium consisting of MEM-Alpha, 10% FBS, 10 µM insulin (Sigma, USA), 200 µM indomethacin (Sigma), 500 µM isobutyl-methylxanthine (Sigma), and 1 µM dexamethasone (Sigma) were used. After differentiation the cells were stained with Oil red O solution. Oil red O stock solution was prepared by dissolving 0.5 g Oil Red O (Sigma) in isopropanol, which was diluted with deionized water at a 3:2 ratio,

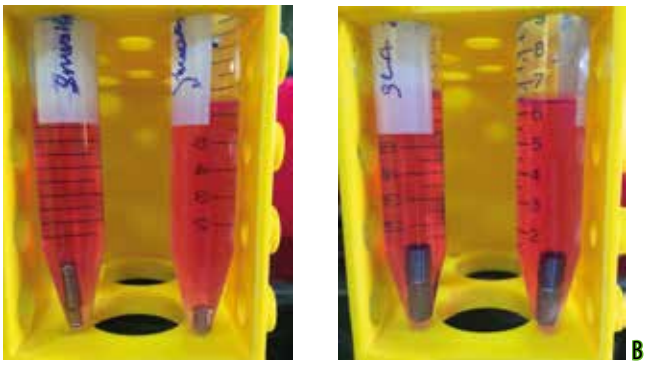


FIG. 2 Collection of Conditioned Media. A Incubation of Smooth implants (Noble pharma Branemark selftap implant of diameter 3.75mm and length 18mm and 8.5mm). B Incubation of SLA treated implant (MIS Seven Internal Hex implant of diameter 5mm and length 13mm with wide platform).

followed by filtration using filter paper. Cells were stained for 15 min at room temperature and excess dye was subsequently removed with 70% ethanol and DPBS. Cells were then observed and images were captured using an inverted phase-contrast microscope (Olympus, Japan).

Cytotoxicity study

The SLA treated implant (MIS Seven Internal Hex implant of diameter 5mm and length 13mm with wide platform) and smooth implants (Nobelpharma Branemark selftap implant of diameter 3.75mm and length 18mm and 8.5mm) were incubated in the standard culture media(MEM-Alpha) for 72 hours (Fig. 2). Post incubation the conditioned media was removed, filter sterilized and used to culture DPSCs. The proliferation rate of DPSCs at 24, 48 and 72 hours post-incubation in the conditioned media obtained from SLA treated and Smooth implant were calculated.

Osteogenic differentiation with titanium implants

Implants were grouped into two groups, a test group containing SLA treated implants (MIS Seven Internal Hex implant of diameter 5mm and length 13mm with wide platform) and a control group containing smooth implants (Nobelpharma Branemark selftap implant of diameter 3.75mm and length 18mm and 8.5mm).

Cell sheet preparation

Cell sheet were prepared, by incubating 15,000 cells of DPSCs in a 6 well plate and cultured till full confluence. The confluent cell culture at passage 4 was harvested and seeded to 100mm dish and cultured till full confluence. After reaching full confluence the DPSC cell sheet were scraped using a cell scraper and wrapped around the implant of test and control group. The implants in test group were grouped based on the type of media used, as group 1 containing osteogenic inducers and group 2 without osteogenic inducers. The SLA treated implant in group 1 was incubated in osteogenic induction media consisting of MEM-Alpha, 15% FBS, 0.1 µM dexamethasone, 10 mM sodium β-glycerophosphate and 100 µM ascorbic acid. The differentiation was performed for 21 days. The SLA treated implant in group 2 was incubated with basal medium. Both osteogenic induction medium and basal culture medium were changed every 3 days. The medium was removed and the cell sheet was fixed with 4% paraformaldehyde (Sigma, m/v) and stained with 40 mM Alizarin Red S for 10 to 30 min at room temperature. Excess dye was removed in case of over-staining by washing three times with DPBS.

The implants in the control group were again grouped based on the type of media used, as group 1 containing osteogenic inducers and group 2 without osteogenic inducers. Smooth implants in group 1 were incubated in osteogenic induction media. The differentiation was performed for 21 days. Smooth implants in group 2 were incubated with basal medium. Both osteogenic induction medium and basal culture medium were changed 3 to 4 days in a week. The medium was removed and the cells sheet was fixed with 4% paraformaldehyde and stained with 40 mM Alizarin Red for 10 to 30 min at room temperature. Excess dye was removed in case of over-staining by washing three times (Fig. 3).

RESULTS

In the present study MSC-like cells were isolated from dental pulp tissues. Of five samples, four samples were successful.

Enzymatic digestion

Cell suspensions were seeded on plastic tissue culture dishes containing a complete medium. Initially cells had round morphology and started proliferating after adhering to the plastic culture dish at 3-5 days. Primary culture reached 80% confluence on day 14 and the confluent cells had fibroblast-like morphology.

Cell morphology

Initial primary cell cultures of DPSCs were round in shape. After getting attached to the cell culture plate they started attaining fibroblast-like morphology. The cells began to proliferate after attaining spindle shaped with fibroblast-like morphology. After passage, the cells were attached to the bottom of the culture dish within 24 hrs and reached 90% confluence within 2 weeks of culture under standard experimental conditions. Sub-cultured cells gradually became flattened and they too acquired a fibroblast-like morphology exhibiting a more homogenous population of cells up to fifth passage.

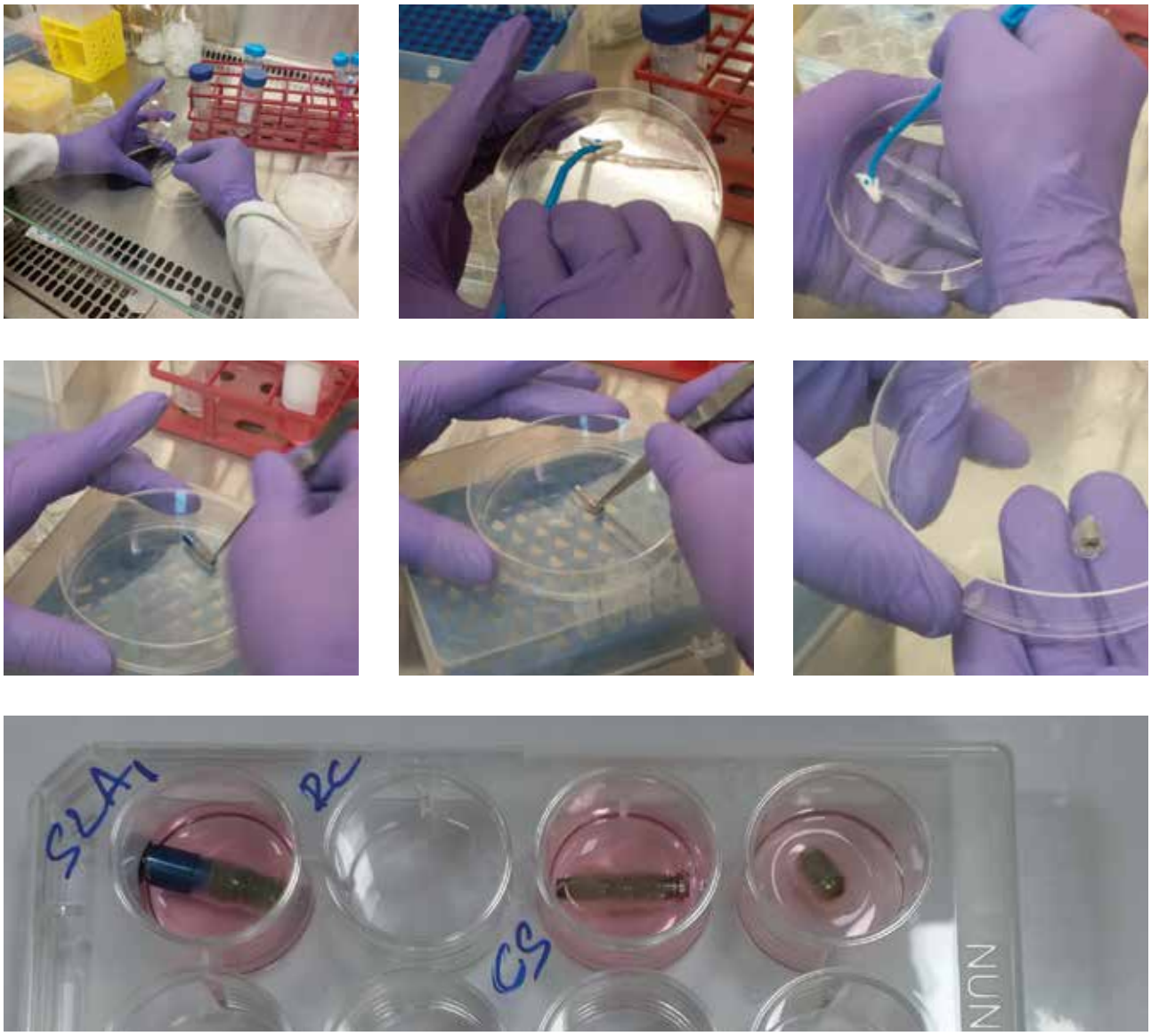


FIG. 3 Cell sheet preparation and wrapping of cell sheet around the implant.

Cell viability

DPSCs showed 85% viability at passage 1, 88% viability at passage 2. The percentage increased to 93% at passage 3 and 92% at passage 4.
Cell proliferation and population doubling time (CPDT) analysis: CPDT of DPSCs is 61.25±34.

Colony-forming assay

The colony-forming ability of DPSCs was conducted by culturing single-cell suspensions in plastic dishes for a period of 15 days. Cells were fixed in 4% paraformaldehyde and stained with crystal violet solution. Presence of colony aggregates that contained 50 or more cells were observed in both the groups.

Karyotyping

Karyotyping of freeze thawed DPSCs showed no chromosomal abnormalities at Passage 5 and 4 respectively.

Flow cytometry analysis

DPSCs showed positive expression of CD73 and CD44, whereas it showed higher level of expression of CD90. DPSCs showed very low expression of CD34 and CD45

Osteogenic differentiation

To test the osteogenic differentiation ability of DPSCs, the cells were cultured in osteogenic induction medium for three weeks. Alizarin red S staining of DPSCs cultured in osteogenic induction media showed formation of mineralized calcium nodule at day Alizarin red S staining of DPSCs cultured without osteogenic induction media showed formation of mineralized calcium nodule at day 21.

Adipogenic differentiation

To assess the adipogenic differentiation potential of DPSCs, the cells were cultured in adipogenic induction medium for three weeks. Oil red O staining of DPSCs cultured in adipogenic induction media showed the accumulation of neutral lipid globules or clusters at DA. DPSCs cultured without adipogenic media also showed accumulation of lipid globules at day 21.

Cytotoxicity test

There was no significance between proliferation rate of DPSC between SLA treated group and smooth surfaced implant groups.

Osteogenic differentiation with ti implants

The DPSC cell sheet was divided into test and control group. The test group was again divided into two group based on the type of induction media used as group 1 (with osteogenic inducers) and group 2 (without osteogenic inducers). The DPSC cell sheet obtained with the above mentioned method is wrapped around the implants of both test and control groups. The implants in group 1 of both test and control groups were incubated in osteogenic medium for 21 days and the implants in group 2 of both test and control groups were cultured only in basal media for 21 days. After 21 days the implants in test and control groups were stained using Alizarin red S stain. The implants in both the test and control group took up the stain (Fig. 4).

SEM analysis

The morphology of osteoblast cells grown on Ti surface were studied at SEM. The cells were actively adhered on the surface of the material which is depicted in figure 5. The sample 1 (SLA treated implants in the presence of osteogenic media) exhibited slimy monolayer of osteoblast cells. whereas in sample 2 (SIA treated implants without the presence of osteogenic media) the cells were fully grown on the material. Mass density of cells could be observed on the material. Sample 3 (smooth surfaced implant in the presence of osteogenic media) supports the formation of extracellular secretion along with cell attachment and proliferation. Nanofiber like collagen structures were observed on the material. The same phenomenon was observed in sample 4 (smooth surfaced without the presence of osteogenic media), it supports cell adhesion and extracellular secretion of the osteoblast cells.

DISCUSSION

Improving bone formation at the implant site is the current thrust area of research in the field of implant dentistry in order to downgrade the healing time. Surface modification of implants have been done since decades to increase the surface area and to improve the cellular response in order to hasten the process of osseointegration. Physical and chemical modification were done to improve the property of the titanium implants to enhance the process of osseointegration. In areas where the inherent quality of the bone is poor modifying the implant surface will no longer help in improving the osseointegration.

Recently, biological modification of implants is being done by coating the implant surface with bioactive molecules like ECM, peptides, etc to enhance the process of osseointegration in a poor quality bone (18). Cell therapy at the implant site was also done to improve bone formation. Shayesteh et al. (30) loaded *ex vivo* expanded MSC derived from the bone marrow onto the scaffold made of tri-calcium-phosphate (TCP) and used it during sinus elevation procedure. They postulated that bone marrow-derived stem cells have both angiogenic and osteogenic property, thereby accelerating the process of bone formation. Later it was found that the scaffold material induced a toxic effect on the loaded stem cells (30).

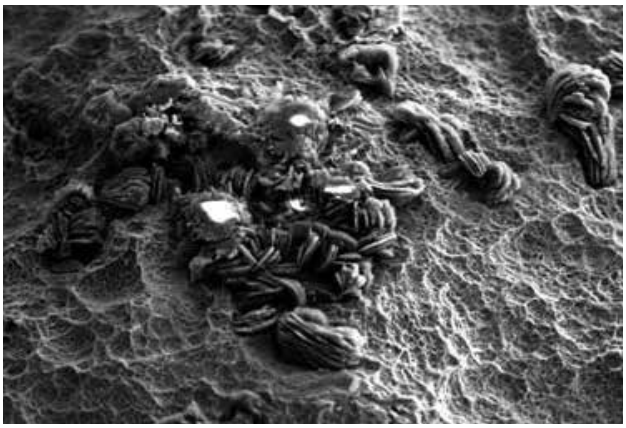
GROUP 1
(with osteogenic inducer)
MIS Seven Internal Hex Implant
(5 x 13 mm)

GROUP 2
(without osteogenic inducer)
MIS Seven Internal Hex Implant
(5 x 13 mm)

GROUP 3
(with osteogenic inducer)
Noble Pharma Branemark Implant
(3.75 x 18 mm)

GROUP 4
(without osteogenic inducer)
Noble Pharma Branemark Implant
(3.75 x 18 mm)

FIG. 4 Osteogenic differentiation study with Titanium.



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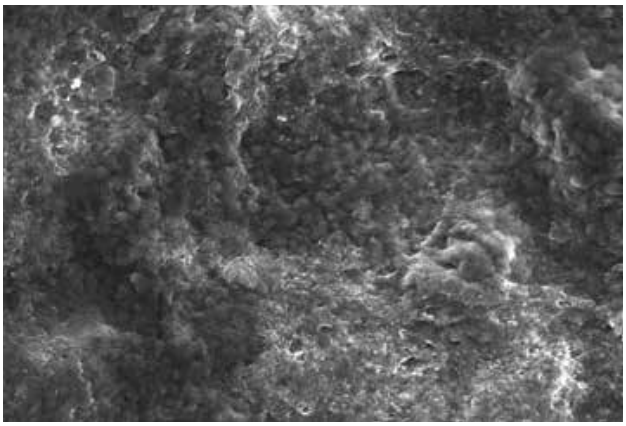

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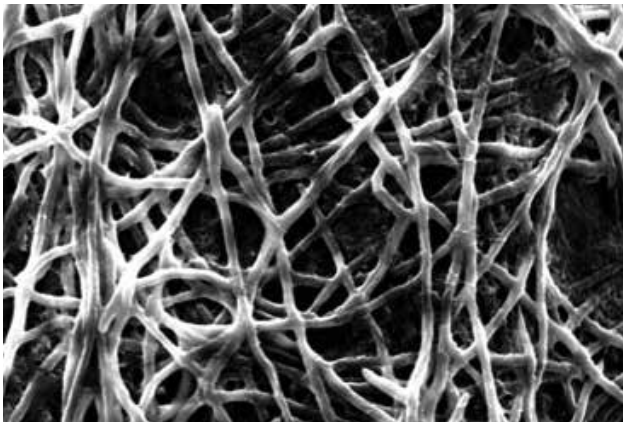

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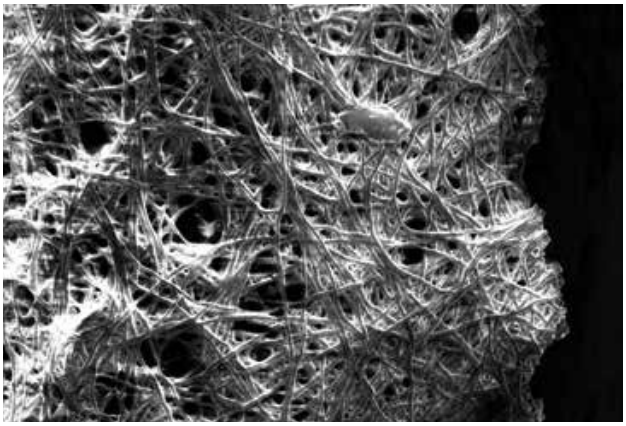

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FIG. 5 A Exhibits slimy monolayer of osteoblast cells(SLA treated group1) B The cells were fully grown on the material (SLA treated gropu 2) C Formation of extracellular secretion along with cell attachment and proliferation. Nanofiber structures were observed on the material.(smooth implant group 1) D Supports the cell adhesion and extracellular secretion of the osteoblast cells (smooth implant group 2).

Wei Zhou et al. (5) constructed a biologically modified implant by wrapping commercially available implants with the MSCs-cell sheet. He postulated that the cell sheet acted as a scaffold to hold the stem cells in place and also helps in maintaining the viability of the cells due to the presence of cellular derived ECM. ECM help in attracting the endogenous stem cells and also helps in enhancing the differentiation of migrated stem cells (1). Due to the additional less invasive procedure for sample collection in case of BM-MSCs, stem cells from dental tissue emanated as an alternative source for cell therapy. Jo et al. (31) found that the stem cells isolated from different dental tissues, such as dental pulp, dental follicle, apical papilla, showed MSCs-like properties and had the ability to differentiate into osteogenic, adipogenic and other kind of lineage with varying efficiency. Muraliramamoorthi et al. (32) conducted a systematic review and they concluded that the dental stem cells have the capacity to undergo osteogenic differentiation. Yusofa et al. (33) stated that DPSCs have angiogenic and osteogenic potential which is essential for bone formation. Naddeoa (34) stated that the implant surface has the ability to promote osteogenic differentiation of DPSCs.

This study aims at investigating the osteoinduction capacity of SLA treated implant using DPSCs. Apart from this, the present study also points towards investigating the interaction of DPSCscell sheet on the titanium implant surface. This supports the procedure of modifying the commercially available implants using dental stem cell sheets derived from dental tissues.

The primary culture of DPSCs showed presence of plastic adherent cells, with fibroblast-like morphology. The International Society for Cellular Therapy (ISCT-MSC) guideline proposes plastic adherent cells as one of the criteria to define the cell as MSCs.

DPSCs showed both osteogenic and adipogenic differentiation. The biological characterization of DPSCs suggests that the cell line retains MSCs properties. Cyotoxicity analysis, performed by culturing the DPSCs in the conditioned media, did not show any significant reduction in proliferation rates of DPSCs. This ascertained that the SLA treated implants did not show any toxic effect on DPSCs and are therefore considered biocompatible.

The interaction of titanium implant with DPSC is studied by wrapping it with cell sheet derived from DPSCs. After

culturing the DPSCscell sheet wrapped around titanium implants for 21 days, depending on the group they belonged, all implant-cell sheet complex were stained using alizarin red S stain. All the implant-stem cell complex took up the stain. Implant-stem cell complex cultured without osteogenic inducion media also took up the stain, this confirms the osteoinductive capacity of titanium implants. SEM analysis also revealed that the surface supports osteoblastic cell proliferation. Smooth surface favours comparatively more cell growth than SLA treated surface. Smooth surface also allowed for the formation of extracellular matrix when compared to SLA treated surface.

CONCLUSION

The present study aimed to investigate the interaction of DPSCs on titanium implants and the osteoinductive capacity of SLA-treated titanium implants reaching the following conclusions.

1. DPSCs exhibited MSC properties and maintained >90% of viability at passage 3-4.
2. Cytotoxicity study revealed that SLA treated implants are biocompatible and did not affect cellular proliferation.
3. Interaction of DPSC cell sheet on SLA treated implant surface revealed the osteoinduction capacity of SLA-treated titanium implants.
4. Also it is possible to create a biologically modified implant surface by wrapping up the dental stem cell sheet to the titanium implant.
5. Smooth surfaced implants favours more cell growth and extracellular matrix secretion onto their surface in comparison to SLA treated implant surface.

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