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POSTGRADUATE DENTAL COLLEGE
ARMY POSTGRADUATE DENTAL SCHOOL
228 EAST HOSPITAL ROAD
FORT GORDON, GEORGIA 30905

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Name of Candidate: CPT Nathaniel Hoang
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Kyung S. Kim
DEPARTMENT OF PROSTHODONTICS, ARMY POSTGRADUATE DENTAL SCHOOL
Committee Chairperson

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16 MAY 2023

Jennifer V. Sabol
DEPARTMENT OF PROSTHODONTICS, ARMY POSTGRADUATE DENTAL SCHOOL
Committee Member

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Steven E. Handel
DEPARTMENT OF PROSTHODONTICS, ARMY POSTGRADUATE DENTAL SCHOOL
Committee Member

Growth of human gingival epithelial cells on dental restorative material: lithium disilicate in vitro

by

Nathaniel Hoang, DDS

Captain, Dental Corps

United States Army

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Growth of Human Gingival Epithelial Cells on Dental Restorative Material: Lithium Disilicate *in vitro*

Nathaniel Hoang, DDS,[a] Henry Chuang, PhD,[f] Douglas Dickinson, PhD,[g] Walter G. Dimalanta, DDS, MS,[b] Kyung S. Kim, DMD, MS, MS,[d] Jennifer V. Sabol, DDS, MS,[c] James C. McPherson, PhD,[f] Jake T. Wilding, DDS, MS,[h] Martin J. Smallidge, DMD, MPH, MS[h]

[a]Resident, U.S. Army Advanced Education Program in Prosthodontics and Army Postgraduate Dental School, Uniformed Services University of the Health Sciences, Fort Gordon, GA.

[b]Former Program Director, U.S. Army Advanced Education Program in Prosthodontics, Fort Gordon, GA.

[c]Program Director, U.S. Army Advanced Education Program in Prosthodontics, Fort Gordon, GA.

[d]Assistant Program Director, U.S. Army Advanced Education Program in Prosthodontics, Fort Gordon, GA.

[e]Former Resident, U.S. Army Advanced Education Program in Prosthodontics, Fort Gordon, GA.

[f]Clinical Researcher, Clinical Investigations, Fort Gordon, GA.

[g]Vice-President for Research and Development, Camellix LLC and former Professor, Department of Dental Research, Army Postgraduate Dental School, Uniformed Services University of the Health Sciences, Fort Gordon, GA.

[h]Former Resident, U.S. Army Advanced Education Program in Prosthodontics, Fort Gordon, GA.

ABSTRACT

Growth of human gingival epithelial cells on dental restorative material: lithium disilicate in vitro

OBJECTIVES: To investigate the biocompatibility of human gingival epithelial cells (HGE) with lithium disilicate (IPS e.max CAD, Ivoclar) with different surface roughness (R_a) values.

METHODS: Thirty-three lithium disilicate (LD) samples were divided into four treatment groups: Group 1 consisted of six discs adjusted with burs of decreasing roughness (CRF to Extra Fine, Brasseler), Group 2 consisted of discs polished with the Dialite kit (Brasseler) (one each Blue, Pink, and Grey), Group 3 consisted of discs polished with the Luster kit (Meisinger), and Group 4 consisted of one disc the sample control with glaze only (e.max CAD Crystall Glaze, Ivoclar). Samples in each group were placed into 11 wells in three separate 12-well plates, and the empty well served as plastic control. Sample roughness (R_a) was measured using a stylus-type profilometer. Scanning electron microscopy (SEM) was used to image the surface of the discs. Primary HGE cells were seeded onto discs in three 12-well plates and grown for 1, 3, or 6 days.

RESULTS: Very inconsistent growth of HGE cells on both plastic and LD was observed, both within an experiment between samples and between experiments. There was no

evident relationship between cell behavior and surface roughness or the polishing methods, or a difference to growth on plastic, with the exception that the polished Luster group demonstrated a statistically significant greater cell population Day 6 compared to all other polishing methods. However, the replicate experimental values for Luster were very heterogeneous.

CONCLUSIONS: The primary HGE cells behaved in a manner inconsistent with normal exponential cell growth in culture and showed large variation in the pattern of growth between experiments. Growth on LD was comparable to growth on plastic, consistent with biocompatibility. With the possible exception of Luster, neither the polishing method nor the achieved R_a value appeared to affect the number of attached cells at any time. No evidence was found that the method of polishing, or the achieved surface roughness, had a marked effect on HGE cell behavior.

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INTRODUCTION

Dental implant-supported restorations have become the standard for replacement of missing teeth. Ideally, the gingival tissue will develop a seal around the implant to prevent infiltration of bacteria into the bone. For this to occur, the implant material must show biocompatibility: the ability of a material to create an appropriate response from host tissues. For soft tissue to form an intimate connection with the implant/prosthesis, the materials used to make either the abutment or prosthesis must minimally not cause injury to adjacent tissues, and at best integrate with them. For the gingiva, this would involve epithelial cell adhesion to the material surfaces, with subsequent proliferation and migration.

Lithium disilicate-based material is a commonly used material for custom transgingival implant-supported restorations. An advantage of lithium disilicate (LD) is that restorations can be made using in-office CAD/CAM technologies for single appointment delivery. Research has shown high levels of biocompatibility for LD, not only due to low plaque retention, but also due to adhesion and proliferation of human epithelial cells (Brunot-Gohin et al.) and human gingival fibroblasts (Jung et al.), particularly when the surface is polished. However, there has been only limited investigation of the adhesion of human *gingival* epithelial cells,[16] specifically non-immortalized primary cells, to LD surfaces, and the effect of surface polishing on the adhesion.

The purpose of this study was to investigate in-vitro the biocompatibility of a LD dental restorative material with primary human gingival epithelial cells. The effect of

surface roughness on the cells' ability to grow on the material surface was also investigated by measuring the viability of cells grown on the surface of samples with different surface topologies prepared by different grinding and polishing techniques.

Since surface preparation, which affects surface roughness, could potentially affect cell adhesion and growth, test surfaces were prepared by grinding or polishing to varying levels of roughness, as measured by R_a (μm). The gold standard surface for in vitro cell growth is tissue culture plastic.

The two hypotheses tested were that

1) Human gingival epithelial (HGE) cell growth on LD would be equal to that on tissue-culture plastic. The null hypothesis is that there would be no difference in growth.

2) HGE cell growth would have a positive correlation to LD surface roughness. The null hypothesis was that there would be no correlation.

MATERIAL AND METHODS

Pre-crystallized LD blocks were sectioned using a water-lubricated diamond cutting blade on a precision saw (Buhler; Uzwil, Switzerland), resulting in rectangular discs 1 mm thick, with a surface area of 14 x 12 mm =168mm^2. The samples were then fully crystallized in an Ivoclar Programat EP 5010 furnace (Schaan, Liechtenstein). To create a range of surface roughness values, samples were prepared with a diamond bur, polishing wheels, or glazed. Four treatment groups were prepared: Group 1 consisted of six discs adjusted with burs of decreasing roughness (CRF to Extra Fine); Group 2

consisted of discs polished with the Brasseler Dialite kit (one each Pink, Blue, and Grey); Group 3 consisted of one disc polished with the Meisinger Luster kit; Group 4 consisted of a control disc with glaze only (e.max CAD Crystall Glaze). A total of thirty-three LD discs were divided into three 12-well plates, with 11 discs within each plate. Each 12-well plate consisted of six bur samples (CRF to Extra Fine), one glaze sample, four polished samples (Luster, Dialite Blue, Dialite Pink, Dialite Grey), and one empty well serving as the plastic control. Each 12-well plate was designated for Day 1, 3, or 6. All three plates (Day 1, 3, and 6) were used in four replicate experiments (Experiments 1-4).

The polished set of samples were polished according to the protocol described by Steiner et al., in which they were polished for 30 seconds in one direction and for another 30 seconds at 90 degrees to the first direction.[35] Similarly, the samples adjusted with diamond burs were held on a flat surface and the long side of the bur was brushed across the sample 5 times going lengthwise. The sample was then turned 90 degrees and the bur was brushed across the sample 5 times going widthwise. This procedure was followed to produce a more homogenous surface texture. For a consistent application of pressure, each sample was placed in a putty matrix jig and placed on an electric scale. The scale was tared, and the samples were then polished with a pressure range of 300-500 gm of pressure (2.94-4.90 N) with the hand piece set at 10,000 rpm.

Another set of three disc samples was polished according to the manufacturer instructions for use with the Brasseler Dialite (Savannah, GA) porcelain extraoral polishers, with each sample polished with blue, pink, or grey. Another set of samples

was polished using the Meisinger Luster (Centennial, CO) intraoral porcelain polishing kit. The protocol was followed for each polishing wheel from coarse to fine. The final LD sample was glazed using a Ivoclar IPS e.max CAD Crystall./Glaze and Crystall./Glaze Liquid for dilution (Schaan, Liechtenstein).

A stylus-type profilometer (Mahr Perthometer M2, Providence, RI) was used to measure surface roughness, determined as Ra (μm) values. Three measurements were made per sample and the mean R_a calculated.

To prepare the disc samples for tissue culture, the samples were steam-cleaned (Triton; BEGO GmbH & Co. KG, Bremen, Germany) for 10 seconds on each surface, and then sterilized in an autoclave. Discs (sets of 11 different surfaces) were then placed in the wells of three sterile 12-well tissue culture plates with the single polished surfaces facing upward. The 12^{th} well served as a tissue culture plastic positive control for growth.

The same three sets of 11 samples (Days 1, 3, 6) were cleaned and sterilized as described above and re-used for a further three replicate experiments (Experiments 2-4). Fluorescence microscopy was used to confirm removal of epithelial cells after cleaning and sterilization in the autoclave.

Following the protocol of Smallidge et al. and Wilding et al., pooled-donor primary cells (CELLnTEC, Bern, Switzerland) were used. PrimaIHGE cells were grown according to the source's instructions, and low passage numbers (3-6) were used for each experiment. Cells were grown in CnT-Prime epithelial culture media (CELLnTEC) to

a confluence of approximately 80% before passaging or harvesting. Passaging and harvesting were performed using Accutase (CELLnTEC) to dissociate cells from the culture surface. Cells were harvested by gently removing the media from the tissue culture flask by aspiration, washing twice with pre-warmed calcium-free phosphate buffered saline (PBS), then cells were incubated at 37°C with 3.0 ml Accutase solution (CELLnTEC) for 12 minutes with tapping the bottom of the flask at the end of the incubation period. Detachment was observed under a light microscope (Carl Zeiss Vision Inc., Oberkochen, Germany). The cell suspension was removed from the flask and mixed with 10 ml media to stop digestion. After centrifugation at 700g for 10 minutes, cells were resuspended in 10 ml of media. A hemocytometer and trypan blue staining were used to determine live cell density.

Samples were seeded at a density of 2-8x10^5 cells/mm^2 onto three replicate 12-well tissue culture plates, with a surface area of 4 cm^2 per well. Eleven wells each contained one disc sample prepared by a different method covering the range of R_a values. One well was used as a plastic surface positive control. One each of the three plates was incubated at 37°C for 1, 3, or 6 days (24, 72 and 144 hours) prior to assay. A total of four replicate experiments were performed.

To quantify cell growth, at each time interval, one 12-well plate was removed for propidium iodide nuclear staining and cell counting. The media was gently removed from each well with a transfer pipette, and each well was washed twice with 2.0 ml of magnesium and calcium-free PBS. One milliliter of a propidium iodide staining solution consisting of 0.1% v/v TX-100, 100 µg/ml RNase A and 10µg/ml propidium iodide in PBS

was added to each well and the plate incubated at 37^{o}C for 20 minutes. The solution was then removed and the wells containing material samples were washed twice with PBS. The samples were transferred to a new plate and placed cell surface down for microscopic observation.

Imaging of the discs and plastic controls was completed immediately following the staining procedure using fluorescence microscopy (Evos inverted microscope; Thermo Fisher Scientific, Waltham, MA), with a Texas red filter set (excitation 535 nm and emission 617 nm) to visualize propidium iodide stained nuclei. Images were photographed at 10x magnification corresponding to a sample area of 1.0 mm^2 per image field. Twenty images were taken of each disc and the control well, distributed over the entire material sample area in a 4 x 5 grid. The Evos imagining software Auto Count Tool (Thermo Fisher, Waltham, MA) was used to obtain stained nuclear counts for each image field after setting thresholds for brightness and particle area. For each of the eleven samples and the plastic control at each incubation period, total cell number per field was determined by averaging the 20 field cell count.

A steam-cleaned and autoclaved sample from each treatment group was analyzed by scanning electron microscopy (SEM). Samples were mounted on aluminum stubs with carbon adhesive tabs and coated with gold-palladium for 6 minutes in a Hummer Sputter Coater 6.2 (Anatech, Union City, CA). Imaging was performed in a JSM IT-500HR Scanning Electron Microscope (JEOL USA Inc, Peabody, MA) at 10kV. Each sample was carbon-coated before investigation under SEM at 330x magnification.

Linear regression modeling and other statistical tests were performed using statistical software (Graphpad Prism 6.0, Graphpad Software, Inc. La Jolla, CA), with alpha = 0.05.

The normality of a distribution was be assessed by two methods: the D'Agostino and Pearson omnibus normality test, and the Shapiro-Wilk test. Specific outlier values were identified by the ROUT method, with higher Q % values being more relaxed, and lower values more stringent in selection.

RESULTS

The polishing, glazing and grinding protocols produced a range of R_a values (0.086-2.798 mm) (Table 1).

1	CRF	2.798 µm
2	Super Coarse	2.425 µm
3	Coarse	2.270 µm
4	Medium	1.661 µm
5	Fine	1.102 µm
6	Extra Fine	0.864 µm
7	Glaze	0.829 µm
8	Dialite Blue	0.442 µm

9	Dialite Pink	0.257 µm
10	Dialite Grey	0.213 µm
11	Luster	0.086 µm
12	Control	0.015 µm

Table 1: Mean R_a values for surfaces used for primary HGE cell culture

1) Evaluation of plastic control cell growth

The initial viability of the cell suspensions was >95%, as determined by trypan blue staining. Cells were initially plated (Day 0) at a density of 500 (Experiments 1, 3), 1000 (Experiment 2) or 2000 cells/mm^2 (Experiment 4).

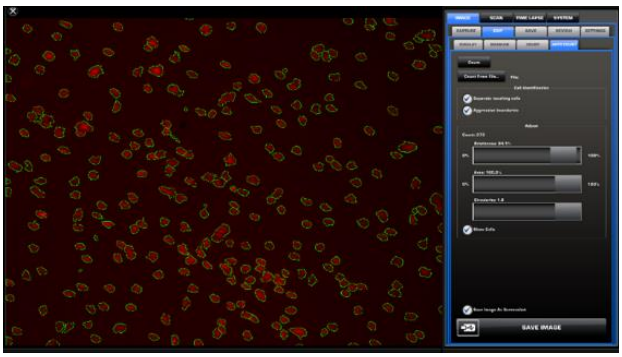


Figure 1: Human gingival epithelial cells visualized under fluorescence microscopy after staining with propidium iodide with Texas red filter. A representative sample is shown.

To examine the general pattern of cell growth on plastic, cells were stained with propidium iodide (Figure 1), and nuclei par unit are determined by microscopy and imaging software. The mean cell count/mm^2 normalized to the

initial load (which differed between experiments) was plotted against time (Figure 2). Experiment 3 showed a modest increase in cell number between Day 1 and Day 6 with the number of cells doubling, consistent with a slow net rate of cell accumulation, and indicating potentially normal growth during the experimental period. In contrast, Experiments 2 and 4 showed a substantial decrease in cell number between Days 1 and 3, with Experiment 4 having lost most of the cells by Day 3. Experiment 2 showed a continued, albeit slower rate of loss through Day 3 and Day 6. Since the media was changed Day 3, this continued loss was not likely due to nutrient exhaustion. Experiment 1 showed a more uniform decline in cell number through the experimental period. Collectively, inconsistent growth between experiments was observed, and three out of four experiments showed no similarity to the expected normal pattern of tissue culture growth.

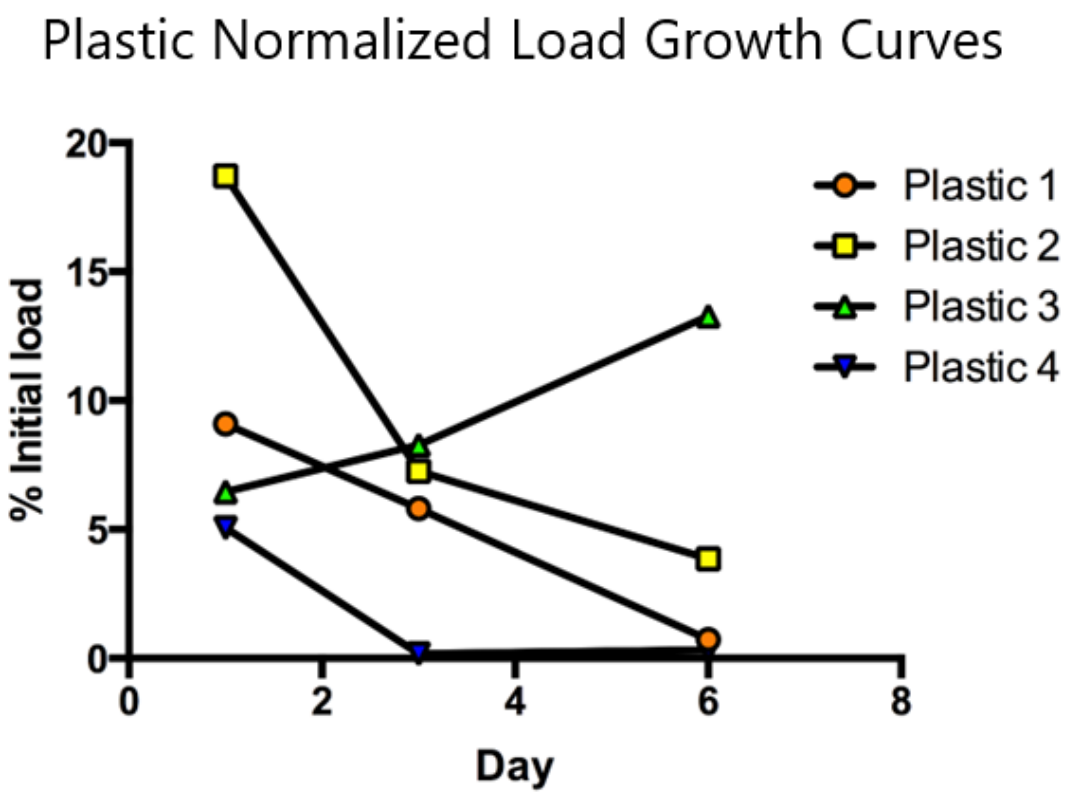


Figure 2: Primary HGE cell growth on plastic control (count/mm^2) during experimental period (Days 1, 3, 6). Values are expressed as the percent of the initial cell density load, with each curve showing results for Experiments 1-4.

(a) Day 1 Mean cell count

(i) First 24 hr time points:

During the initial 24 hr period, cells would attach to the plastic surface, but proliferation would be expected to be relatively low. The four replicate experiments gave a mean percent of initial load cell count on plastic at Day 1 of 9.8%+6.2% (Table 2), indicating a substantial variation between experiments in the proportion of loaded cells showing attachment and survival, and with an overall poor initial attachment to plastic. All four experimental 24 hr values were

significantly lower than 100% (one sample t-test; $p<0.0001$). That is, variance between experiments for the Day 1 estimates was unlikely to account for the discrepancy between initial load and Day 1 values; all were particularly low. Therefore, either about 90% of the cells failed to attach and grow or detached within the 24-hr period, or there was a substantial discrepancy between the hemacytometer cell count estimate for plating density and the cell counting by nuclear staining or both. Consistent with detachment, in Experiment 4, most of the cells were lost by Day 3 and could be seen detaching.

Table 2: Mean and standard deviation (SD) of 24 hr field counts of plastic samples. For each Plastic control sample, the mean/SD of the 20 field counts was determined. The % of initial load was determined as a proportion of cells/mm^2 loaded on Day 0. The overall mean and SD of the % initial load for the experiments (n=4) is also shown.

Experiment initial load/mm^2 (4 cm^2 =400 mm^2 well)	**Mean cell count/SD 24hrs**	**24hr % initial load**
1 - 500	45.5/40.9	9.1
2 - 1000	187.1/132.3	18.7
3 - 500	32.3/23.8	6.5
4 - 2000	101.1/105.7	5.1
Overall mean/SD		9.8/6.2

The mean cell density for the two 500/mm^2 initial load experiments (1, 3) was 38.9/mm^2. The 24-hr cell density for Experiment 2 (initial load 1000/ mm^2) was 4.8-fold higher than the 500/mm^2 load, rather than the 2-fold higher expected. The 24-hr cell density for Experiment 4 (initial load 2000/ mm^2) was 2.6-fold higher than the 500/mm^2 load, rather than the 4-fold higher expected.

These values indicate a lack of proportionality between initial seeding load and measured cell densities at 24 hrs; that is, the proportion of attaching and remaining cells varied considerably between experiments. This could have been due to random variation between cell cultures/media, or there could have been a large difference between experiments in the timing of initiation and/or magnitude of cell detachment.

There appeared to be a large loss of initially viable cells as based on trypan blue staining between loading and staining for counting cells on the surface at 24 hrs. For Experiment 4, the loss appeared to continue through Day 6. Experiment 4 values for Days 3 and 6 were therefore suspect. Experiment 1 showed an apparent loss after Day 3. This loss of cells was consistent with a poor correlation between initial cell load and the cells remaining at 24 hrs and suggested a poor and inconsistent performance of the primary cells. This also precluded the use of the initial plating density values as a Day 0 value for regression analysis of growth curves.

(ii) Variance of cell counts at three timepoints:

The values for the field count coefficient of variance (CV, %) for the plastic samples (one well per plate) are shown in Table 3. This provides an estimate of the homogeneity of cell growth on the plastic surface.

Table 3: Coefficients of variance for field counts of plastic samples. For each sample, the mean and SD of the 20 field counts were used to determine a % CV value.

Experiment/Day	**Day 1**	**Day 3**	**Day 6**
1	90.03	116.45	79.64
2	70.71	42.20	71.23
3	73.59	69.33	85.61
4	104.55	Cells lost	Cells lost
Experiment mean **CV**	84.72	61. 82	36.22

The variance was relatively large (overall average 60.9%), particularly on Day 1, indicating considerable heterogeneity in the distribution of cells at each day and the evenness of growth between days, although the heterogeneity declined through the growing period.

(iii) Normality of Distribution, Skewness, and kurtosis of cell counts:

For the 10 values from growth on plastic (4 experiments, three time points; cells lost Experiment 4 at Days 3 and 6, Experiment 1 Day 6 included for evaluation), 5/10 failed the more robust D'Agostino and Pearson omnibus normality test (Table 4), while 9/10 failed the Shapiro-Wilk test. Of the groups failing the Omnibus test, 5/5 had a positive skewness value >1.0, indicating a long tail to higher values. Five/5 had a kurtosis value >1.0, in two cases

(Experiment 1 and 4 at Day 1), the value was very high, consistent with a larger proportion of values in the tail to the high side. Experiment 2 passed the omnibus test for all three Days.

Identification of outliers using the ROUT algorithm with a relatively stringent Q value of 1% identified 14 potential outliers, with only two below the relevant mean. Experiment 1 gave four high and one low measurement value, Experiment 2 gave six outliers to the high side at Day 1, but none Day 3 or 6. The mean of the outliers Day 1 represented 37.7% of the initial load.

Removal of these outliers gave just 1/10 groups failing the omnibus test for normality (Experiment 1, Day 6), and 2/10 failing the Shapiro-Wilk test, with only Experiment 1 Day 6 showing skewness and kurtosis values >1.0.

Table 4: Normality of distribution, skewness, kurtosis and potential Gaussian outliers of cell counts of plastic samples. Values are shown for samples with all 20 values, no outliers removed. The p values for groups that failed the D'Agostino and Pearson omnibus normality test (significantly different from normal distribution) are shown. ROUT outliers identified with Q=1 are indicated.

EXPERIMENT	**1**			**2**			**3**			**4**		
Day	1	3	6	1	3	6	1	3	6	1	3	6
P value	<0.0001	0.001	0.0006					0.003		<0.0001	ND	ND
Skewness	2.752	1.872	1.787					1.734		2.499		
Kurtosis	9.33	2.359	3.324					4.641		8.718		
Outliers	1	3	1	6								
	Hi	Hi	Lo	Hi								

The above results indicated an inconsistent and abnormal pattern of behavior of the cells on plastic, beginning Day 1 for replicate Experiments 1 and 4.

The cultures used in the four experiments gave markedly different growth patterns on plastic during the course of the experiment. For half of the ten Day sample measurements (Experiment 4 Day 3 and 6 excluded), the replicate area cell counts showed a tail to the high side. These data suggested that there were patches on the plastic surface with relatively high numbers of cells, especially in Experiment 2, and that the proportion of these patches did not fit the random (Gaussian) distribution to be expected from random deposition of cells during plating. These data are consistent with an initial

random plating of cells, albeit with apparent poor initial adhesion, followed by loss of cells in areas in a non-random manner.

Only Experiment 3 showed evidence for growth on plastic during the experimental period; however, a non-linear regression fit to an exponential curve was not possible due to the limited number of timepoints.

(b) Comparison of growth on plastic to growth on samples.

If the different, generally poor, growth patterns on plastic for each experiment were a reflection of the cell culture and/or media batch, then a similar pattern would be expected for growth of the same cells on the different material samples subject to modulation by any effect of the material itself. Remarkably, this was not the case (Figure 3).

For Experiment 1, which shows a uniform decline in cells per mm^2 with plastic from an initial low load, all but the Extra Fine bur show a similar low initial adhered cell population Day 1, with plastic the highest in this subset. However, Extra Fine showed a mean Day 1 load of 85.8 cells/mm^2 (17.2% initial load), almost 2-fold greater than the Experiment 1 Day 1 plastic control (45.5 cells/mm^2, 9.1%). Extra Fine and Fine both appeared to show growth between Day 1 and Day 3, reaching a plateau through Day 6. After a lag in growth through Day 3, CRF and Super Coarse showed a substantial increase in cell number through Day 6, with these four materials having 100-150 cells/mm^2 Day

6, in contrast to plastic at 3.7 cells/mm^2. Super Coarse and Medium showed a modest, continuous rate of cell accumulation during the six-day period.

Dialite, Luster, and Glaze all showed a modest initial cell number similar to plastic Day 1. The cell count through Day 3 for Luster and Dialite Blue showed a decline that paralleled that for plastic. However, between Day 3 and Day 6, Dialite Blue showed a moderate increase, and Luster a dramatic increase, to 6.6-fold the Day 1 value. Dialite Pink, Grey, and Glaze all showed a modest increase between Day 1 and Day 6, followed by a further rise through Day 6, consistent with an exponential growth. For Dialite Pink, the rise was close to that seen for Luster.

In Experiment 2, all burs showed a broadly similar initial load, and a similar progressive decline in number through Day 6. Coarse, Medium, and Fine showed a rapid decline through Day 3 that paralleled the Plastic decline. Super Coarse show a slower rate of decline through Day 3, while CRF and Extra Fine remained relatively unchanged. Between Day 3 and 6, Fine and Medium were relatively unchanged, while the remaining materials showed a decline comparable to that for plastic. Although there were moderate differences in the path through Day 3, with the exception of Dialite Pink (which was relatively unchanged), Dialite, Glaze and Luster materials all had lower cells at Day 6 than Day 1.

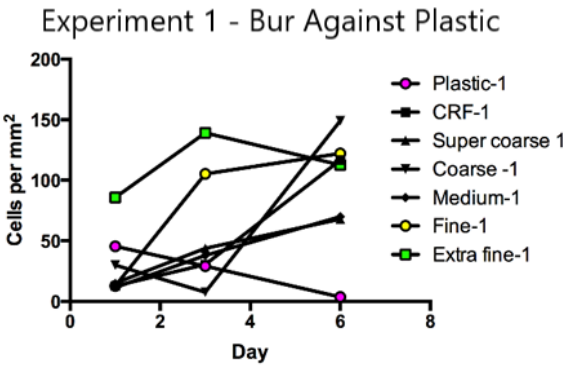
Experiments 3 and 4 showed a complex variation between materials. For Experiment 3 burs, Coarse, Fine, and Extra Fine showed a substantially higher Day 1 load, about 3-fold higher than the load with CRF, Super Coarse, Medium, and Plastic. Through Day 3, CRF showed a rapid rise, Super Coarse and Medium showed a modest rise that paralleled the increase with Plastic, while Coarse showed a slow decline through Day 3. Coarse showed a modest decline, and Fine and Extra Fine showed a rapid decrease to about half the level with Plastic at Day 3. Through Day 6, Fine, and Extra Fine showed a rapid large increase, and Medium a less dramatic increase, all reaching levels comparable to Plastic. CRF and Super Coarse showed a parallel moderate decline, while Coarse showed a large and rapid decline. All Dialite, Glaze, and Luster samples had a Day 1 value about threefold higher than Plastic. However, through Day 3, unlike plastic (which showed a modest increase), these materials showed a decline to match the level with plastic. All but Dialite Pink remained relatively unchanged through Day 6, while Dialite Pink declined modestly.

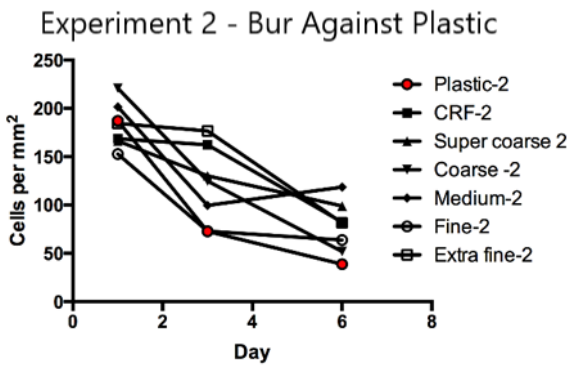
Experiment 4 with burs showed about half the initial number of cells Day 1 compared to plastic, but whereas Plastic showed a rapid decline through Day 3 to near zero, all the materials showed a rise that was substantial in the cases of CRF and Super Coarse. Through Day 6 Super Coarse and Fine showed a decline to low levels, Coarse and Medium remained relatively unchanged, CRF showed a modest rise, and Extra Fine showed a large rise to a high number of cells, 10.7-fold greater than the Day 1 load. The Dialite, Luster, and Glaze samples all had

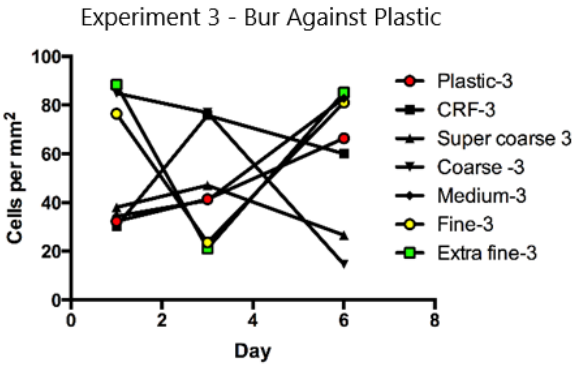
Day 1 cell numbers close to those for Plastic, except for Dialite Pink and Glaze, which were rather low. While Plastic dropped to near zero at Day 3, Dialite Grey and Blue remained fairly constant. Glaze, Dialite Pink, and Luster rose at a similar modest rate through Day 3. Through Day 6, Glaze remained relatively unchanged, while Luster and Dialite Pink rose considerably, with Luster reaching 6.2-fold the Day 1 value.

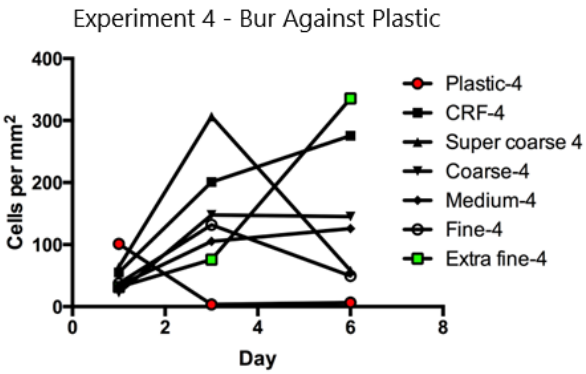
Figure 3: Graphs compare growth on samples vs. plastic expressed as mean cell count/mm^2 during the experimental period for each experiment (Experiments 1-4). For each sample, the means of the 20 field counts are shown (i.e., not normalized to initial load). Panels (a)-(d): Experiments 1-4 show average cell growth on bur sample. Panels (e)-(h): Experiments 1-4 show average cell growth on polished and glazed samples.

a

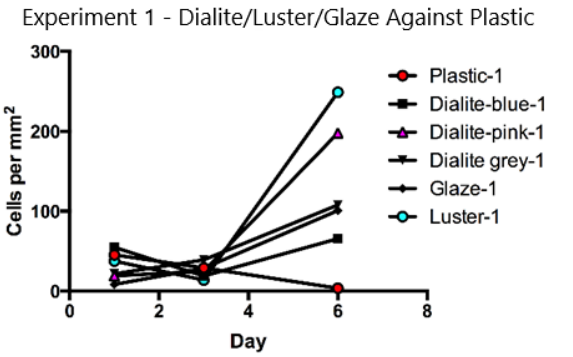


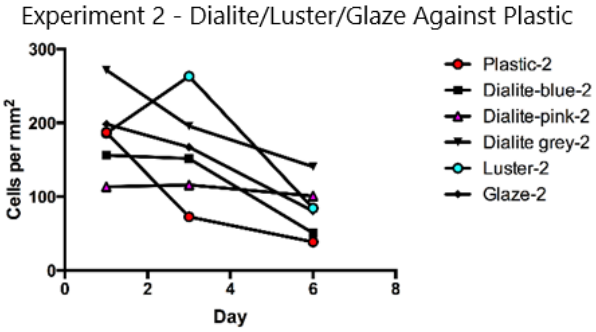


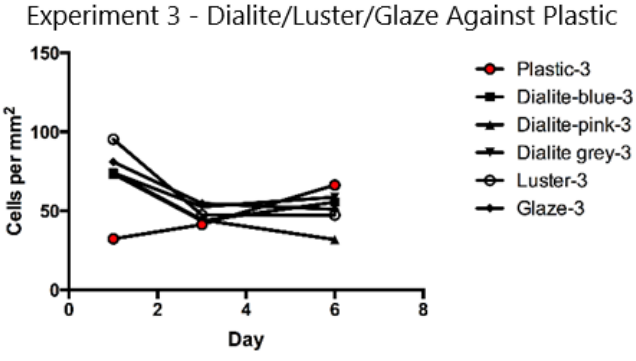


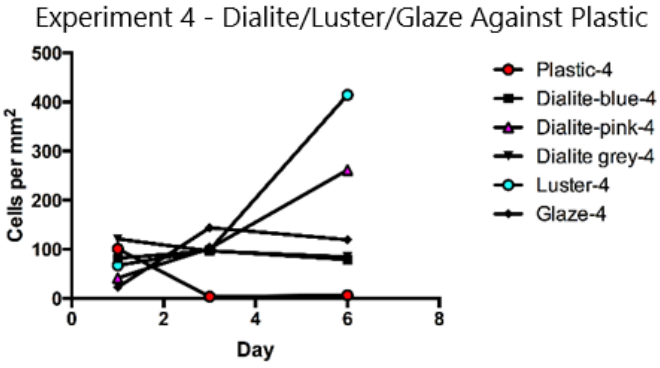


b









(c) Analysis of Day 1 cell counts (adhesion) on samples:

The Day 1 cell counts for the 11 material samples were examined in the same way as performed for the plastic control wells. Since six different roughness levels were available for the same polishing method (bur), bur adjustment was examined first to exclude polishing method as a variable. Inspection of a plot of the four experimental values for each material as replicates for the six polished surfaces revealed a similar low attachment and a large spread comparable to that seen for plastic (Figure 4).

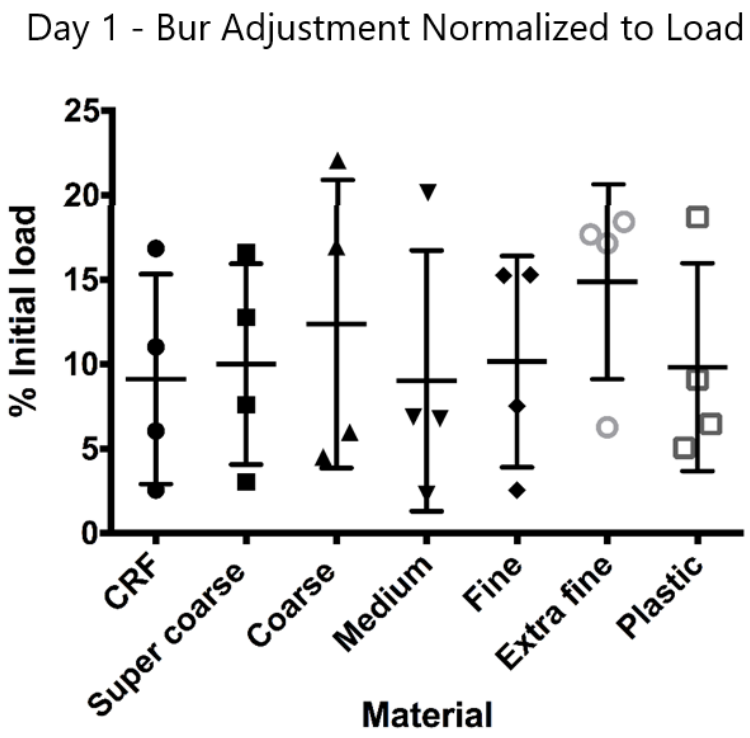


Figure 4: Graph of LD adjusted with burs and plastic Day 1 mean cell count/mm^2 normalized to initial load. The vertical bars show the mean and standard deviation.

The distribution of values tended to be to the extremes, suggesting a poor fit to a normal distribution, and potential heterogeneity (i.e., more than one population sampled). However, n=4 replicates was too low for application of normality tests.

To further evaluate the Bur data, a one-way ANOVA was performed to compare the mean values (i.e., a single sampled population with a normal distribution was assumed; ANOVA is relatively robust for deviations from normality). No significant differences in mean values were found (p=0.87). That is, across the four experiments and the seven surfaces evaluated, there was no significant difference between surfaces in the Day 1 cell density.

Given an absence of a significant effect of surface at Day 1, all 28 Day 1 Experimental/Surface normalized cell density values were evaluated for a fit to a normal distribution (Figure 5). The overall mean was 10.8%. The dataset failed both normality tests (DP p=0.004, SW p=0.016), with low skewness (0.238), but high negative kurtosis (-1.46); a symmetric distribution about the overall mean, but a deficiency of values in the tails.

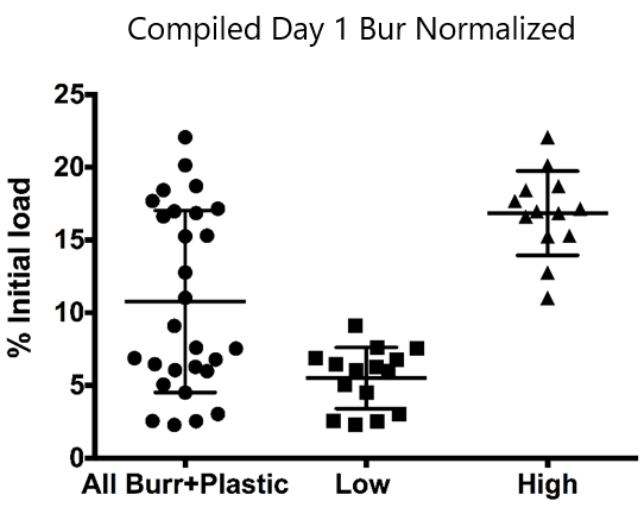


Figure 5: Scatterplot graph of LD adjusted with burs and plastic Day 1 mean cell count/mm^2 normalized to initial load. The All Bur+Plastic incorporates all 28 data values. The Low and High groups show the 28 values divided into below (Low) and above (High) the mean of 10.8%. The vertical bars show the mean and standard deviation.

Inspection of a scatterplot of the 28 values reveals a clear pattern of two populations, with 13 values at or above the mean, and 15 below. Both subgroups passed both normality tests, with low skewness and modest kurtosis. The Low mean was 5.5%+2.1% and the High mean was 16.8%+2.9%. Inspection of the distribution of these values across the experiments showed High/Low values of 1/6, 7/0, 3/4 and 2/5 for Experiments 1-4 respectively. This distribution of values was unlikely to be random (alpha =0.05, Fishers Exact test p=0.007). That is, the different experiments could show high, low, or mixed Day 1 values across the seven materials considered here. Thus, Experiment 2 showed uniformly (relatively) high net attachment (mean 18.3%+2.3%), Experiment 1 showed a low attachment (6.1%+5.5%), and Experiments 3 and 4 were respectively 11.0%+5.4% and 7.7%+3.1%. The high value in Experiment 1 was with Extra Fine (17.2%), for Experiment 3 they were with Coarse, Fine and

Extra Fine (22.1, 15.3 and 18.4% respectively), and for Experiment 4 with CRF and Super Coarse (11.0 and 12.8).

Given the original split between High and Low was based on the overall mean, revising the assignment of the two Experiment 4 values to Low gives a new means of 6.3%+2.9% and 17.7%+2.0% respectively (2.8-fold difference). The mean values of the two groups were significantly different (unpaired t-test, $p<0.0001$).

Collectively, there were significant differences between the experiments for initial (net) attachment Day 1; Experiments 1 and 4 both showed low attachment for Bur polished materials and plastic, while Experiment 2 showed uniformly higher attachment, and Experiment 3 was mixed.

(d) Analysis of Day 1 cell counts (adhesion) on Bur samples by R_a value

The revised division of Bur and Plastic experimental values was used to plot the mean normalized cell density against R_a value. As shown in Figure 6, there was no indication of a relationship between cell density (primarily reflecting net attachment) and R_a value.

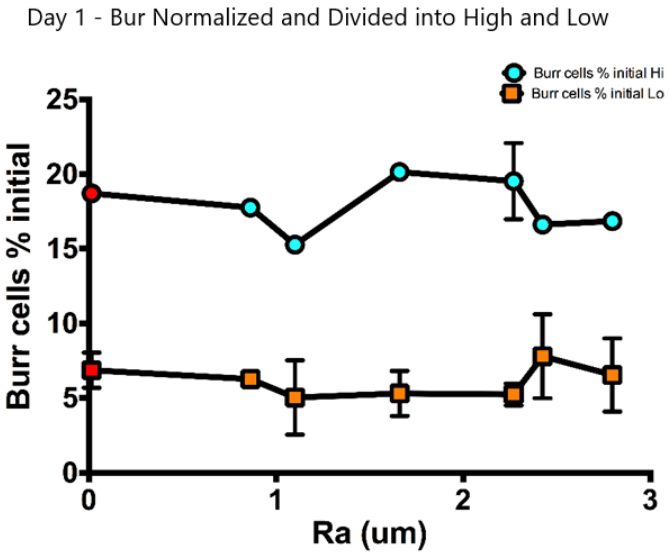


Figure 6: Plot of Day 1 mean cell count/mm^2 for samples adjusted with burs and plastic normalized to initial load against R_a value, divided into High and Low attachment experimental values. Red symbols denote plastic values. Vertical bars show SEM.

A linear regression fit to a line found no significant deviation from a zero slope for each line (i.e., no relationship) $p>0.8$.

Thus, taking into account the different performance of cells in the experiments, there was no evidence for an effect of Bur adjustment to different R_a values on Day 1 cell density.

(e) Analysis of Day 3 and Day 6 cell counts on Bur samples

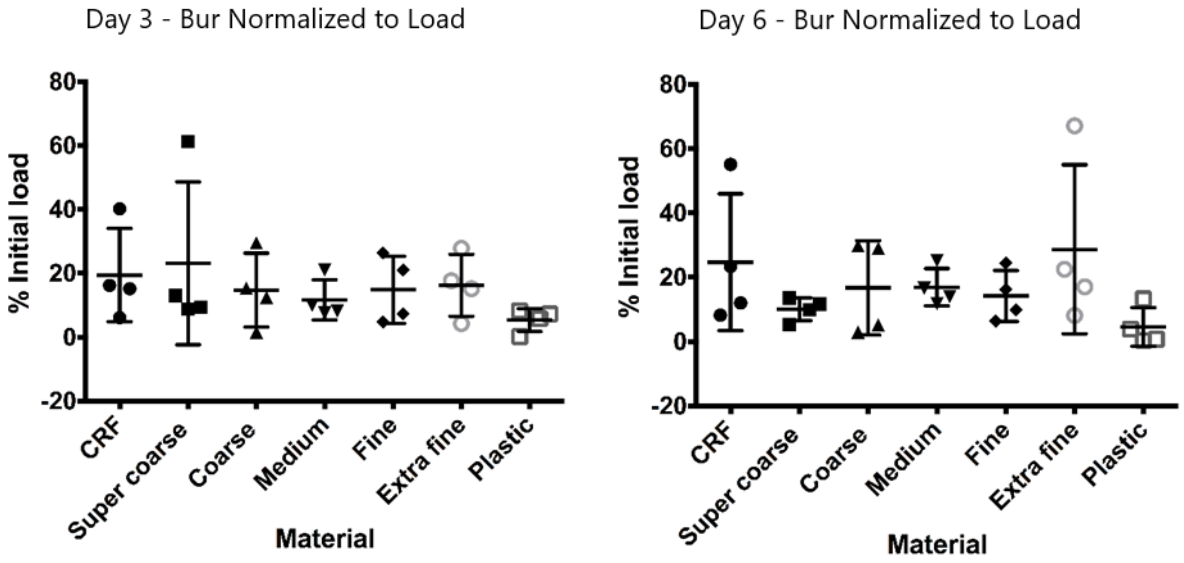
The complex pattern of changes in cell densities during the time course of each experiment, and the differences between experiments, suggested an interaction between multiple factors, such as initial adhesion, activation of cell division, and detachment (with time of initial of division and detachment, and extent of each, being further variables). Therefore, a routine growth analysis of

cell density during the time course of the experiment was precluded. Days 3 and 6 were therefore evaluated independently, using the same strategy as for Day 1.

By Day 3, most samples showed a cell density comparable to that at Day 1, although two values were markedly higher, consistent with cell growth (Figure 7). However, the four experimental values for a material were no longer biased towards the extremes. Day 6 showed no marked change from Day 3.

Figure 7: Graph of LD samples adjusted with burs and plastic Day 3 (a) and Day 6 (b) mean cell count/mm^2 normalized to initial load. The vertical bars show the mean and standard deviation.

a) b)

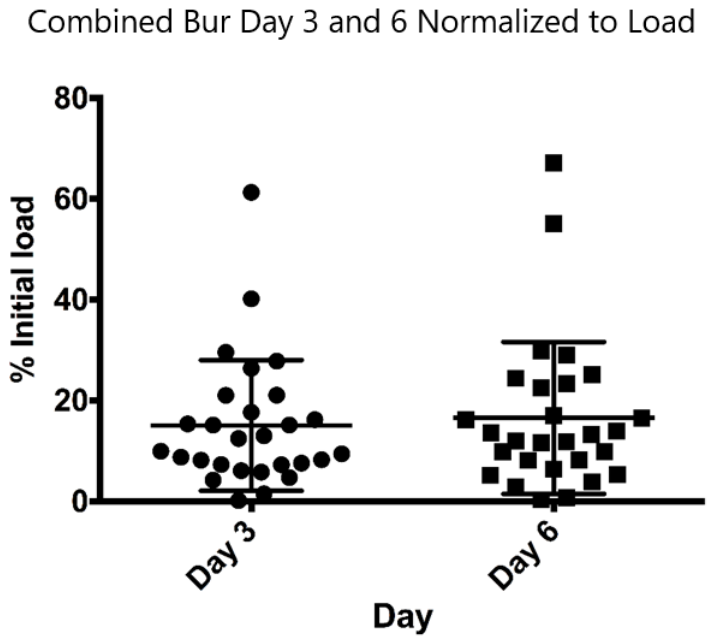


To further evaluate the Bur data, a one-way ANOVA was performed to compare the mean values (i.e., a single sampled population with a normal distribution was assumed). No significant differences in mean values were found for Day 3 ($p=0.65$) or Day 6 ($p=0.32$). That is, across the four experiments and

the seven surfaces evaluated, there was no significant difference between surfaces in the Day 3 or Day 6 cell density.

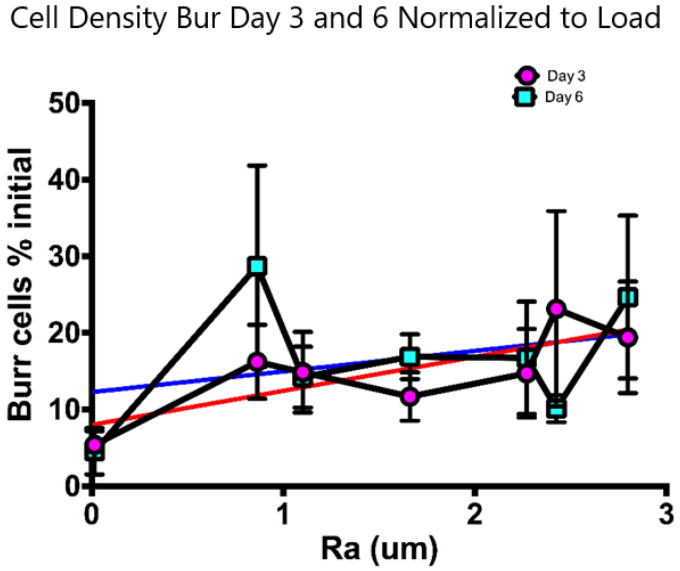
Given an absence of a significant effect of surface at Day 3 and Day 6, all 28 Day 3 and 28 Day 6 Experimental/Surface normalized cell density values were evaluated separately for a fit to a normal distribution. The overall means for Days 3 and 6 were respectively 15.1+13.0% and 16.6+15.1%, indicating an overall lack of net growth of cells during the six-day period (Figure 8). Both datasets failed both normality tests (DP $p<0.0001$, SW $p<0.0002$), and showed a high positive skewness (2.02, 2.01) and high positive kurtosis (5.20, 4.76), indicating a long tail to higher values. ROUT outlier analysis at a conservative Q=1 identified one potential outlier in Day 3 (Experiment 4, 61.3%, Super Coarse), and two high outliers in Day 6 (Experiment 4, 55.1%, CRF; 67.1%, Extra Fine). After removal of these outliers, Day 3 still failed both tests for normality ($p<0.03$), but Day 6 passed both ($p>0.27$). Day 3 showed skewness and kurtosis values just over 1 (1.12 and 1.19), while Day 6 values were 0.46 and -0.63. Since ANOVA is relatively robust towards minor deviations from normality, normality can be assumed for testing. There was no significant difference between means of these two groups (unpaired t-test, $p=0.69$). That is, overall, there was no net change in cell density between Days 3 and 6.

Figure 8: Scatterplot graph of LD samples adjusted with burs and plastic Day 3 and 6 mean cell count/mm^2 normalized to initial load. All 28 data values for each day are plotted. The vertical bars show the mean and standard deviation.



The Bur and Plastic experimental values were used to plot the mean normalized cell density against R_a value. As shown in Figure 9, there was no indication of a relationship between cell density and R_a value. Linear regression analysis showed no significant difference from zero for the Day 3 (p=0.10) or Day 6 (p=0.40).

Figure 9: Plot of LD samples adjusted with burs and plastic Day 3 and Day 6 mean cell count/mm^2 normalized to initial load against R_a value. The regression lines for the Day 3 and Day 6 curves are red and blue respectively. Vertical bars show SEM.



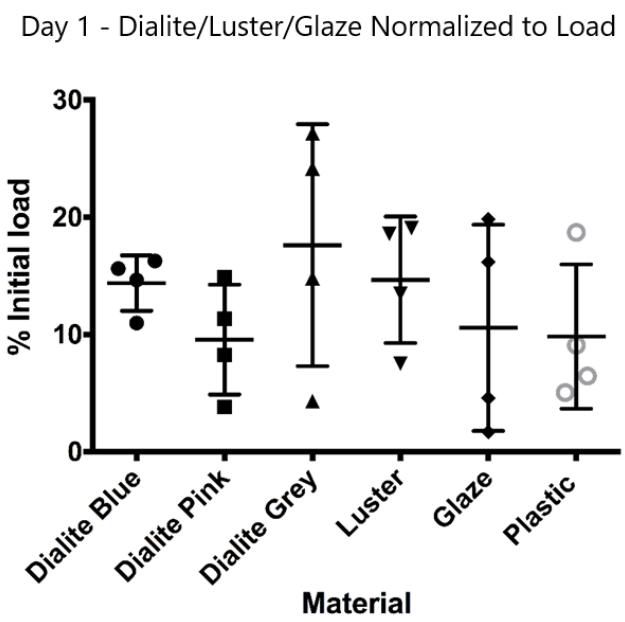
(f) Analysis of Day 1 cell counts on Dialite, Luster, and Glaze:

The same strategy employed to analyze Bur polished samples was used for these materials. The Glaze control was also compared to plastic. Inspection of a plot of the four experimental values for each material as replicates for the four polished surfaces revealed a similar low attachment (range of means 9.6-17.6%) and a large variance comparable to that seen for plastic, and for Glaze (unpolished) (Figure 10).

As before, the distribution of values tended to be to the extremes, suggesting a poor fit to a normal distribution and potential heterogeneity. However, n=4 replicates was too low for application of normality tests.

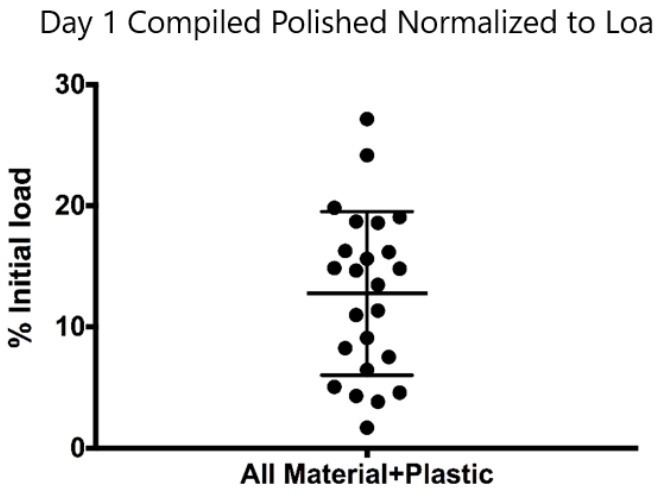
To further evaluate this material data, a one-way ANOVA was performed to compare the mean values. No significant differences in mean values were found (p=0.49). That is, across the four experiments and the six surfaces evaluated, there was no significant difference between surfaces in the Day 1 cell density.

Figure 10: Graph of Dialite, Luster polished material, Glaze, and plastic Day 1 mean cell count/mm^2 normalized to initial load. The vertical bars show the mean and standard deviation.



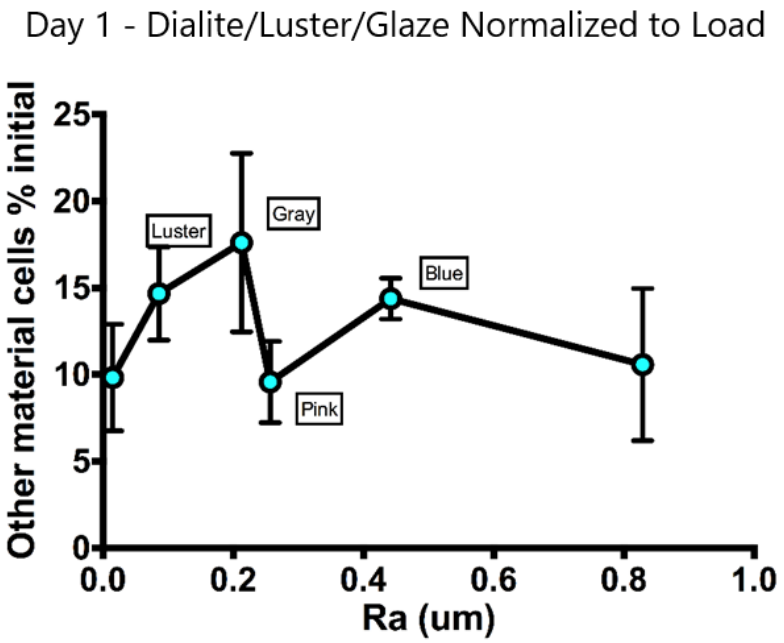
Given an absence of a significant effect of surface at Day 1, all 24 Day 1 Experimental/Surface normalized cell density values were evaluated for a fit to a normal distribution (Figure 11). The overall mean was 12.8+6.8%. The dataset passed both normality tests (DP p=0.74, SW p=0.97), with low skewness (0.21), and moderate negative kurtosis (-0.60).

Figure 11: Scatterplot graph of Other polished material, Glaze and plastic Day 3 and 6 mean cell count/mm^2 normalized to initial load. All 24 data values for each day are plotted. The vertical bars show the mean and standard deviation.



The Material, Glaze, and Plastic experimental values were used to plot the mean normalized Day 1 cell density against R_a value. As shown in Figure 12, there was a somewhat complex relationship between cell density primarily reflecting net attachment and R_a value. Cell density appeared to increase modestly at the lowest R_a values up to about 0.2 µm, then there was a sharp drop with Pink Dialite, then a rise with Blue Dialite. However, the previous one-way ANOVA test showed no significant difference between the groups.

Figure 12: Plot of Dialite and Luster polished material, Glaze, and plastic Day 1 mean cell count/mm^2 normalized to initial load against R_a value. Vertical bars show SEM.



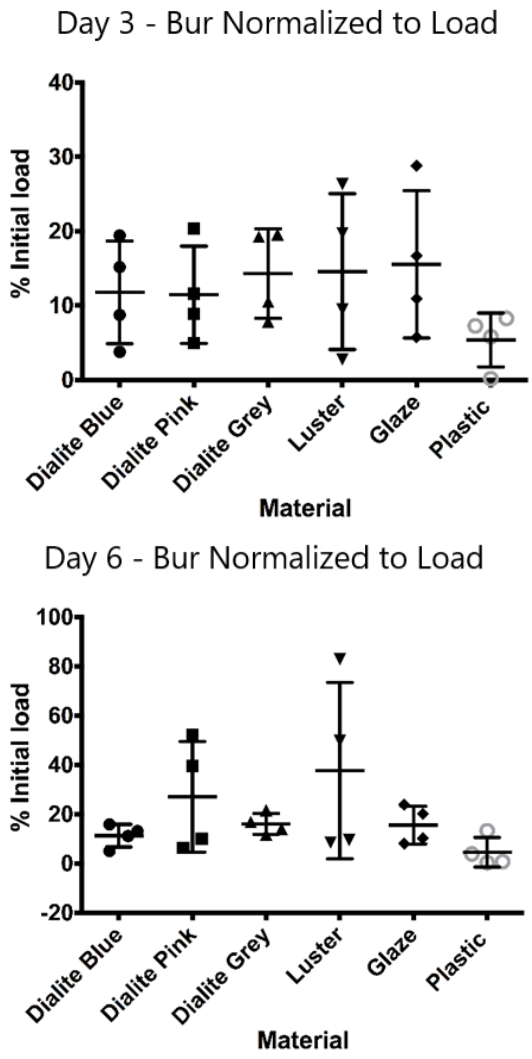
(g) Analysis of Day 3 and Day 6 cell count on Dialite, Luster, and Glaze samples

Days 3 and 6 were evaluated independently, using the same strategy as for Day 1. By Day 3, several samples showed a cell density rather higher than that seen with Bur samples at Day 1, but there was a wide range in values for a material and the mean values were not much higher than for Bur material (range 5.4-15.5%) (Figure 13). Day 6 showed no marked change from Day 3, although Luster showed one remarkably higher value (82.9%). There was an increase in the range of values for the means (4.6-37.6%), with Luster being the highest.

To further evaluate the Other Material data, a one-way ANOVA was performed to compare the mean values. No significant differences in mean

values were found for Day 3 (p=0.48) or Day 6 (p=0.17). That is, across the four experiments and the six surfaces evaluated, there was no significant difference between surfaces in the Day 3 or Day 6 cell density.

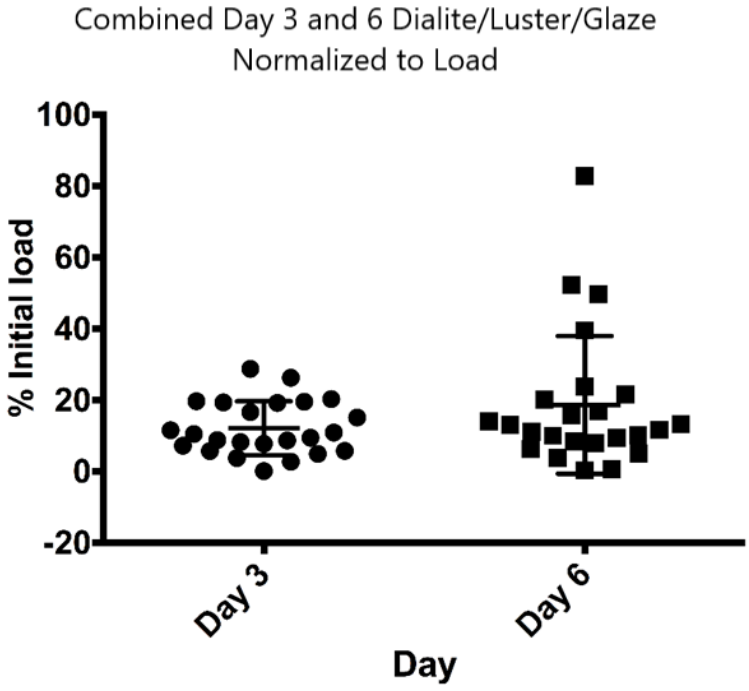
Figure 13: Graph of Other polished material, Glaze, and plastic Day 3 (a) and Day 6 (b) mean cell count/mm^2 normalized to initial load. The vertical bars show the mean and standard deviation



Given an absence of a significant effect of surface at Day 3 and Day 6, all 28 Day 3 and 28 Day 6 Experimental/Surface normalized cell density values were evaluated separately for a fit to a normal distribution. The overall means for

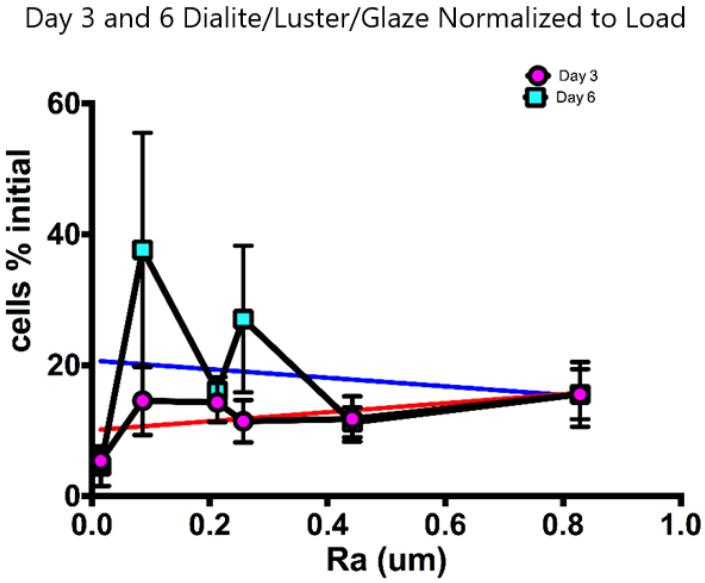
Days 3 and 6 were respectively 12.2+7.6% and 18.7+19.3%, indicating an overall lack of net growth of cells during the six-day period (Figure 14). The Day 3 dataset passed both normality tests (DP p=0.43, SW p=0.20), with modest skewness and kurtosis (0.58, -0.44). Day 6 failed both tests (both p<0.0001). ROUT outlier analysis at a conservative Q=1 identified three high outliers in Day 6 (Experiment 4, 52.3%, Dialite Pink; 82.9% Luster; Experiment 1 49.8% Luster). After removal of these outliers, Day 6 still failed both tests for normality (p<0.04). Day 6 excluding outliers showed skewness and kurtosis values of 1.41 and 3.37. The adjusted Day 6 mean was 12.6+8.8%, very similar to the Day 3 mean. That is, overall, there was no net change in cell density between Days 3 and 6.

Figure 14: Scatterplot graph of Other polished material, Glaze, and plastic Day 3 and 6 mean cell count/mm^2 normalized to initial load. All 24 data values for each day are plotted. The vertical bars show the mean and standard deviation.



The Other Material and Plastic experimental values were used to plot the mean normalized cell density against R_a value. As shown in Figure 15, there was no indication of a relationship between cell density and R_a value. Linear regression analysis showed no significant difference from zero for Day 3 (p=0.24) or Day 6 (p=0.66). Excluding plastic values from the analysis does not give a significant slope.

Figure 15: Plot of Other polished material, Glaze, and plastic Day 3 and Day 6 mean cell count/mm^2 normalized to initial load against R_a value. The regression lines for the Day 3 and Day 6 curves are red and blue respectively. Vertical bars show SEM.



(h) Analysis of Polishing modality at Day 1, 3, and 6.

Plastic was excluded from this analysis so that all comparisons involved LD. Glaze and Luster were initially excluded as there were only 4 experimental values for each day. All experimental values for the Bur and Dialite polishing modality were pooled giving 24 samples for Bur adjustment, and 12 for Dialite per Day.

Day 1, one-way ANOVA showed a highly significant effect ($p<0.0001$) comparing Bur High, Bur Low, and Dialite. Bur Low was significantly lower than Bur High (Tukey's multiple comparisons test; $p<0.0001$) and Dialite ($p=0.0004$), but Bur High and Dialite were not significantly different ($p=0.0004$).

Day 3 and Day 6, there was no significant difference between Bur and Dialite polishing (t-test with Welch's correction for different variance, p=0.20 and 0.52).

Lastly, the mean polishing method values were compared to the single mean Luster and glaze values Day 1, 3, and 6 by a one-sample t-test.

Day 1, the Bur High and Bur Low sample cell densities were respectively significantly higher and lower than the Luster value (p=0.0016, <0.0001), but Dialite was not different (p=0.69). Similarly, the Bur High and Bur Low sample cell densities were significantly higher and lower than the Luster value (p<0.0001, =0.0001) but Dialite was not different (p=0.13).

Day 3, the Bur and Dialite samples were not significantly different from Luster (p>0.26) or Glaze (p>0.11).

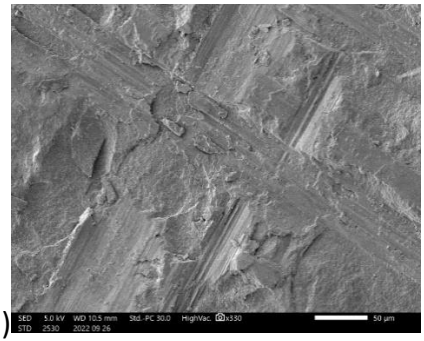
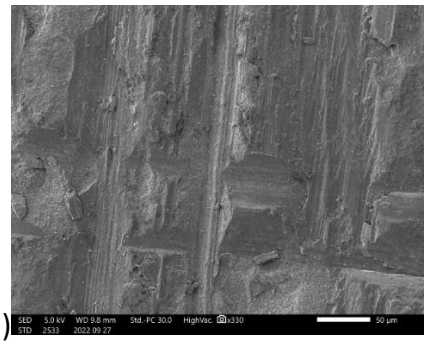
Day 6, the Bur and Dialite samples were significantly lower than the Luster value (p<0.0005), but not significantly different to Glaze (p>0.54).

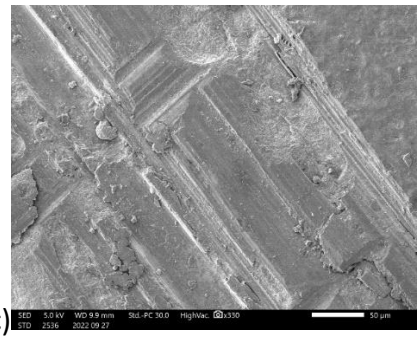
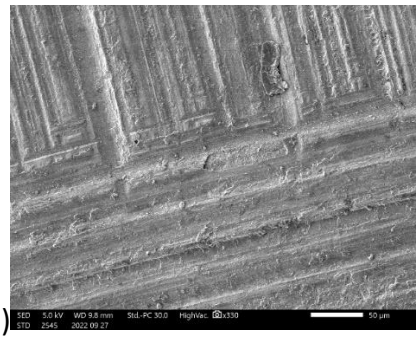
It should be noted that Luster Day 6 showed two lower values (8.0 and 10.2%) and two very high values (49.8 and 82.9), raising the possibility of the high/low cell pattern previously seen for Bur samples Day 1.

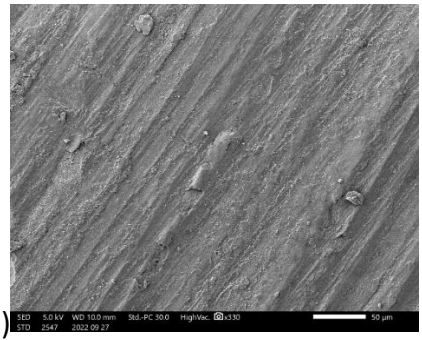
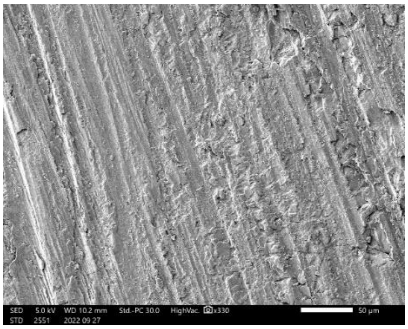
2) Scanning Electron Microscopy

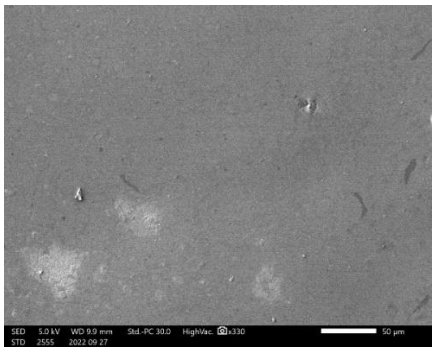
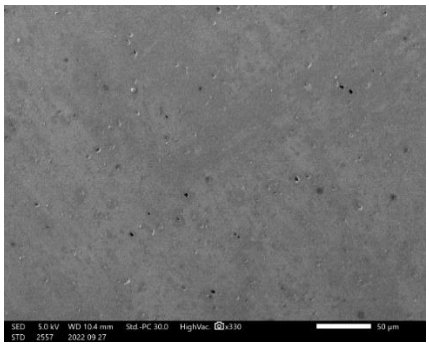
The images captured with SEM are shown in Figure 16.

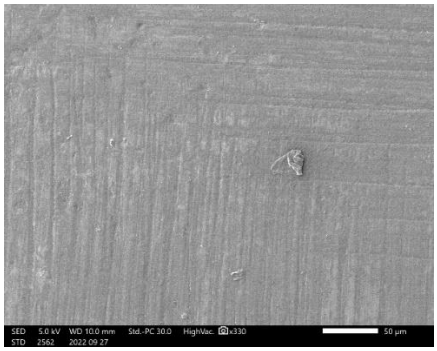
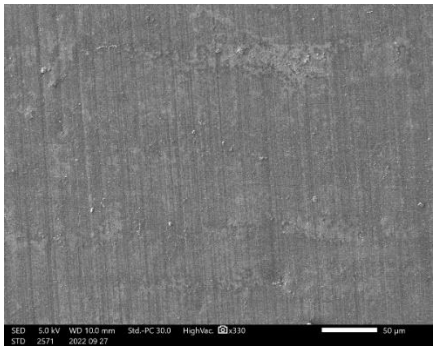
Figure 16. SEM images of LD surfaces under 330x magnification. Images (a)-(f) correspond to bur samples, (g) corresponds to the glazed sample, and (h)-(k) correspond to polished samples (h)-(k). From decreasing roughness of bur samples: CRF (a), Super Coarse (b), Coarse (c), Mehem (d), Fine (e), Extra Fine (f). Polished samples: Luster (h), Dialite Blue (i), Dialite Pink (j), Dialite Grey (k).

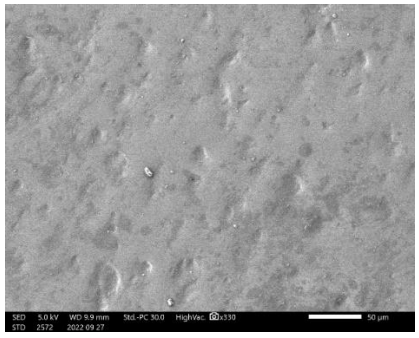
a)  b) 

c)  d) 

e)  f) 

g)  h) 

i)  j) 

k) 

DISCUSSION

This study investigated the growth of primary HGE cells on LD surfaces. Samples were polished with a polishing wheel method, a diamond bur, and a glaze. Samples with a range of roughness values, quantified as R_a, were tested over a 144 hr period. Growth on the samples were compared to the growth on tissue culture plastic, a control used routinely to characterize cell growth under laboratory conditions. Normally, tissue

culture cells will attach during the initial 24 hr period, but generally will not divide efficiently. During a following period of growth, cells enter an exponential growth phase, with the rate of cell division depending upon the cell line. Then as cells reach a higher density they often undergo contact inhibition and cease dividing, although cancer cells generally show poor, if any, inhibition.

Primary cells become subject to senescence over a certain number of passages, and the media can be unstable, resulting in poor growth. Cells grown on plastic should display exponential growth during at least part of the 144 hour growth period. The tissue culture plastic was used as the control and to determine the validity of the experiment design. The control was also used to assess the cells' behavior on a test material.

Measurement of the primary HGE cell density on plastic over the six-day experimental period in four independent experiments gave a pattern that was inconsistent with an exponential growth model; overall, no similarity to the normal pattern of tissue culture growth was observed.

The cells and/or media showed considerable inconsistency between experiments, both for growth on plastic and the LD surfaces. Moreover, examination of the Day 1 overall cell density on individual material discs or plastic revealed a divergence into two groups of values, one with relatively high and the other with relatively low cell densities. This suggested heterogeneity in cell behavior both between and within experiments. Such heterogeneity could

indicate different populations of cells either in the primary culture seeded into the wells, or selection for different populations during replicate experiments.

At least four factors appeared to combine to influence the cell number per unit area at any time:

1) Initial cell adherence (which was generally relatively poor)

2) Cell detachment (which could vary in timing and magnitude)

3) Cell proliferation (which will be primarily exponential following a lag period until a plateau phase is entered, dependent partially on initial cell load)

4) The material substrate.

Future testing of the relationship between growth of primary human gingival epithelial cells and LD surfaces would require validation of the growth pattern of the cells, which would need to show efficient, uniform cell attachment, low detachment, consistency in cell numbers between replicates, and exponential growth.

This number of variables and the observed inconsistency precluded a statistical testing of the null hypothesis of no relationship between growth and surface roughness. However, a qualitative evaluation and the limited statistical testing that could be performed, suggested that LD can be as good as plastic for initial attachment, and

depending on the surface treatment, could be better. Similarly, LD could potentially be better than plastic at promoting net growth.

The confounding factors relating to erratic cell behavior (initial cell adherence, detachment, and proliferation) may be related to the combining of HGE cells from different passages. An optimal number of viable cells were needed to conduct each experiment, and to do so required harvesting the requisite number of cells by growing multiple batches of cells simultaneously. The heterogeneity of cell growth patterns may be, in large part, due to the presence of different cell batches in each Experiment.

Aside from the number of variables and inconsistency, as in studies by Smallidge et al. and Wilding et al., a limitation of this study regarding growth pattern was that sampling was confined to three time points, thus limiting the power of regression analysis. Further clarification of the specific effects of R_a on cell growth would require more frequent sampling. Another limitation of the current study is that it only described the topology of the experiment surfaces with a linear R_a value. A further limitation is that stylus-based measuring instruments, rather than optical instrumentation, were used to measure surface roughness. The size of the physical stylus limits the instrument's scanning resolution and could possibly affect the measurements, especially on smoother surfaces. Average peak and valley heights to include area measurements like S_a could be used to better understand the cells' interaction with material surface.

The current study was done in a sterile environment and some samples had an R_a at 0.086 μm, which is much lower than the 0.2 μm as previously suggested in the literature to be a minimal cut off for soft tissue attachment to implant abutments.[6]

There is a possibility that Luster polishing could allow for better cell performance, but further work would be required to test this.

Surfaces polished using the extraoral Luster polishing kits showed an R_a value of 0.086 µm, which is below the threshold value. The glazed samples gave an R_a of 0.829 µm. The surfaces treated with grinding gave a range of 0.864-2.798 µm. Even with such a small number of samples, following manufacturer's recommendations for polishing still produced R_a values below the threshold for cell attachment and growth. It is likely that clinicians will be able to polish LD to a surface smooth enough to maximize he growth.

Although the results of this current study could not fully test the null hypothesis, it is important to consider that microbial biofilm development is inhibited by smooth surfaces. LD should be polished to the lowest reasonable R_a value to maximize the desired outcome for hygiene, therefore the HGE cells will respond to the material and not to a build-up of biofilm.

CONCLUSIONS

There was no consistent pattern of growth or attachment of cells in relation to surface roughness of LD, and growth on LD was comparable to growth on plastic. Initial cell adherence, detachment, proliferation, and material substrate were confounding factors limiting statistical analysis. Broadly, within the limitations imposed by the cells, no evidence was found that the method of polishing LD, or the achieved surface roughness, had a marked effect on HGE cell behavior, and growth on LD was

comparable to growth on plastic. Further studies are required to address the detailed effects of LD surface roughness and polishing methods on primary HGE cell biocompatibility. However, such studies will require the identification of sources of cells that show consistent and reproducible growth.

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