TWO COLORIMETRIC ASSAYS VERIFY THAT CALCIUM SULFATE PROMOTES PROLIFERATING ACTIVITY OF HUMAN GINGIVAL FIBROBLASTS

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Statement of problem. The role of calcium sulfate in stimulating the growth of gingival soft tissue has been reported in few studies. Such a unique property of calcium sulfate could serve as a trouble-solving broker in compensating for the lack of soft tissues in various oral surgeries.

Purpose. The purpose of this study was to compare the proliferating activities of human gingival fibroblasts seeded on various bone graft barrier materials of calcium sulfate, collagen, and polytetrafluorethylene (PTFE).

Material and methods. Two calcium sulfates (CAPSET® and CalForma®, Lifecore Biomedical Inc., St. Paul, Minnesota, USA), a resorbable natural collagen (Bio-Gide®, Geistlich Pharma Ag., Wolhusen, Switzerland), and a non-resorbable PTFE (TefGen-FD®, Lifecore Biomedical Inc., St. Paul, Minnesota, USA) served as the human gingival fibroblasts' substrates and comprised the four experimental groups, whereas the untreated floors of culture plastics were used in the control group, in this study. Cells were trypsinized, seeded, and incubated for 48 h. The proliferating activities of fibroblasts were determined by XTT and SRB assay and absorbance (optical density, OD) was measured. One-way ANOVA was used to analyze the differences in the mean OD values between the groups of CAPSET, CalForma, Bio-Gide, TefGen, and the control ($p<0.05$).

Results. From the XTT assay, the mean OD value of the control group, the highest, was significantly greater than that of any of the four experimental groups followed by CalForma, CAPSET, TefGen, and Bio-Gide. Further, the mean OD value of CalForma, was significantly greater compared to that of Bio-Gide. From the SRB assay, Calforma showed the highest mean OD value, which was significantly greater than that of any other groups, followed by the control, CAPSET, Bio-Gide, and TefGen. The mean OD values of both the control and CAPSET were significantly greater compared to that of TefGen ($p<0.05$).

Conclusion. Assessment of the viability and proliferation of cultured fibroblasts seeded and incubated for 48 h on various barrier-material substrates using XTT and SRB assay showed that calcium sulfate CalForma® promotes the proliferating activity of human gingival fibroblasts.

Key Words

Calcium sulfate, Human gingival fibroblast, XTT assay, SRB assay

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Medically graded calcium sulfate hemihydrate (MGCSH), as a bone-graft material, has been widely used in oral surgery. However, few studies have reported the use of calcium sulfate as a barrier between the gingival soft tissue and the underlying bone graft material. Among those, one study reported the outstanding role of calcium sulfate in stimulating the growth of gingival soft tissue[1]. In the field of soft tissue esthetics in Dental Implantology, such a unique property of calcium sulfate could serve as a trouble-solving broker for augmenting soft tissue implant coverage or in preventing peri-implant soft tissues from degenerating under various unfavorable clinical conditions. The purpose of this study was to compare the proliferating activities of human gingival fibroblasts plated on various bone graft barrier materials of calcium sulfate, resorbable collagen, and non-resorbable polytetrafluorethylene (PTFE).

MATERIAL AND METHODS

CELL CULTURE

Healthy gingival tissues were obtained from patients who underwent oral surgery for removing impacted wisdom teeth at St. Vincent's Hospital Department of Dentistry. In all cases, tissues were obtained from subjects following informed consent as prescribed in an approved St. Vincent's Hospital Institutional Review Board (IRB) protocol. Tissues were incubated for 16-22 h in Hank's balanced salt solution (HBSS, Gibco BRL, Life Technologies, Grand Island, NY, USA) at 4℃ for the purpose of separating connective tissue from epithelium. Obtained connective tissues were cut into small pieces and placed in Petri dishes (direct explant method) in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Life Technologies, Grand Island, NY, USA) supplemented with penicillin G sodium (50 IU/ml), streptomycin sulfate (50mg/ml), and amphotericin B and were kept overnight at 4℃.

Cells or explants were washed 3 times in phosphate-buffered salines (PBS, Gibco BRL, Life Technologies, USA) and suspended in DMEM supplemented with 10 % fetal bovine serum (FBS, Sigma-Aldrich Co., St. Louis, MO, USA), penicillin G sodium (50 IU/ml), streptomycin sulfate (50mg/ml), and amphotericin B. The composition and concentration of the solution were maintained to be used as the culture medium through the entire procedure of this study (DMEM supplemented with 10% FBS and antibiotics). Suspended fibroblasts were seeded into a T-75 flask (enzymatic dissociation) and incubated in a humidified incubator at 37℃ with 5% CO_2 in 95% air. When cells reached 80% confluence (about once per week), they were removed and suspended using a trypsin-EDTA solution (0.25% trypsin and 0.1% glucose dissolved in 1 mM of EDTA-saline, Sigma-Aldrich Co., St. Louis, MO, USA), washed, centrifuged and resuspended. Finally, human gingival fibroblasts were seeded for subculture at a cell population density of 2×10^4 cells/ml in 6-well plastic culture dishes in DMEM supplemented with 10% FBS and antibiotics. In all experiments in this study, the culture medium was changed every second day after seeding.

PREPARATION OF SUBSTRATES

Powders of medically graded calcium sulfate hemi-hydrate bone graft barriers (CAPSET® and CalForma®, Lifecore Biomedical Inc., St. Paul, Minnesota, USA) were mixed with hydrated liquids according to the manufacturers' recommendations and solidified for 1 h on the floors of 24-well plates. The bone graft barrier membranes

of resorbable natural collagen (Bio-Gide®, Geistlich Pharma Ag, Wolhusen, Switzerland) and non-resorbable PTFE (TefGen-FD®, Lifecore Biomedical Inc., St. Paul, Minnesota, USA) were trimmed to an appropriate size and shape to be attached onto the floors of 24-well plates using a cyanoacrylate adhesive and a silicone bonding agent. The commercially available barrier materials served as the fibroblasts' substrates and comprised the four experimental groups, whereas the untreated floors of 24-well plates were used in the control group, in this study.

XTT ASSAY

The viability and proliferation of fibroblasts were determined by an XTT assay (Cell Proliferation Kit II, Roche Applied Science, Mannheim, Germany) as described by Roehm et al.2 Cultured fibroblasts (3rd-4th passage) were trypsinized and seeded on the prepared substrates of CAPSET, CalForma, Bio-Gide, and TefGen as well as on the control substrates at a cell population density of 4×10^4 cells / ml in DMEM supplemented with 10% FBS and antibiotics. Fibroblasts were incubated in a humidified incubator at 37℃ with 5% CO_2 in 95% air for 48 h.

XTT (soium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6- nitro)benzene-sulfonic acid hydrate) labeling reagent and electron coupling reagent (N-methyl dibenzopyrazine methyl sulfate, PMS in phosphate-buffered salines, PBS) were thawed. Each vial was thoroughly mixed and a clear solution was obtained. An XTT labeling mixture was prepared by mixing 50 μl of XTT labeling reagent and 1 μl of electron coupling reagent. 50 μl of XTT labeling mixture was added per well and incubated for 2 h in a humidified incubator at 37℃ with 5% CO_2 in 95% air. Absorbance (optical density, OD) of produced formazan transferred to 96-well plates was measured using ELISA analyzer (Spectra MAX 250, Molecular Devices Co., Sunnyvale, CA, USA) at 470 nm with a reference wavelength at 650 nm. All experiments were independently repeated in triplicate using newly prepared substrates.

SRB ASSAY

To assess the proliferation of fibroblasts by measuring their total protein content, a sulforhodamine B (SRB) assay was performed (sulforhodamine B sodium salt, Sigma-Aldrich, St. Louis, MO, USA). Materials and methods used in fibroblast plating and incubation on five different substrates were identical to those used in the XTT assay. 50 ml of cold (4℃) 50% TCA (trichloroacetic acid) were gently added in each well. Plates were left for 30 min at 4℃ and subsequently washed 5 times with distilled water. Plates were then left to dry at room temperature for 24 h. 100 μl 0.4% SRB dye solution (0.4% SRB dissolved in 1% acetic acid solution) was added to each well and left at room temperature for 30 min. SRB was removed and the plates were washed 5 times with 1% acetic acid before air drying. Bound SRB was solubilized with 150 μl 10 mM unbuffered Tris-base solution and transferred to 96-well plates. Plates were left on a plate shaker for 10 min. Absorbance was measured using ELISA analyzer (Spectra Max 250, Molecular Devices Co., Sunnyvale, CA, USA) at 470 nm with a reference wavelength at 650 nm. All experiments were repeated independently in triplicate using newly prepared substrates.

STATISTICAL ANALYSIS

The mean OD values and the standard deviations of the data from both the XTT and SRB assays were calculated. One-way analysis of variance (ANO-

VA) was used to analyze the differences in the mean OD values between the groups of CAPSET, CalForma, Bio-Gide, TefGen, and the control ($p<0.05$).

SCANNING ELECTRON MICROSCOPY

Adhesion and morphology of fibroblasts on the surfaces of various bone graft barriers was analyzed under scanning electron microscopic (SEM) observations. At 24 h incubation, samples were fixed in 4% paraformaldehyde for 2 h and rinsed twice in 0.1M PBS for 10 min. Samples were again fixed for another 2 h in 1% osmium tetroxide. Samples were then dehydrated by immersing for 10 min in each of 20, 50, 60, 70, 80, 90, and 100% ethanol dilutions followed by drying with a critical point dryer (Jumb, Bio-Rad Hercules. CA, USA). Samples were sputter coated with a 10-nm gold film (SEM coating system, E 5150, Bio-Rad Hercules. CA, USA) and imaged with JSM-5410 LV® SEM (Jeol, Tokyo, Japan).

RESULTS

ANALYSIS OF VARIANCE

In ANOVA, the mean OD values from both the XTT and SRB assays at 48 h incubation were significantly different between and within all groups ($p<0.05$). It was noted that, according to the data using different substrates, the results of the two colorimetric analyses were significantly related (Table I).

MULTIPLE COMPARISON TESTS

Multiple comparisons of the fibroblasts' viability / proliferation data from the XTT assay showed the mean OD value of the control group (0.22), the highest among all groups, to be significantly greater than that of any of the four experimental groups using barrier-material substrates such as CalForma (0.16), CAPSET (0.12), TefGen (0.11), and Bio-Gide (0.08) ($p<0.05$) (Fig. 1). Further, the mean OD value of CalForma was significantly greater compared to that of Bio-Gide ($p<0.05$). All other comparisons between groups were not statistically significant.

Comparative analysis on the fibroblasts' total protein content from the SRB assay revealed that CalForma showed the highest mean OD value (1.07), which was significantly greater than that of any other groups ($p<0.05$) such as the control (0.43), CAPSET (0.38), Bio-Gide (0.35), and TefGen (0.15) (Fig. 2). Also, the mean OD values of both groups of control and CAPSET were significantly greater compared to that of TefGen ($p<0.05$). All other comparisons between groups were not statistically significant.

Table I. Analysis of variance ($p<0.05$)

		Sum of Squares	Degree of Freedom	Mean Square	F-Statistcs	Significance
XTT	Between Groups	0.034	4	0.008	25.402	0.000
	Within Groups	0.003	10	0.000		
	Total	0.037	14			
SRB	Between Groups	1.048	4	0.370	66.087	0.000
	Within Groups	0.056	10	0.006		
	Total	1.536	14			

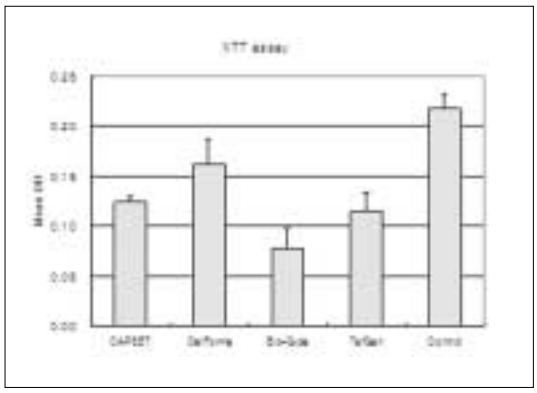

Fig. 1. Mean optical density values from XTT assay.

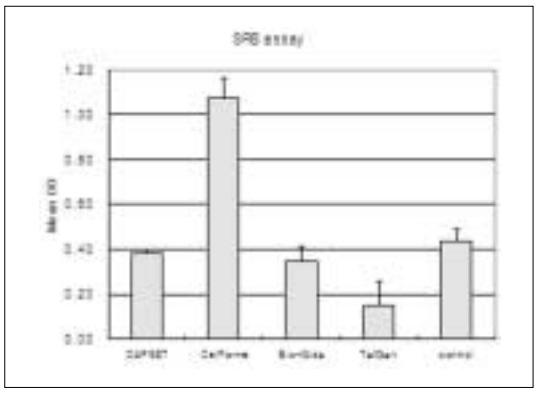

Fig. 2. Mean optical density values from SRB assay.

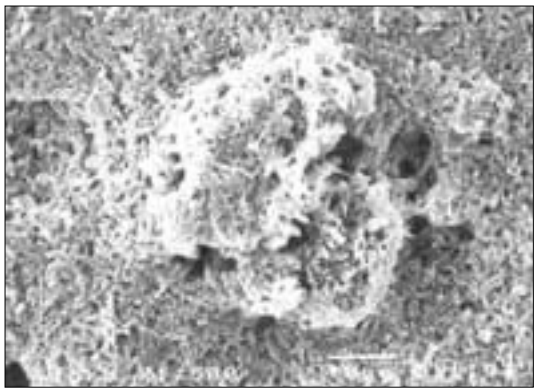

Fig. 3. SEM view of human gingival fibroblasts adhered to CAPSET.

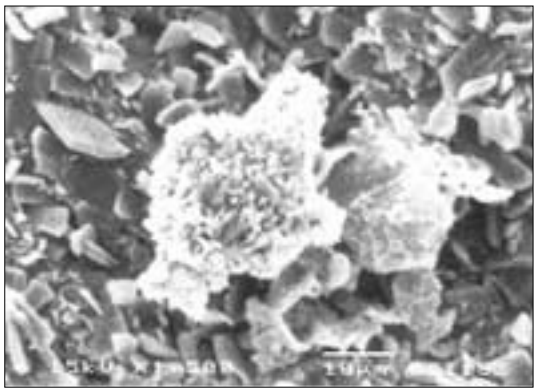

Fig. 4. SEM view of human gingival fibroblasts adhered to CalForma.

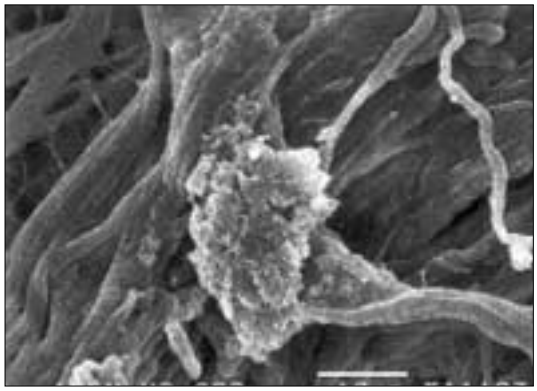

Fig. 5. SEM view of human gingival fibroblasts adhered to Bio-Gide.

SCANNING ELECTRON MICROSCOPY

Human gingival fibroblasts plated on CAPSET (Fig. 3), CalForma (Fig. 4), and Bio-Gide (Fig. 5) appeared nearly ovoid. Roughness was observed on the surfaces of CAPSET and CalForma with the latter appearing rougher. Collagen fibers were observed on the surface of Bio-Guide.

DISCUSSION

The two colorimetric assays, XTT and SRB, used in this study are methods well established in investigating the influence of specific substance and/or material on cell survival. The

results of the XTT assay for cell viability and proliferation are influenced not only by the number but also by the metabolic activity of cells. Cleavage of XTT by dehydrogenase enzymes of metabolically active cells yields a highly colored formazan product which is water soluble. This feature enables the product to be diluted in medium, eliminates the need for formazan crystal solubilization prior to absorbance measurements as is required in MTT assay, protects cellular membranes from being damaged, and leads the cells to survive throughout the test process. The development of sulforhodamine B (SRB) protein staining assay for the in vitro measurement of cellular protein content of adherent and suspension cultures was established by Skehan et al. [3]. The dye binds to basic amino acids of cellular proteins and colorimetric evaluation provides an estimate of total protein mass which is closely related to cell number.

The advantages of SRB assay over tetrazolium-based assays include higher sensitivity as well as better linearity to cell number and allow a correct estimation of cell proliferation. Tetrazolium-based assays such as the XTT assay used in this study tend to exhibit a slightly hyperbolic cell number against the optical density curve in adherent cells, leading to an error in the results. Since adherent fibroblasts are frequently used in *in vitro* assays, they, too, can display such an error. The results from SRB assay could be considered more reliable than those from XTT assay in relation to changes in proliferation (cell number) rather than changes in metabolic activity (viability). Indeed, our result from SRB assay strongly suggests that calcium sulfate CalForma® stimulates human gingival fibroblasts' proliferation related to cell number. On the other hand, in SRB assay, an addition of 50 μl 50% cold trichloroacetic acid (TCA) to the medium is necessary for cell fixation and this TCA fixes both cells and proteins such as fetal bovine serum contained in the medium, a phenomenon called background staining. Together with any shearing force generated to dislodge the adherent cells at any step in SRB assay, background staining causes an error in the results[4].

PTFE, Teflon, was originally developed in the industry to replace polyamides (nylons) in that it displays superior resistance to chemicals and friction. These characteristics are considered to yield another characteristic related to a decrease in wettability. Barely adhered cells to the poorly wettable PTFE surface were assumed to be easily dislodged by forces generated from any manipulation such as adding or stirring up liquid solutions to / in the wells. Indeed, fibroblasts were rarely discovered on the surface of PTFE barrier membrane in SEM which is again, verified from XTT and SRB assays. On the contrary, cells were assumed to be strongly adhered to the highly wettable calcium sulfate substrate, basically a gypsum product, which easily absorbs water even after solidification. These assumptions are based on the hypothesis corresponding with Altankov et al. that cell proliferation increases with increasing substrate surface wettability[5].

The results of this study provide further evidence to support the findings by Hanein et al. that even subtle differences in inorganic substrata can have major effects on cell adhesion6. In this study, these differences can be considered in several ways. A thorough review by Discher et al. made it clear that the contractile state of a cell can be strongly influenced by the stiffness of the anchoring substrate and that the contractile traction forces exerted by a cell tend to increase with the stiffness of its substrate[7]. These intracellular forces, also called cytoskeletal tension per se or cytoskeletal prestress, together with extracellular matrix (ECM) and cytoskeletal structure are considered to play decisive roles in the control of various biological activities, including cell proliferation and growth[8]. Among the materials used as the substrates for cell culture in this study, the

floors of untreated culture plastics were considered to be the stiffest followed by the solidified calcium sulfate materials. Results from both assays support the role of the substrate stiffness in altering fibroblasts' viability and proliferation. Considering cell migration as one of the essentials in proliferation, our result from the XTT assay corresponds with that of a previous study[9]. Using various barrier materials as substrates, the study concluded that human gingival fibroblasts migrated most extensively over the normal culture plastic followed by calcium sulfate compared with PTFE or polylactic acid.

A hypothesis of cellular mechanotransduction, where alterations in substrate surface topography may lead to changes in the probability of gene transcription, is another point to be considered in this study[10]. The fact that a significant difference in the mean OD values from the SRB assay was present between CAPSET and CalForma, which are both calcium sulfates, suggests that substrate surface topography actually altered biological activities of cells which might have resulted from changes in gene expression. In SEM observations, surface particles of CalForma appeared greater in size compared with those of CAPSET suggesting that the surface of CalForma is rougher.

Substrate chemistry, one of the important factors in cell-substrate interactions, is inappropriate for discussing the differences in the fibroblasts' proliferating activities between the groups because some of the materials used in this study were organic while others were not. However, it would be very interesting to analyze the surface chemistry of various organic as well as inorganic barriers to determine the differences in the biological activities of cells that selectively adhere to such materials.

CONCLUSION

Cultured fibroblasts were plated and incubated for 48 h on 1) two medically graded calcium sulfate hemi-hydrate bone graft barriers CAPSET® and CalForma®, 2) a resorbable natural collagen barrier membrane Bio-Gide®, and 3) a non-resorbable polytetrafluorethylene barrier membrane TefGen-FD®. Assessment of the viability and proliferation using XTT and sulforhodamine B assay showed that calcium sulfate CalForma® promotes the proliferating activity of human gingival fibroblasts.

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