Introduction

One of the main challenges that are encountered in rehabilitating edentulous area is insufficient bone height or width. Several techniques for alveolar ridge augmentation have been introduced; however, with continual search for the most predictable regenerative outcome and least complications, the concept of tissue engineering was introduced. For successful tissue engineering, two important factors should be considered. First, engineering features related to biomechanical properties of the scaffold, architectural geometry, and space maintaining properties. Second, biological features related to the biological functions of the engineered matrix, including cell recruitment, permission of neovascularization, and delivery of the requisite growth factors for tissue regeneration. ¹

Regarding the engineering features, several materials have been used including bone grafts as a template to support cellular migration and space maintenance. ² Different types of bone grafts including autografts, allografts, and xenografts are available according to its source of origin. Earlier studies have showed that autogenous bone is the “gold standard” for bone augmentation procedures. However, shortcomings like donor-site morbidity, bone resorption, and high operative and hospital costs have urged the exploration of alternative materials, such as allografts, xenografts, and bone-mimetic synthetic materials. Allograft is a source of bone substitute of the same species that has been introduced mainly due to the complications associated with harvesting autogenous bone. Unlike the autografts, it lacks the osteogenic cells and consequently acts only as an osteoinductive and osteoconductive material. ³

Xenograft is a source of bone substitute from another species such as coral, bovine, and equine. During the processing of xenografts, organic components such as cells and proteinaceous materials are removed to produce an inert absorbable bone scaffold, which assists in revascularization, osteoblast migration, and new bone formation, and so it mainly act as an osteoconductive material only. ³ ⁴

Regarding the biological features of tissue engineering, mesenchymal stem cells (MSCs) are considered proper candidates due to their extensive expansion rate and its potential to differentiate into different types of cells. Of these MSCs, periodontal ligament stem cells are now considered as a more appropriate cell
type for developing novel periodontal tissue engineering strategies, as it displays high expression of runt-related transcription factor 2 (RUNX-2) and alkaline phosphatase which are closely associated with the differentiation into hard tissue-forming cells, and it also has the ability to differentiate into clonogenic colonies that are self-renewable and have the ability to produce a range of dental and non-dental tissues, including cementoblast-like cells, adipocytes and collagen-forming cells. 5

Proliferation and differentiation of human periodontal ligament stem cells (hPDLSCs) is also affected by the surrounding microenvironment that will direct the process toward a specific differentiation pathway. One culturing medium for the periodontal ligament stem cells is the regular MSC medium. The main characteristic component in it is the fetal bovine serum (FBS) which plays a major role in facilitating cell attachment to culture dishes, cell growth, and cytodifferentiation. 6 Another culture medium is the osteogenic medium, which is supplemented by dexamethasone. Dexamethasone stimulates the RUNX-2 which is associated with bone formation and regulation of osteoblast differentiation. 7 Knowing that there are different types of culturing medium for periodontal ligament stem cells (PDLSCs), the choice of the best scaffold culture medium remains a major issue to imitate the extracellular matrix architecture and biological functions. The aim of this study is to evaluate the ability of osteogenic culture media in comparison with regular culture media in enhancing the osteoblastic cell differentiation of hPDLSCs through the use of confocal laser microscopy to examine cell adhesion, intercellular continuity, and cytoskeletal development and further with the use of scanning electron microscopy (SEM) to assess the cellular morphology of the newly formed bone cells.

Materials and Methods

Ethical Guidelines
This study was conducted in accordance with protocols of King Saud University.

Approval of the Ethical Committee was obtained from College of Dentistry Research Center and the Institutional Review Board.

Work protocol of the study was conducted using facility and support of “Molecular and Cell Biology” Laboratory at the College of Dentistry, King Saud University, Riyadh, Saudi Arabia.

Scaffold Material
The scaffold material used was xenograft OsteoBiol SP-block Norm (Tecnoss Dental, Coazze TO, Italy), an equine bone block graft that combines the mineral properties of cancellous particulate with the space maintenance of collagen providing a convenient approach to filling periodontal and maxillofacial defects. All samples were unified in a dimension of 5 × 5 × 5 mm.

Cell Culture
Human periodontal ligament stem cell line was supplied from Sciencell Company, isolated from human periodontal tissue, cryopreserved, and delivered frozen in vial containing >5 × 10^6 cells in 1 mL volume. Human periodontal ligament stem cells were cultured at passage 4 in regular growth media supplied with FBS and placed in a T-75 flask; cells were allowed to grow, and the media was changed every other day until it reached a confluency of 85% within 14 days of culture.

After that, cell counting was done using a hemocytometer to ensure that cell proliferation and growth has reached the desired confluency. Subsequently, PDLSCs were seeded with an amount of 250,000 of cells on each block in accordance with the following groups:

Reg-group where PDLSCs were cultured with a xenograft block in regular growth media; Osteo-group where PDLSCs were cultured with a xenograft block in osteogenic media.

In each group, six blocks of xenograft bone blocks were cultured with PDLSC in either type of the growth media (Fig. 1). For osteogenic induction of osteo-group, cells were maintained in regular growth media for 24 hours and then it was replaced by an osteogenic media. All samples were cultured in a 48-well plate, one block in each well supplemented by 500 mL of the assigned culture media which was changed every other day until the day of assessment. Subsequently, all samples were imaged through confocal laser microscope as a whole block at 21 days, after that the blocks were sectioned into thin sections to be assessed by the SEM.

Analysis

PDLSCs Sustainability and Adhesion

Confocal laser scanning microscope: To stain the samples, the culture media was discarded and cells were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate-buffered saline (PBS). Afterward, permeabilization was done through adding 0.5% Triton X-100 for 10 minutes and then blocking with skimmed milk for 30 minutes. Primary monoclonal antibody to vinculin (Acris, Rockville, MD, USA) was added and incubated overnight at 4°C and then material was discarded and samples were washed with PBS three times. That was followed by incubation with the secondary antibody, Alexa Fluor 488 goat anti-mouse IgG1 (Molecular Probes; Invitrogen, Eugene, OR, USA) for 1 hour at room temperature to stain the focal adhesions of vinculin and then incubated with Alexa Fluor 594 phalloidin (Molecular Probes) also for 1 hour at room temperature to mark the actin cytoskeleton. After that, samples were washed three times with PBS and imaged with Nikon C2 Confocal Microscope system consuming an argon laser beam with excitation lines at 488 nm and a helium–neon source.

Morphological Assessment

SEM: Preparation of the samples for SEM analysis started by washing away any proteinaceous fluid. After that, samples were fixed in...
2.5% glutaraldehyde for 48 hours in room temperature, dried in increasing ethanol concentrations, and then critical point-dried to ensure that the samples are free of any volatile solvents. They were then mounted on aluminum stubs and gold coated to visualize the shape of the cells; the most important thing to consider during gold sputter coating is to start with a short burst of coating lasting only one or a few seconds at a low coating current and allow the sample surface to cool again before continuing with the main coating operation.

RESULTS

Confocal Laser Microscopy

Confocal images showed positive representations of osteogenic differentiation by identifying the actin cytoskeleton in red color and the focal adhesions of vinculin in green color and confirming cellular adhesion on the bone blocks through intercellular continuity and cytoskeletal development. The green fluorescent signals outlined the scaffolds’ margin; however, these signals were more intense in the groups cultured in osteogenic media and showed even more spreading of cells in comparison with groups cultured in regular growth media. In fact, in the osteogenic media group, the samples displayed even more yellow color that signify the overlapping of high intensities of both green and red signals, but predominantly green color indicating superior osteogenic differentiation (Fig. 2).

Scanning Electron Microscopy

At first, imaging of both types of scaffolds was done prior to culture, to show its topographical morphology. The images reveal the porous architecture of the scaffold. Results shows that cultured hPDLSC had grown within the scaffolds as illustrated by the cell colonies that are formed and adhered to the scaffolds. The morphological characteristics of the newly formed cells show a flat, spread-out, and polygonal appearance with extending cytoplasmic processes indicating their osteoblastic differentiation. These processes were closely linked with the pores of the scaffold indicating the biocompatibility between the cells and the scaffold and the positive anchorage of the cells on the scaffold. It was noticed that cells growth and infiltration was more pronounced in the samples cultured in osteogenic media compared with the samples cultured in regular growth media, as the cells were more coalesced with higher expression of extending cytoplasmic processes that increased the cell-to-cell contact and cellular bridging covering almost the majority of the scaffolds’ surface area (Fig. 3).

DISCUSSION

In an attempt to find the best bone-tissue engineering model, the classic paradigm suggests three main components: biocompatible scaffold, cells, and signaling molecules. The acquisition of scaffolds to supply stem cells with a media that mimics the microenvironment is an attractive alternative for the use of a combination of cytokines and growth factors all in one construct.3 The aim of this study was to evaluate the ability of osteogenic culture media in comparison with regular culture media in enhancing the osteoblastic cell differentiation of hPDLSCs. In this study, xenograft bone was used as a scaffold and carrier for these stem cells in all groups. In the present study, all group samples were evaluated by confocal laser microscopy, and all images showed fluorescent signals positive for new bone cells. The images illustrated green signals that outlined the scaffold margin indicating the focal adhesion of vinculin and red signals indicative for the actin cytoskeleton. Boyde has used confocal images as an assessment method for evaluating the expression of actin and focal adhesion of vinculin, which are critical proteins for cell attachment and sustainability; these images give indications for intercellular continuity, cytoskeletal development, adhesion properties, and potential damage.9 The green fluorescent marking on the images represents the focal adhesions of vinculin, which are membrane-associated complexes that serve as nucleation sites for actin filaments, and it links between the cell exterior and the actin cytoskeleton inside.10 The accumulation of green fluorescence signals has been reported to be a positive sign for the anchoring vinculin junction that binds the new bone cells to the material, and it is also indicative for the osteogenic nature of the differentiated cells and their adhesion.11,12

Our study findings showed that hPDLSCs cultured in osteogenic media demonstrated superior osteogenic differentiation in comparison with those cultured in regular growth media. Superior osteogenic differentiation was shown by displaying more yellow florescent signals indicating the plausibility of the culturing conditions and the osteogenic culturing media used. Superior osteogenesis in the osteogenic media groups could be attributed to

Figs 2A and B: Confocal laser microscopy images: (A) 21 days of cultured hPDLSC in regular growth media; (B) 21 days of cultured hPDLSC in osteogenic media
the presence of dexamethasone content, as it was confirmed to be the upstream regulator of the RUNX-2, an essential gene responsible for stimulating proper differentiation along the osteoblast lineage; however, its level must be tightly regulated to keep it committed to this desired lineage and the presence of dexamethasone insures for appropriate regulation.

It is important to emphasize some characteristics of the differentiated cells in this study that confirm the osteogenic differentiation due to the use of osteogenic growth media. That was shown on the SEM images, where all samples displayed evidence of flat, spread-out, and polygonal cells with extending cytoplasmic processes and filopodia that connects between the newly formed cells and the scaffold indicating a positive osteoblastic differentiation. Matsuoka et al. have showed that cell geometry is highly correlated with the differentiation into osteogenic lineages making the SEM images a popular method for assessment of cellular morphology. The osteoblastic characteristics in our findings are consistent with previous evidence, where seeded bone marrow stem cells on different scaffold materials and cultured in osteogenic media have displayed the differentiated cells as a flattened polygonal shape with an intercellular bridging and anchorage through extensions of cytoplasmic process and filopodia.

Superior formation of new bone cells was evident with samples cultured in osteogenic culture media and after a time period of 21 days signifying the advantage of the osteogenic media. This superior advantage is related to the dexamethasone content in the media, where it has been shown that bone-like formations process increased in a timely fashion in cultures supplemented by dexamethasone. Although other studies show no difference in the osteogenic process with the use of dexamethasone in cultures, that could be attributed to the concentrations of the dexamethasone used, other contents in the media, or even other factors that affect the culture media like the temperature used. This study was limited to investigating the osteogenic differentiation under the effect of only one concentration of dexamethasone in osteogenic media, and so further studies are needed to investigate the effect of the different dexamethasone concentrations in culture growth media and other factors on the osteogenic potential of hPDLSCs.

**Conclusion**

Xenograft bone blocks are biocompatible scaffold for the osteogenic differentiation of seeded hPDLSCs. Osteogenic culture media enhances and increases the osteogenic differentiation of hPDLSCs into new bone cells more than regular growth culture media. Periodontal ligament stem cells are a predictable biological input as a cell-based tissue-engineered construct and biologically acceptable when it is cultured in a suitable growth media that mimics the intended environment.

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