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https://www.usuhs.edu/pdc

THESIS APPROVAL PAGE FOR MASTER OF SCIENCE IN ORAL BIOLOGY

Title of Thesis: "Human Gingival Epithelial Growth In-Vitro on a Polymer-Infiltrated Ceramic Network Restorative Material"

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Master of Science Degree
June 13, 2018

THESIS/MANUSCRIPT APPROVED: DATE:

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Human gingival epithelial growth *in vitro* on a polymer-infiltrated ceramic network restorative material.

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The views expressed in this manuscript are those of the authors and do not necessarilyreflect the official policy of the Department of Defense, Department of Army, US Army Medical Department, or the US Government.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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ABSTRACT

Statement of Problem. Soft tissue health around an implant restoration is important in preventing infection of peri-implant tissues. There has been little investigation of the effect of surface roughness of implant restorations on gingival cell growth.

Purpose. To investigate the growth of primary human gingival epithelial (HGE) cells on polymer-infiltrated ceramic network (PICN) material (Vita Enamic) with different surface roughnesses.

Material and Methods. PICN material samples were polished with either silica carbide paper (grit-polished) or the manufacturer's polishing wheels (wheel polished), and the surface roughness (R_a) measured. HGE cells were seeded and grown for 1, 3, or 6 days. Growth on tissue culture plastic was used as a control. Non-linear regression analysis was used to examine the effect of surface roughness on cell growth.

Results. HGE cell growth on tissue culture plastic fitted an exponential growth model over the six day experimental period ($r^2 = 0.966$). Through Day 6, cell density on PICN decreased with increasing surface roughness, with a fit to an exponential decay model ($r^2 = 0.666$). A threshold R_a value of 0.254 μm (95% CI 0.177-0.332) was determined as an upper limit for exponential growth. Samples polished by the manufacturer's method produced surface roughness below 0.245 μm.

Conclusions. PICN material (Vita Enamic) polished to a smooth surface ($R_a < 0.25$μm) provided improved epithelial cell growth compared to rough surfaces. The manufacturer's polishing method achieved a sufficiently smooth surface. These *in vitro* results suggest that smoother restorative material surfaces could improve peri-implant soft tissue health.

CLINICAL IMPLICATIONS

Human gingival epithelial (HGE) cells were found to grow exponentially and more effectively on increasingly smooth surfaces of a polymer-infiltrated ceramic network material. These findings suggest that the subgingival portion of implant-supported restorations should be polished to a R_a value of less than 0.127 μm to improve soft tissue integration.

INTRODUCTION

Computer-aided design/computer-aided manufacturing (CAD/CAM) technologies have made possible the manufacturing of implant-supported restorations in the dental office. A challenge for this technology has been the availability of materials that can be milled and delivered in just a single appointment. In 2013, a polymer-infiltrated ceramic network (PICN) material (VITA Enamic; VITA Zahnfabrik, Bad Säckingen Germany) was introduced to the market for CAD/CAM milled indirect restorations. PICN consists of a resin polymer network structure comprised of triethylene glycol dimethacrylate (TEGDMA) and urethane dimethacrylate (UDMA), surrounded by a leucite-reinforced, zirconia-containing feldspar ceramic.[1] The physical properties of the material have been investigated and it is now marketed for fabricating full contour implant-supported crowns.[1-5] The manufacturer has developed specific polishing kits for laboratory and intraoral polishing.

Implant-supported restorations replace the crown portion of the tooth but also have a transgingival component extending to the platform of the supporting implant. A biologic seal, similar to the periodontal structure surrounding natural teeth, forms around the restoration and implant, preventing bacterial invasion and adverse host responses that can lead to implant loss.[6-10] Biocompatibility is a critical aspect of the interaction between the restoration surface and surrounding tissues.[11] It is important to understand the biocompatibility of our restorative materials to achieve optimal clinical outcomes. Studies have shown that PICN material is not cytotoxic due to the processing method of the resin components.[12]

Surface roughness is a component of biocompatibility affecting host cellular adhesion and proliferation (net growth) across a material surface, and potentially adverse bacterial colonization. A review by Bollen, Lambrechts, and Quirynen found that reducing the surface

roughness of the surface of various restorative materials below a threshold R_a of 0.20 µm would not have an impact on reducing bacterial adhesion.[13] An *in vivo* study from this group found surfaces with roughness below the 0.20 µm threshold had no further reduction in biofilm.[14] Regarding soft tissue response to surface roughness, another *in vivo* study showed that different material types can affect the soft tissue health surrounding dental implants.[15] Other *in vitro* studies have suggested that differences in surface roughness of commercially prepared titanium surfaces did not affect soft tissue outcomes.[7] More research is needed to understand the influence of ceramic materials on soft tissue integration.

The purpose of this study was to investigate *in vitro* the effect of surface roughness (R_a) of a PICN material on the growth of primary human gingival epithelial (HGE) cells. The effect of surface roughness on cell growth on the material surface was observed compared to cell growth on tissue culture plastic. The null hypothesis was that PICN surface roughness would have no effect on cell growth.

MATERIAL AND METHODS

Blocks of the PICN material were sectioned using a water-lubricated diamond cutting blade on a precision saw (Buhler; Uzwil, Switzerland), resulting in rectangular disks with sample dimensions of 14 mm x 12 mm x 1 mm. To investigate the general effect of roughness on cell growth, silica carbide sand paper (150-2500 grit) was used to generate grit-polished samples all prepared by the same sanding method (grit-polished). This polishing method created a set of samples with a wide range of surface roughness values. To evaluate the effect of the manufacturer's polishing instruments (wheel-polished) on cell growth, coarse and fine wheels (Vita Enamic Polishing Kit; VITA

Zahnfabrik, Bad Säckingen Germany), were used to polish seven disk samples. A stylus-type profilometer (Mahr Perthometer M2, Providence, RI) was used to measure surface roughness, determined as R_a (μm) values. Three measurements were made per sample and the mean R_a calculated. After preparation and measurement, the samples were assigned, at random, into three groups of samples, such that each group had a uniform distribution of roughness values covering the broadest R_a range (n=9, 8, and 9 disks for Days 1, 3 and 6 respectively).

To prepare the disk samples for tissue culture the following cleaning protocol was developed. First, the samples were steam-cleaned (Triton; BEGO GmbH & Co. KG, Bremen Germany) for 10 seconds on each surface followed by sonication in distilled water for 10 minutes. Samples were placed in 70% ethanol in a sealed sterile container and sonicated for 10 minutes. The ethanol wash was then repeated once. The samples were placed into an antibiotic solution of 100 U/ml penicillin 100 ug/ml streptomycin (Atlanta Biologicals, Flowert Branch, GA 30542), 50 ug/ml gentamicin (Atlanta Biologicals, Flowert Branch, GA 30542) and 1.25 ug/ml Amphotericin B (Corning Mediatech, Inc. Manassas, VA 20109) in sterile saline and sonicated for 10 minutes. Following the cleaning protocol, disks were placed in the wells of sterile 12-well tissue culture plates with the single polished surfaces facing upward. The same set of samples was cleaned, using the cleaning protocol described above, and re-used for replicate experiments. Fluorescence microscopy, as described below, was used to confirm removal of epithelial cells following the cleaning protocol. No bacterial contamination was observed through the study.

Pooled-donor primary HGE cells (CELLnTEC, Bern, Switzerland) were used. Primary HGE 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), and then incubating cells with 3.0 ml Accutase solution (CELLnTEC) for twelve minutes while tapping the flask at two minute intervals and observing detachment 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. Cell density was determined using a hemocytometer.

Samples were seeded onto 12- well tissue culture plates. Eleven wells contained one disk sample each and one well was used as a plastic surface positive control. Samples were placed with the polished side up. Cells were seeded at a density of 500 cells/mm^2. The cells were incubated at 37^{o}C for 1, 3, or 6 days prior to assay in a CO_2 tissue-culture incubator.

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 wells of a new 12-well plate were filled with 2.0ml of

magnesium and calcium free PBS, and the samples were transferred to the new plate and placed cell surface down for microscopic observation.

Imaging of the disks 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 disk and the control well, distributed over the entire material sample area in a 4x5 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 counts.

Non-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. Exponential growth of HGE cells on tissue-culture plastic was evaluated using fit to an exponential model, for both replicates within a single experiment (to evaluate the quality of cell growth within the experiment), and for replicate experiments (n = 3). To evaluate growth of cells on PICN material at different surface roughness, an exponential decay model was fitted to the Day 6 data, according to the following equation:

$$Y\ [\text{cell density}] = (Y_0\ [\text{cell density at } R_a \text{ zero}] - \text{plateau [cell density at infinite } R_a]) * \exp(-K * R_a) + \text{plateau, where K is the rate constant } (1/\mu m)$$

Further analysis was completed using a segmental linear regression to estimate the limit R_a value for inhibition of cell growth.

RESULTS

Low passage number primary HGE cells grown on tissue culture plastic gave similar numbers of cells on Day 6 in three replicate experiments, and the cell numbers fit an exponential growth model over the six days of growth ($r^2 = 0.966$, Fig. 1), with a mean doubling time of 1.72 days (95% confidence interval (CI) 1.37-2.31 days).

For the grit-polished PICN material, there was no evident effect of surface roughness on cell growth on Day 1; all R_a values showed similar limited growth (fit to a linear model showed no significant difference from slope = 0; F-test, $p = 0.33$), with the cell density comparable to that seen on plastic on Day 1 (one-sample t-test; $p = 0.15$), consistent with initial cell attachment to both surfaces. By Day 3, there were indications of a trend to higher cell densities on lower R_a surfaces. On higher R_a surfaces, the cell densities were not markedly higher than those seen at Day 1, suggesting cessation of growth on rougher surfaces. However, on Day 3, the increases in cell growth at different R_a values were not statistically significant ($p = 0.13$). The overall mean cell density on Day 3 across all Ra values was 209 cells/mm^2, which was significantly lower (one sample t-test, $p < 0.0001$) than the regression mean for Day 3 growth on plastic of 339 cells/mm^2 (209/339 = 61.7%), consistent with overall slower growth on PICN compared to plastic.

By Day 6, the growth on PICN samples with lower R_a surfaces was markedly greater than the growth on higher R_a surfaces (Fig. 2). To further analyze the Day 6 growth with respect to R_a, non-linear regression was used to fit the data to a one-phase exponential decay model (Fig 3). The data from three replicate experiments showed a good fit ($r^2 = 0.666$), with a determined plateau value (i.e. limit cell density at high R_a) of 199 cells/mm^2 (95% CI 67-331), not different from the mean cell density at Day 3 (209), indicating that minimal growth occurred between Day

3 and Day 6 on high R_a surfaces. This value was only 17.7% (199/1122) of the mean Day 6 cell density on plastic estimated by regression analysis. Y_0 (cell density at $R_a = 0$) had a best fit value of 989 (95% CI 670-1307), not significantly different from the estimated Day 6 growth on plastic (1122 cells/mm^2), indicating that growth on low R_a surfaces was comparable to growth on plastic. To estimate the R_a value at the transition from growth inhibition (compared to plastic) to continued growth through Day 6, segmental linear regression (a simplification of the exponential decay model) was performed, constraining the slope of the line at high R_a values to zero (Fig. 4). A R_a value of 0.254 μm (95% CI 0.177 - 0.332) was determined as the point of intersection between lines of best fit of high R_a values and low R_a values. That is, surfaces with a Ra $\geq$ 0.254μm would be expected to show an inhibition of growth.

The effect of R_a value on growth inhibition was further examined over the six day growth period by dividing the disk samples into three roughness categories (low $R_a \leq 0.127$, medium $R_a > 0.127$-≤ 0.254, and high $R_a > 0.254$), and non-linear regression was used to fit the cell density data at Days 1, 3 and 6 to an exponential growth model (Fig. 5). Growth on both low and medium R_a surfaces gave a very good fit to the model ($r^2 = 0.884$ and 0.841). The high R_a gave a poorer fit to this model ($r^2 = 0.439$), and gave a comparable fit ($r^2 = 0.445$) to a straight line, slope 20.2 cells/mm^2/day (95% CI 0.04583 to 40.36), consistent with poor growth on high R_a surfaces over the experimental period, as determined above. Growth on low and medium Ra surfaces gave doubling times of respectively 2.37 days (95% CI 1.68 - 4.02) and 2.97 days (2.05 to 5.38), which were not significantly different to the estimated 1.72 days (95% CI 1.37 - 2.31) doubling time for growth on plastic.

Samples polished with the manufacturers recommended instruments (wheel-polished) showed a relatively narrow range of R_a values (0.066 - 0.253 μm), all below the 0.254 μm cut-

off identified above for growth inhibition. Similar to grit-polished disks, cell growth on wheel-polished disks was modest through Day 3. The two wheel-polished surfaces (R_a values 0.100 and 0.253 μm) tested on Day 6 (n = 3 replicate experiments) showed mean cell densities of 650 and 376 cells/mm^2 respectively. The segmental linear regression equation for grit-polished low R_a samples was used to estimate predicted cell density values of 608 and 237 cells/mm^2 respectively for 0.100 and 0.253 μm; these values were not significantly different from those measured for the wheel-polished surfaces (one-sample t-tests; $p \geq 0.07$). That is, wheel-polished surfaces and grit-polished surfaces of comparable R_a gave similar growth, indicating no marked differences in growth for the two polishing methods.

DISCUSSION

In this study, the growth of primary HGE cells on PICN material surfaces was investigated. Samples were polished with either a grit-polishing or wheel polishing method. Samples with a range of roughness values, quantified as R_a, were created. Cell growth was measured over a six day period, and results were compared to growth on tissue culture plastic, a control used routinely to characterize cell growth under laboratory conditions. HGE cell density on plastic over the six day experimental period was found to fit an exponential growth model, consistent with cell growth.

The results of this study showed that cell growth on PICN material was affected by R_a value, with Day 6 cell density versus R_a values showing a good fit to an exponential decay model. By Day 6, HGE cells grown on PICN samples with low R_a values had a cell density comparable to growth on plastic, and substantially greater than that of cells grown on PICN samples with high R_a surfaces. Evaluation of growth rates over the six day period was consistent

with a lower rate of cell division at higher R_a value, and potentially cessation of growth between Days 3 and 6, as evidenced by lack of an overall significant increase in cell number between these days. One limitation of this study regarding growth patterns was that sampling was confined to three time points, limiting the power of regression analysis. Further clarification of the specific effects of R_a on HGE cell growth would require more frequent sampling, and measurement of mitotic index. Nonetheless, a clear effect of R_a on cell growth was observed, and therefore, the null hypothesis of no relationship between growth and roughness was rejected.

Segmented linear regression analysis of Day 6 data revealed a threshold value of 0.254μm, above which maximal inhibition of cell growth occurred. HGE cells grown on PICN material with surface roughness below a R_a value of 0.254μm showed an exponential increase in growth with respect to decrease in Ra. Since maximum growth of epithelial cells on the transgingival surface of implant supported crowns is desired, PICN material should therefore be polished to a R_a value below 0.254 μm to avoid maximal inhibition of growth. This is in contrast to the findings of previous studies. [13-14] Within the limitations of this study, to maximize HGE growth, PICN material should be polished to a roughness ideally below 0.127μm.

One limitation of the current study is that it only described the topology of the experiment surfaces with a linear Ra 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 instruments scanning resolution and therefore measurement values, 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.

Surfaces polished using the VITA Enamic polishing kit followed by diamond paste on a Robinson brush gave R_a values ranging from 0.049-0.253 μm, all below the threshold value; those polished with the VITA Enamic fine wheel had values between 0.049-0.118 μm. Although only a small number of disks were examined, growth on these wheel-polished surfaces was comparable to that seen on grit-polished surfaces. Therefore, if the manufacturer's recommendations for polishing are followed, it is likely that clinicians would obtain a sufficiently smooth surface to maximizing epithelial cell growth.

The current study did not quantitatively evaluate the growth of HGE cells on surfaces smoother than 0.049 μm, leaving open the possibility that growth could be inhibited on smoother surfaces. However, this value of 0.049 μm is much lower than the 0.2 μm previously suggested in the literature to be a minimal cut off for soft tissue attachment to implant abutments.[14] Since biofilm development is inhibited by smooth surfaces and the present study shows improvement in epithelial growth on smooth surfaces, it is possible that the PICN material should be polished to the lowest reasonable R_a value to maximize the desired outcome for both hygiene and host response.

CONCLUSIONS

Polishing PICN material as smooth as possible (below a R_a of 0.127μm) was found to maximize epithelial cell growth on the PICN material surface. Following the manufacturer's polishing protocol was found to achieve this goal. Further research is needed to further understanding of the soft tissue integration to implant prosthetic surfaces.

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FIGURES

Fig. 1. Non-linear regression fit of an exponential growth model to mean cells/mm^2 (average of 20 fields) from replicate experiments (n = 3). The mean and SEM are shown.

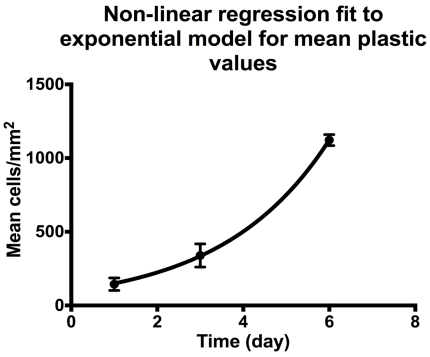

Fig. 2. Cell growth on PICN material surfaces of different roughnesses. Mean cells/mm^2 values at Days 1, 3 and 6 over the tested R_a range are shown. Symbols and bars show mean and SEM (n = 3 experiments).

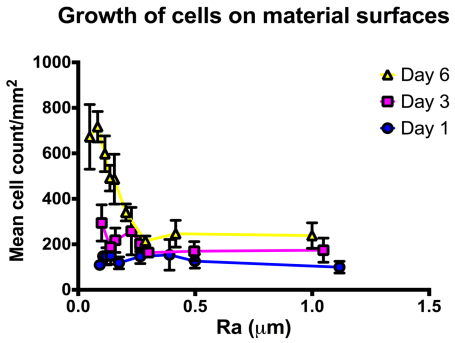

Fig. 3: Non-linear regression analysis of exponential decay model fit for Day 6 cell growth. Each symbol represents a mean field value for each tested disk within one of the three experiments (each experiment represented by a different color). The curve shows the regression fit.

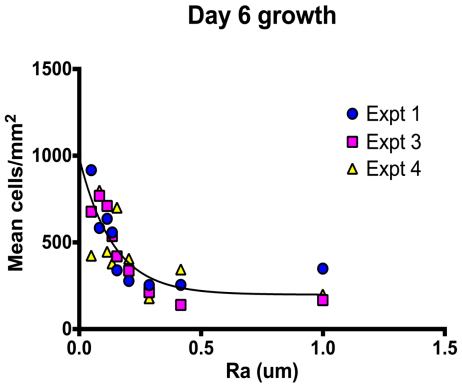

Fig. 4: Segmental linear regression analysis of fit for Day 6 growth. Each symbol represents a mean field value for each tested disk within one of the three experiments (each represented by a different color). The two lines show the segmental linear fits to the data.

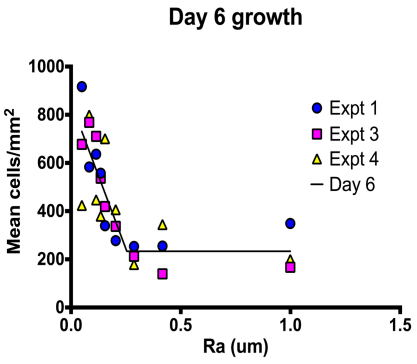

Fig. 5: Non-linear regression analysis of fit to exponential growth for cells growing on groups based on a range of Ra. The mean (n = 3 experiments) and SEM are shown. The curves show the regression fit of the exponential growth model.