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Evaluation of Human Periodontal Ligament and Osteosarcoma Cell Attachment and Viability on Particulate Bone and Dentin Allografts

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Abstract

Purpose: *Various bone grafting substitutes have been used in the periodontics for bone regeneration which include autografts, allografts, xenografts and alloplasts. Autogenous particulate dentin has been used successfully as a bone grafting substitute. The aim of present study was to evaluate the effect of demineralized and mineralized freeze-dried bone allograft and particulate dentin on osteoblasts-like cells and human periodontal fibroblasts.*

Materials and methods: *Demineralized freeze-dried bone allograft (DFDBA) and freeze-dried bone allograft (FDBA) and ground dentin was used in the study. Particulate dentin was divided into four groups according to the size of the particles and demineralization - small dentin (particle size less than 200 µm), small dentin demineralized, large dentin (particle size 250-1200 µm), large dentin demineralized. Effect of all the specimens was checked on osteoblast-like cells (MG63) and human periodontal ligament cell lines. Percentage of surviving cells was measured using colorimetric MTT assay spectrophotometrically on 7th and 14th day of the cell culture. Scanning electron microscopy (SEM) was used to check the cellular attachment.*

Results: *Demineralized dentin matrix has shown significantly enhanced viable cell percentage for both the cell lines. DFDBA and demineralized dentin has reported comparable percentage of surviving cells. Dentin seems to be more compatible with osteoblasts-like cells than fibroblast. FDBA has shown the least favorable results. Cellular attachment for both the cell lines can be appreciated on SEM images.*

Conclusion: *Demineralized particulate dentin has reported considerable percentage of cell viability making it a reasonable option for bone grafting substitute.*

Introduction

Periodontal diseases are one of the prime reasons behind loss of alveolar bone and a resultant loss of teeth [1]. Regenerative periodontal therapy has revolutionized the field of dentistry. Various bone grafting materials are being used for the purpose of regeneration of alveolar bone which has been lost due to periodontitis. Autografts are the bone obtained from the same patient and implanted at the desired site. They are considered to be the gold standard because of lack of antigenicity and great osteogenic, osteoinductive and osteoconductive potential [2,3]. The use of autografts is limited because of the requirement of second surgical procedure, donor site morbidity, potential complications and risk of infection and limited availability. Allografts such as mineralized freeze-dried bone allografts (FDBA) and demineralized freeze-dried bone allografts (DFDBA) have shown positive results in cases of regenerative therapy and preservation of alveolar ridge [4,5]. Drawbacks of FDBA and DFDBA include patient acceptance, antigenicity, and the possibility of disease transmission. Other bone grafting substitutes are xenografts and synthetic bone grafting materials known as alloplasts. Osteogenic potential and osteoinductive properties of these graft materials are still in question [6].

Patients are becoming increasingly aware of dental procedures and have started considering the importance of natural dentition and many times they opt for dental implants to replace the missing teeth. It is essential to preserve the height and width of an alveolar ridge for future planning of dental implants. The quality and quantity of alveolar bone are important factors in determining physical and aesthetic success in implant therapy. Numerous bone grafting materials and techniques have been adopted to achieve the goal of regeneration, ridge augmentation, and ridge preservation.7

Many commercially available bone grafting materials have issues related to cost, making it difficult for several practitioners to use bone grafts in a day to day practice. Human dentin has similar composition as that of bone. Dentin is composed of 70% mineral, 20% organic matrix and 10% water by weight [8]. It contains bone morphogenetic proteins (BMPs) [9], insulin-like growth factors (IGFs) I and II and fibroblasts growth factor (FGF) [10]. Use of dentin as an autograft was first reported in 2003 for maxillary sinus augmentation in a clinical case report [11]. Various studies have shown positive results for bone regeneration when particulate dentin is used as a novel bone grafting substitute. Animal studies in which demineralized dentin is used as a xenograft have shown bone regeneration [12]. Autogenous demineralized dentin matrix (DDM) has been successfully used as bone grafting substitute in last decade [13]. Many times we come across the condition where extraction of teeth is indicated prior to full mouth rehabilitation. If we can use these teeth, which would be discarded, for ridge preservation and augmentation, that will be the most biocompatible, innovative and cost-effective option for bone grafting [14].

The aim of the present in vitro study is to check the effect of particulate dentin on human osteoblast-like cells (MG63) and periodontal fibroblasts growth culture and compare it with DFDBA and FDBA. Scanning electron microscopy and light microscopy is used for enhanced visualization.

Materials and methods

Preparation of study material

Commercially available demineralized freeze-dried bone allograft (DFDBA), freeze-dried bone allograft (FDBA) and powdered dentin were used in the study as study material. DFDBA and FDBA (Surgical EstheticsTM, Northridge, California) with particle size 250-1000 µm were used in the study. Particulate dentin was obtained from extracted teeth. Periodontally compromised, non-treatable, non-carious teeth and teeth which were indicated for extraction orthodontic purposes were used in the study. After extraction teeth were thoroughly cleaned and soft tissue attachment was removed using ultrasonic instrumentation. Teeth were decoronated using a diamond disc. The remaining portion of the teeth was ground using the Smart Dentin Grinder (KometaBio) to obtain dentin powder of particle size 200-1200 µm. Particulate dentin was subjected to a series of chemical treatment for proper cleansing and sterilization. Powdered dentin was washed using a cleansing solution i.e. 0.5 M of NaOH and 20% ethanol for 10 minutes. Excess solution was absorbed using gauze and cleansed dentin particles were washed in phosphate buffered saline (PBS) solution for 3 minutes. Half of the specimens underwent a process of partial demineralization. They were treated using 17% ethylene diamine tetra-acetic acid (EDTA) for 2 minutes. Demineralized dentin particles were washed with PBS three times for 3 minutes to remove excess EDTA. Particulate dentin then divided into four groups according to the size and mineralization of dentin i.e, small dentin (particle size less than 200 µm), small dentin demineralized, large dentin (particle size 250-1200 µm), large dentin demineralized.

Cell lines

Human periodontal ligament cell line and human osteosarcoma cell line (MG63) as a substitute for osteoblasts [15] was procured from National Centre for Cell Sciences, Pune, India. The cell lines were cultured in Dulbecco Modified Eagle Medium (DMEM) with low glucose (Cat No-11965-092). The medium was enriched with 10% heat-inactivated fetal calf serum (FBS) (Gibco, Invitrogen) Cat No -10270106 and 1% Antibiotic-Antimycotic 100X (Thermo Fisher Scientific) Cat No-15240062 solution.

Cell culture

Both the bone grafts and chemically treated ground dentin were weighed to obtain the study samples of 50 mg each using electronic precision balance. Eight specimens of each group of study material were used. Half of the samples were used to check cell activity by 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. From the remaining half samples, two sets of samples were used for light microscopy (Inverted microscope, Model-TCM 400, Magnification 20x, Labomed, USA) and two sets for scanning electron microscopy (SEM). They were aseptically transferred to 24 well plates in a laminar hood. The wells were named and numbered prior for proper identification and to avoid confusion. The study specimens were irradiated by UV rays for 30 minutes to assure proper sterilization. The cells (human periodontal ligament cell line and human osteosarcoma cell line - MG63) were seeded at a density of approximately 2×10^3cells/ well in a 24-well flat-bottom microplate and maintained at 37^0C in 95% humidity and 5% CO_2 for overnight. Control wells contained the same cell suspension except the study materials and underwent similar treatment as that of test groups. Transfer of graft materials into the wells and seeding of cells on the study samples were carried out inside the biological safety cabinet. The cells were incubated for 7 and 14 days and every alternate day fresh media was added. Bone grafts acted as a scaffold on which the cellular growth was analyzed.

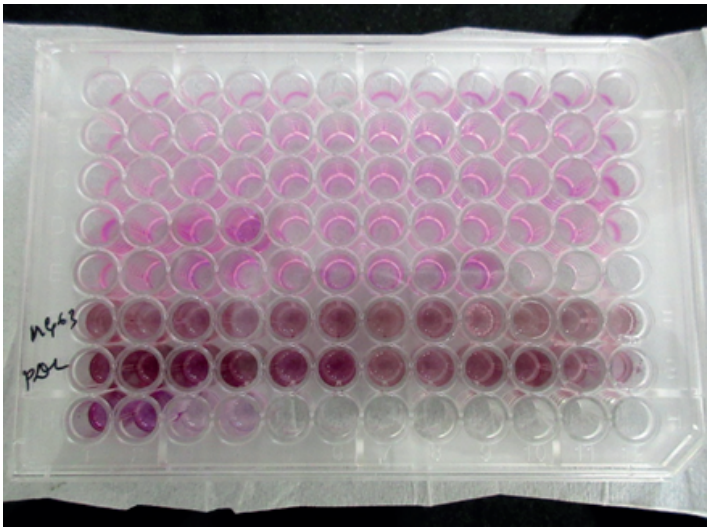

Figure 1.*Purple color of study cultures due to formation of formazan product*

Cell viability evaluation

Viability and survival of the seeded PDL cells and osteosarcoma cells were evaluated using yellow MTT assay. After incubation, specimens in the wells were washed twice with phosphate buffer solution and 20 µL of the MTT staining solution (5mg/ml in phosphate buffer solution) was added to each well. Specimens were incubated at 370C for four hours. Di-methyl sulfoxide (DMSO) 100 µL was added to each well to dissolve the formazan crystals, and absorbance was recorded with a 570 nm using microplate reader.

Principle of MTT assay: The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was the first homogeneous cell viability assay which determines the cell activation in the cell culture independent of cell proliferation. Viable cells with active metabolism convert MTT into a purple colored formazan product with an absorbance maximum near 570 nm (Figure 1). When cells die, they lose the ability to convert MTT into formazan, thus color formation serves as a useful and convenient marker of only the viable cells. Cell culture without any study material was considered as a normal control and their viability was standardized to 100% and readings from test groups compared with the control colorimetrically.

Percentage of viable cells was calculated by using the formula

Surviving cells (%) = (Mean OD of test compound/ Mean OD of Normal control) ×100 (OD- optical density) (Figure 1).

Specimen preparation for scanning electron microscopy (SEM)

Previously prepared two sets of specimens and cell cultures using the same procedure motioned above were used for SEM. But MTT assay was not performed on them. After incubation of 7 and 14 days, the cells were fixed to visualize them under the scanning electron microscope. Bone grafts with cultured cells were washed with PBS three times. They were fixed in 2.5% glutaraldehyde for 15-20 minutes. Specimens were washed using PBS three times and distilled water once. These treated samples were soaked for 5 minutes at room temperature consecutively in absolute ethanol (20%, 50%, 75%, 90%, and 100%) in distilled water. The specimens were observed under SEM after drying.

Result

MTT assay results

All the specimens which were used in the study had a particle size ranging from 250-1000 µm except for small dentin group which was less than 200 µm. The mean percentage of surviving cells was calculated using colorimetric MTT assay and it is described in table 1. Cell culture without any test material was used as normal control (NC) with a percentage of surviving cell standardized to 100%. None of the study materials has reported cytotoxicity. Demineralized dentin matrix has shown significantly positive results for both the cell lines (Figure 1). Dentin seems to be more compatible with osteoblasts-like cells than fibroblast cells. Highest readings were recorded with osteoblasts-like cell lines in case of demineralized small dentin particles. Demineralized dentin particles have shown a greater percentage of viable cells than non-demineralized specimens. Percentage of surviving cells decreased from 7th day to 14th day for osteoblasts-like cells for all the test materials except for partially demineralized dentin. No significant difference was reported even after 14 days for demineralized dentin (Table 1).

DFDBA displayed good compatibility with both the cell lines and intermediate results. Mean percentage of surviving cells was more than FDBA, large dentin particles, and mineralized small dentin specimens but less than demineralized small dentin specimens. FDBA has shown the least favorable results overall and significantly decreasing cell viability was reported at the time of subsequent readings. Although it is difficult to produce statistics due to limited sample size, a definite trend is clearly understandable. After analyzing the data, it seems that dentin was interacting positively with osteoblasts and not so with fibroblasts indicating differential interaction. The other substrates were indifferent to cells.

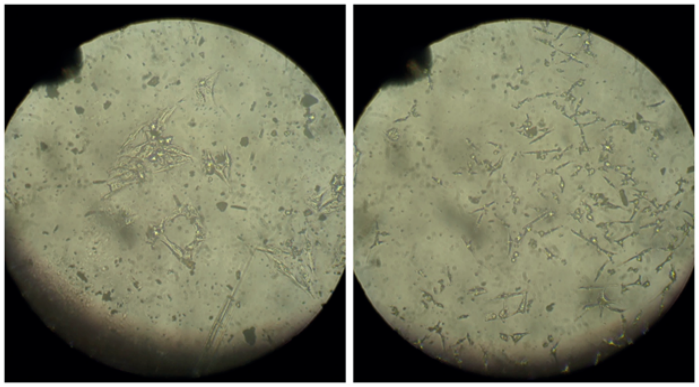

Figure 2. *Light microscopic images of osteoblast-like cells (right) and fibroblasts (left) seen in dentin group*

Light microscopic observations

Spindle-shaped morphology of both the cells lines could be appreciated in light microscopic images. Fibroblasts looked smaller and slender than osteoblasts with more cytoplasmic processes. Osteoblast-like cells were clustered together whereas fibroblasts appeared to be dispersed (Figure 2).

SEM observations

Partial demineralization has improved the surface structure and porosity of dentin specimens which can be clearly seen on SEM images (Figure 3). Noticeable organic matter indicative of clusters of cells was detected in dentin specimen groups. Microporosities and open dentinal tubules were seen in the images of demineralized dentin specimens (Figure 4). Higher magnification images of demineralized dentin particles have shown cellular attachment of both periodontal fibroblasts and osteoblast-like cells (Figure 5,6). Microcracks and Haversian system was seen in FDBA and DFDBA specimens along with dispersed organic matter (Figure 7). Osteoblasts like cells and periodontal fibroblasts attachment were visible on demineralized small dentin and DFDBA. Considerably less organic matter was detected in the FDBA group.

(To check the active cellular attachment and observe the viable cells we need to carry out environmental SEM and other attachment assays which was not possible for us due to unavailability of advanced laboratory support. The SEM used in this study was meant to observe the surface characteristics of the material. We had to fix the cells after 7th and 14th day of the culture for SEM. The process of fixation caused some alterations and most of the cells formed flat layer and got adhered to the base of wells and fewer cells got attached to study materials. We were not able to see morphological features of all the cells as they formed clumps. We carried out an extensive search to identify attached cells on the test materials).

Discussion

The aim of the present study was to analyze and compare the effect of demineralized freeze-dried bone allograft (DFDBA), freeze-dried bone allograft (FDBA) and ground dentin on osteoblasts-like cells (MG63) and periodontal fibroblasts in a growth culture. Colorimetric MTT assay was used to check the viability of cells. Only metabolically active cells can reduce MTT to solubilized formazan which was measured using spectrophotometer. Hence, the number of viable cells would be directly proportional to the

Table1.*Mean of percentage of surviving cells for osteoblasts-like cells and periodontal ligament fibroblasts cell cultures*

No	Test material	Particle size	Mean of active cellular activity %			
			MG63 – osteoblasts like cell line		Periodontal ligament fibroblasts cell line	
			7th day	14th day	7th day	14th day
	Normal control (NC)	-	100.00	100.00	100.00	100.00
1.	DFDBA	250-1000 µm	137	123	127	125
2.	FDBA	250-1000 µm	107	91	111	120
3.	Dentin large	220-1200 µm	128	117	103	105
4.	Dentin large demineralized	220-1200 µm	137	136	110	110
5.	Dentin small	<200 µm	146	120	110	111
6.	Dentin small demineralized	<200 µm	149	147	128	113

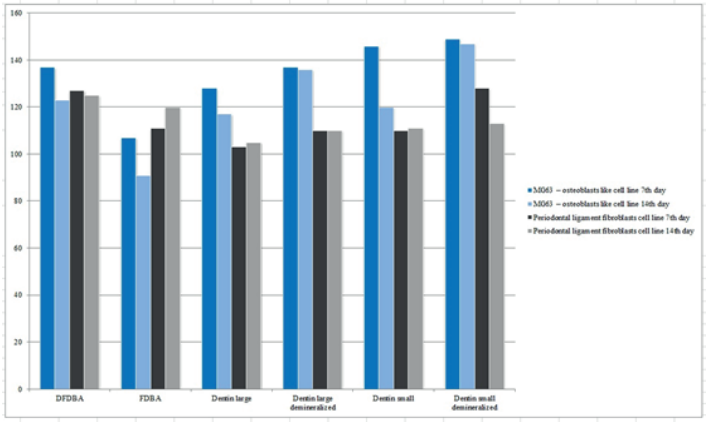

Figure 3. *Graph showing mean of percentage of surviving cells for osteoblasts-like cells and periodontal ligament fibroblasts cell cultures for all the test materials. (Normal control – 100%)*

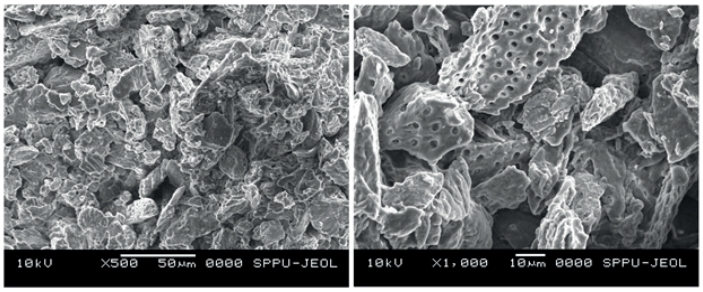

Figure 4. *Partially demineralized dentin showing open dentinal tubules, microporosities and cellular components at lower and higher magnification*

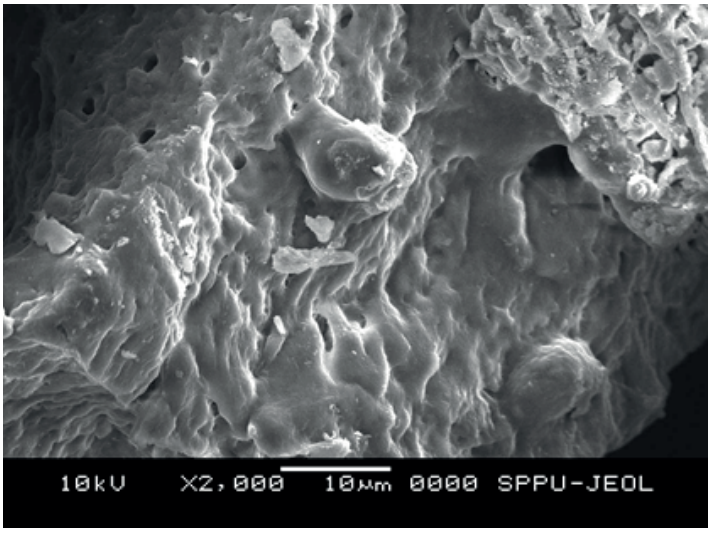

Figure 5. *Periodontal fibroblast attachment on demineralized small dentin*

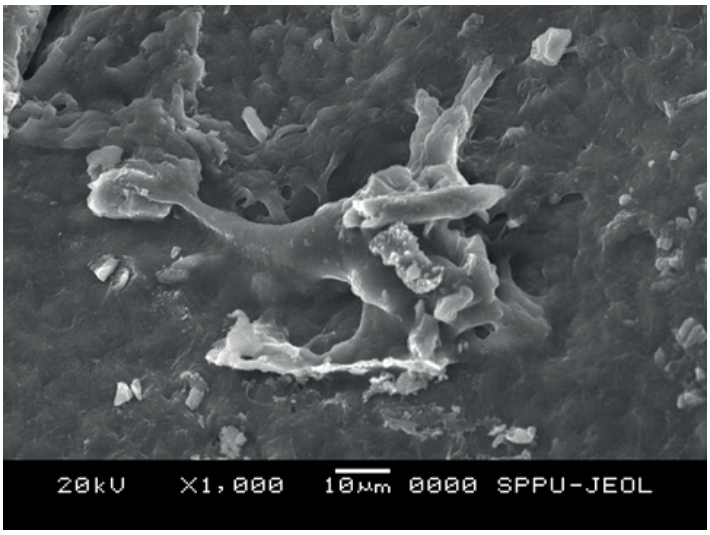

Figure 6. *Osteoblasts-like cells attachment on demineralized small dentin*

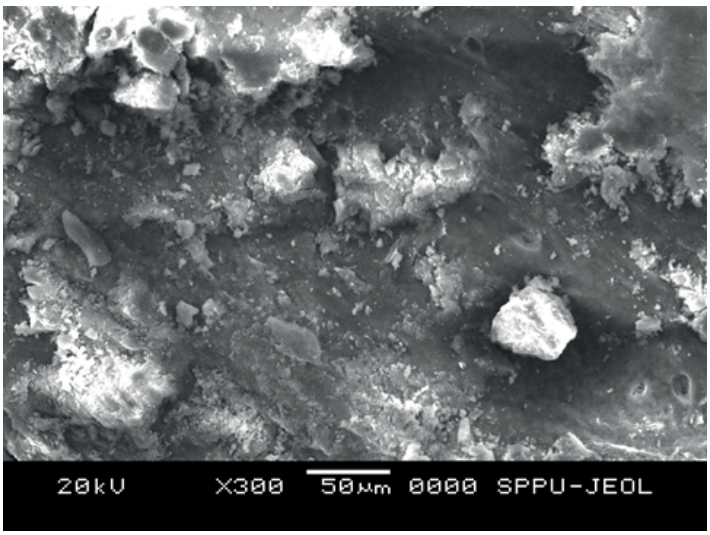

Figure 7. *Organic cellular matrix seen on the surface of DFDBA (MG63 cell line)*

intensity of the purple color obtained after the reduction reaction [16]. The results acquired from this study can be discussed according to the differences in particle sizes, type of graft material and presence or absence of demineralization. Overall results for all the test materials were favorable except mineralized bone allograft i.e. FDBA in which reduced percentage of viable cells was reported.

Human bone and dentin possess similar characteristics and composition. Dentin consists of 70% mineral, 20% organic matrix and 10% water by weight and 45%, 33% and 22% by volume, respectively. The organic portion of dentin mainly composed of type I collagen up to 95% and type III collagen [9]. Presence of bone morphogenetic proteins (BMPs) in dentin was first reported by Urist in 1965 [17]. Various growth factors including transforming growth factor (TGF)-β, insulin-like growth factors (IGF) I and II, platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) are reported in the dentin matrix [11]. Dentin also contains non-collagenous proteins such as osteocalcin, osteonectin and dentin phosphoprotein. BMPs [18] and growth factors are known to induce the osteogenic potential

in mesenchymal stem cells and contribute major role in the bone mineralization process [10]. The inorganic portion of dentin consists of hydroxyapatite crystals. Dentin is more phosphorylated than bone. These phosphophoryn are related to mineralization front. (Dentin phosphoprotein, or phosphophoryn, is one of three proteins formed from dentin sialophosphoprotein and is important in the regulation of mineralization of dentin) [19]. All these osteoinductive and osteoconductive properties of dentin make it a viable option for bone grafting substitutes [20].

The particle size of the bone graft material influences the effectiveness of the bone graft. It plays a major role in anchorage-dependent cell proliferation and differentiation. DFDBA and FDBA which were used in the study had a particle size of 250-1000 µm. Particulate dentin was divided into two groups according to particle sizes. Powdered dentin with a particle size of 250-1200 µm was categorized into large dentin particles and particles less than 200 µm into small dentin group. Studies have reported different results for the effective particle size of the graft materials. It was reported that smaller particle size (100-300 µm) enhanced bone formation for FDBA [21] while DFDBA showed inhibition of bone formation when used in the form of small particles [22]. Sampath and Reddi used smaller particle size (74-420 um) graft material and reported bone differentiation in surrounding area histologically [23].

In this current study DFDBA, FDBA and ground dentin have a similar range of particle size. A comparison of cell survival on these materials showed that DFDBA has consistently shown the highest percentage of surviving cells. The results were comparable with the study done by Mellonig and Levy in 1984 in which they had reported more osteogenic potential of DFDBA with a particle size between 250-1000 µm than particles below 250 µm [24]. The present study reported that FDBA has shown the least favorable results. The reason could be attributed to the larger particle size used in the study. In case of small and large dentin specimens, smaller size specimens reported more viable cells. Limited studies are available in which osteogenic potential of particulate dentin is compared according to the size of the particles. Optimum particle size will provide a beneficial pore size that will, in turn, allow proper cell proliferation, attachment, and ingrowth of blood vessels [25]. A recent study by Koga T. et al in 2017 has demonstrated the effect of degree of demineralization and particle size of dentin used as a bone graft on osseous regeneration [14]. Partially demineralized dentin with larger particle size i.e. 1000 µm had induced noticeably more bone regeneration than mineralized and smaller particle size dentin. It should be noted that results obtained from in vitro studies cannot be directly applied to in vivo interpretations. In vitro studies are conducted in a sterilized environment and the body's protective mechanisms are absent. On the contrary, in vivo studies on bone grafting particles have to interact with inflammatory cells and other mechanisms. Smaller particle size graft will be subjected to strong macrophage activity in vivo and disintegrated quickly.

Demineralization of bone allografts and particulate dentin has shown the enhanced osteoinductive potential of these materials. Demineralization causes exposure of BMPs and collagen fibers. Both have the capacity to induce bone regeneration. The only cell type which has the capacity to transform into another normal cell phenotype is mesenchymal fibroblasts. The fate of these connective tissue fibroblasts can be change to chondroblasts or osteoblasts by exposing them to demineralized tooth and bone [26]. Like many other studies, the present study has also demonstrated more favorable results with demineralized dentin and bone allografts than non-demineralized/mineralized specimens [14]. On the other hand, using fresh mineralized particulate dentin in extraction sockets of mongrel dogs did not enhance the bone regeneration [27]. A significant finding was reported in case of demineralized dentin group. All the test materials showed a decreased percentage of surviving cells for osteoblasts-like cells from 7th day to 14th day of the cell culture but not demineralized dentin group. Irrespective of the particle size, demineralized dentin showed a similar percentage of surviving cells on 7th as well as on 14th of the growth culture.

Demineralized dentin matrix has been successfully used as an autogenous bone grafting substitute for sinus floor augmentation, socket, and alveolar ridge preservation, guided bone regeneration [28]. Animal studies in which demineralized particulate dentin as used as an allogenic or xenogenic bone graft material have reported promising results analogous to autogenous graft [29].

Conclusion

The present study has provided an overview of the effect of DFDBA, FDBA and particulate dentin on osteoblasts-like cells and periodontal fibroblasts. Demineralized dentin matrix has a shown favorable effect on both the cell lines and enhanced cell viability. Demineralization and smaller particle size provided added advantage and have shown more favorable results for both the cell lines for particulate dentin. More *in vitro* and *in vivo* studies are required to obtain predictable outcomes using various particles sizes and degree of demineralization.

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Data availability statement

The observations of the present study are not published anywhere. The documents used to support the findings of this study are included in the references section in the form of authors' names, published article names and journal names. Previously published relevant data was used to support this study and is available in the journals. These prior studies (and datasets) are cited at relevant places within the text as references.

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