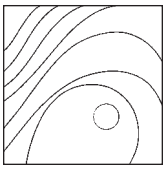
Biologic Behavior of Pressed Lithium Disilicate Ceramic and Zirconia on Human Gingival Fibroblasts: An In Vitro Study



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This study evaluated in vitro the biologic profile of manually polished surfaces of pressed lithium disilicate (LD) ceramic compared to zirconia (Zir) in human gingival fibroblasts. Samples with a 10-mm diameter and 3-mm thickness were used. After manual polishing, the average roughness (Ra) of the samples was measured. The cell proliferation and viability of gingival fibroblasts on the surfaces were assessed at 24, 48, and 96 hours. Additionally, the morphology, cell adhesion, and type III collagen (COLIII) and vimentin (VIM) expression by fibroblasts plated onto these surfaces was analyzed. Polystyrene (Pol) was used as control for all assays. The mean Ra was 0.261 ± 0.053 μm for Zir and 0.345 ± 0.130 μm for LD. Both surfaces presented similar cell proliferation and viability (P > .05). The cell morphology demonstrated that, for both surfaces, the cells were occasionally spindle-shaped, parallel to the direction of the grooves. Compared to Pol, an upregulation of COLIII and VIM gene expression was observed by fibroblasts cultured on Zir and LD at all time points (P < .05). The characteristics presented by LD and Zir surfaces after manual polishing protocol were similar and had biologically favorable performances, thus suggesting LD as a suitable alternative to Zir in the peri-implant region for esthetic purposes.
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The surface of the material used for an abutment should allow the gingival tissue to form a barrier around the implant abutments, which will serve as a seal between the oral environment and the bone adjacent to the implant, as the integrity of this region is a crucial aspect of long-term treatment success.[1,2] However, the esthetic component may require materials other than titanium in the peri-implant region; ceramic abutments have been used instead, especially in the anterior maxilla.[3]

In recent years, increasing interest in metal-free dental restorations has led to innovative research and development of ceramic materials with excellent optical properties and enhanced mechanical characteristics in comparison with the first feldspathic dental ceramics. The most visible advantages of such ceramics are translucency, natural esthetics, shade stability, low retention of bacterial biofilm, low fluid absorption, high hardness, wear resistance, low thermal conductivity, and chemical inertia.[4]

In particular, the use of lithium disilicate (LD) and zirconia (Zir) have become increasingly widespread in dental practice for supporting crowns, structures, and abutments, especially in areas of high esthetic need. In vitro and in vivo studies have been carried out comparing titanium and yttria-stabilized

polycrystalline tetragonal zirconia abutments.[5,6] Both surfaces showed adequate conditions for healing of the peri-implant tissues,[6] with similar clinical results in terms of tissue biocompatibility and biomechanics.[7] Although the mechanical parameters and the clinical performance of LD have been extensively studied, little information is available in the literature regarding the biologic properties of the ceramic surfaces used in the peri-implant region in contact with the gingival tissue. Some studies have shown that these materials may threaten tissue homeostasis.[8–11]

This in vitro study aimed to evaluate the biologic profile of the LD ceramic surface and compare it to that of Zir used in the peri-implant region. A manual polishing protocol was established in order to obtain parallel grooves, and human gingival fibroblasts were cultured on the surfaces.

Materials and Methods

Experimental Groups

Ceramic discs (10-mm diameter, 3-mm thickness) were prepared for this study, both in LD (n = 36; IPS e.max Press, Ivoclar Vivadent) and Zir (n = 36; Block Zirconn, VIPI Odonto Products), in compliance with the manufacturers' protocols.

After obtaining the discs, the surfaces were manually polished to a standard that could be reproducible in a clinical setting. Using a sequence of two types of polishing discs for high shine (Europol Plus, Eurodental), a surface pattern was achieved that included parallel grooves to guide cell growth.[12–14]

To polish the surfaces, an electric motor (LB2000, Beltec) was used at the speed of 10,000 rpm in a standardized, straight vertical or horizontal direction.

The discs were subsequently washed and thoroughly rinsed in deionized water three times for 5 minutes each in an ultrasonic bath (Ultrasonic Cleaner, Cristófoli Biossegurança), packaged and autoclave-sterilized (Autoclave Vitale 12, Cristófoli Biossegurança).[14] In all experiments, polystyrene (Pol) was used as a control (n = 36 Pol discs).

Average Surface Roughness

The surface roughness (Ra) after manual polishing was determined using a stylus instrument (Surftest SJ-200 profilometer, Mitutoyo). Four linear measurements were performed on each sample according to ISO standards,[15] and the arithmetic average roughness was calculated for each sample.

Cell Cultures

Gingival fibroblasts were obtained from explants of healthy attached human gingiva from three different donors, which were harvested during periodontal surgery for crown lengthening.[16,17] This study was approved by the São Leopoldo Mandic Review Board (no. 13683713.0.0000.5374).

The cells were cultured in Dulbecco's Modified Eagle Medium (Sigma-Aldrich) supplemented with 1% antibiotic antimycotic solution (Sigma-Aldrich) containing 10% donor calf serum (Gibco, Thermo Fisher Scientific), then plated onto plastic culture dishes (60-mm diameter) and incubated under standard cell culture conditions (37°C, 100% humidity, 95% air, and 5% CO_2). Once the cells reached subconfluence, they were detached using 0.05% trypsin and subcultured at a density of 110 cells/mm^2. The cells were used at subculture levels 3 or 4 for all experimental assays.

Cell Proliferation and Viability Assays

The cells were cultured on 96-well plates at an initial concentration of 110 cells/mm^2. After 24, 48, and 96 hours, cells from three random wells were detached using 0.05% trypsin and counted in a hemocytometer (Neubauer chamber, Global Optics) to calculate proliferation indices. Using a different set of plates, with the same conditions as previously described, 10 µL of MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazolium bromide; 5 mg/mL in phosphate-buffered saline [PBS]) and 90 µL of base medium were added to each well. Cells were incubated at 37°C for 3 hours in 5% CO_2, 95% O_2, and 100% humidity. After 3 hours, the MTT solution was removed and replaced with 100 µL of dimethyl sulfoxide. The plate was incubated for another 15 minutes at room temperature, and the optical density of the wells was determined using a SpectraMax Plus microplate

reader (Molecular Devices) at a test wavelength of 590 nm. The experiments were performed twice under the same conditions to ensure accuracy.

Cellular Morphology

SEM analysis

For analysis of cell growth morphology, cells seeded onto different surfaces after 48 hours were covered with Karnovsky fixative solution (2% paraformaldehyde, 2% glutaraldehyde, and 3% sucrose), followed by a buffer solution (0.1 M sodium cacodylate, pH 6.4 to 7.4). The specimens were then rinsed in the same buffer (0.05 M), post-fixed in 2% osmium tetroxide for 1 hour, then dehydrated through a graded series of ethanol. The samples were mounted on stubs and placed on a sputter coater (EM ACE600, Leica) to receive an 8-nm platinum coverage prior to scanning electron microscopy (SEM) analysis (Quanta 250 FEG, FEI).

Indirect immunofluorescence

The morphology was also evaluated using vimentin (VIM) cytoskeleton protein expression. Cells grown on samples during 48 hours were fixed in methanol for 6 minutes at 20°C and rinsed in PBS, followed by blocking with 1% bovine albumin in PBS for 30 minutes at room temperature. The primary antibody used was vimentin (V9, mouse, 1:300; Dako). Control staining reaction was performed using PBS as nonimmune immunoglobulin G (IgG) at the same dilution used for the primary antibody. The secondary antibody used was biotinylated anti-mouse IgG (Vector Laboratories). The preparations were washed and mounted using Vectashield with DAPI (4′,6-diamidino-2-phenylindole; Vector) and observed on an Axioskop 2 conventional fluorescence microscope (Zeiss), equipped with 405-nm and 488-nm lasers and ×063 and ×100 Plan Apochromatic 1.4 NA objectives in standard conditions.

Expression of COLIII and VIM

Reverse transcription of total RNA (ribonucleic acid) to cDNA (complementary deoxyribonucleic acid) was performed using DNase (Turbo DNA-free, Ambion). The reaction was carried out using the First-Strand cDNA synthesis kit (Roche Diagnostic) following the manufacturer's recommendations. The real-time polymerase chain reaction was performed using SyberGreen as the detection system. The primer sets (Invitrogen, Thermo Fisher Scientific) were as follows: F5′-TGGCAAAGTGGAGATTGTTGCC-3′ and R5′-AAGATGGTGATGGGCTTCCCG-3′ for type III collagen (COLIII); F5′-TCTGGATTCACTCCCTCTGGTT-3′ and R5′-TCGTGATGCTGAGAAGTTTCGT-3′ for VIM; and F5′-TGGCAAAGTGGAGATTGTTGCC-3′ and R5′-AAGATGGTGATGGGCTTCCCG-3′ for GAPDH (glyceraldehyde 3-phosphate dehydrogenase), used as internal gene reference. The reactions were carried out on a 7500 Fast Real-Time PCR System (Applied Biosystems). All samples were run in triplicates, normalized to internal GAPDH and calibrated to the control group (Pol) levels.

Statistical Analysis

Results are expressed as mean ± standard deviation. In order to compare the results between the different conditions, Student *t* test was applied for the roughness analysis. For the biologic assays, the two-way analysis of variance with post hoc Bonferroni test was applied, with a significance level of .05.

Results

Surfaces Roughness

The results showed that the two test surfaces exhibited similar average surface roughness ($P = .081$). The mean Ra was 0.261 ± 0.053 μm for Zir and 0.345 ± 0.130 μm for LD.

Cell Proliferation and Viability Assay

The results showed that both test surfaces presented a similar cell proliferation and viability as Pol in almost all period times ($P > .05$). However, after 96 hours, Pol surfaces revealed an increase in cell viability compared to LD and Zir (Table 1).

Cell Morphology Analysis

SEM analysis

The cell morphology analysis showed that for all surfaces, the

Table 1 Cell Proliferation and Viability of Fibroblast Cell Cultures After 24, 48, and 96 Hours

	24 h	48 h	96 h
Cell proliferation			
Pol	1.55 ± 0.76^{A,a}	2.00 ± 0.85^{A,a}	3.81 ± 1.00^{B,a}
Zir	0.77 ± 0.46^{A,a}	1.31 ± 1.04^{A,a}	2.77 ± 1.05^{B,a}
LD	1.03 ± 0.59^{A,a}	1.77 ± 0.69^{A,a}	3.18 ± 1.10^{B,a}
Cell viability			
Pol	0.19 ± 0.06^{A,a}	0.25 ± 0.11^{A,a}	0.47 ± 0.04^{B,a}
Zir	0.13 ± 0.03^{A,a}	0.16 ± 0.03^{B,a}	0.32 ± 0.09^{C,b}
LD	0.14 ± 0.03^{A,a}	0.19 ± 0.06^{A,a}	0.37 ± 0.08^{B,b}

Pol = polystyrene (control); Zir = zirconia; LD = pressed lithium disilicate.
Values are presented as mean ± SD. Call proliferation is expressed as the number of cells × 10^4, and cell viability was expressed as arbitrary units. Different uppercase letters indicate significant differences at different time points. Different lowercase letters indicate significant differences among the groups at each experimental time point.

cells were spread and occasionally spindle shaped. For the LD and Zir surfaces, the cells grew parallel to the direction of the grooves. In contrast, cells on Pol surfaces exhibited random growth (Figs 1a to 1c).

Indirect immunofluorescence

The immunostaining for VIM exhibited uniform labeling in the cytoplasm in all conditions studied (Figs 1d to 1f). On the LD surface (Fig 1e), cells presented with numerous cytoplasmic projections with a dendritic appearance. On the Zir surface (Fig 1f), the fibroblasts exhibited a fusiform morphology with parallelism of the cytoplasm.

COLIII and VIM gene expression

The results of COLIII and VIM messenger RNA (mRNA) analysis are presented in Table 2. Compared to Pol, an upregulation of COLIII and VIM gene expression was observed by fibroblasts cultured on Zir and LD in all studied periods ($P < .05$).

Discussion

Despite its extensive clinical use, there is little evidence in the literature on the biologic behavior of the LD surface when used as a prosthetic abutment in highly esthetic areas. The objective of this study was to evaluate the possibility of using this material for customized abutments in peri-implant regions. The present results of in vitro cell proliferation assays indicate that the LD surface showed a biologic profile similar to the Zir surface. These findings corroborate those by Tetè et al,[11] who observed increased cell proliferation for the polished Zir after a period of 24 hours and 72 hours of cell culture, but it was not statistically significant in relation to the LD surface.

In the present study, LD and Zir surface polishing was done superficially—using routine clinical instruments because the abutments made from these materials cannot be morphologically altered—with the goal of creating a surface pattern that could allow guided cell growth.[11–13] This method of limiting surface treatment to polishing only was instrumental to obtaining the results, as it favored cell adhesion and proliferation on both surfaces. The surfaces were not glazed; according to Brunot-Gohin et al,[16] abrasion provides rough surfaces that permit enhanced wettability, thus allowing a strong attachment of epithelial tissue. This type of surface would be ideal for gingival attachment around the crown. Relatedly, diamond pastes were not used for polishing, because chemical waste materials could interfere with cell spreading, according to Brackett et al.[9]

The ultrastructural morphology of the analyzed surfaces showed grooves and ridges (created by the polishing discs) in unidirectional strokes (vertical or horizontal), which enabled the adsorption of extracellular matrix proteins[16,17] and the adhesion of fibroblasts following a patterned cell-spreading direction. In

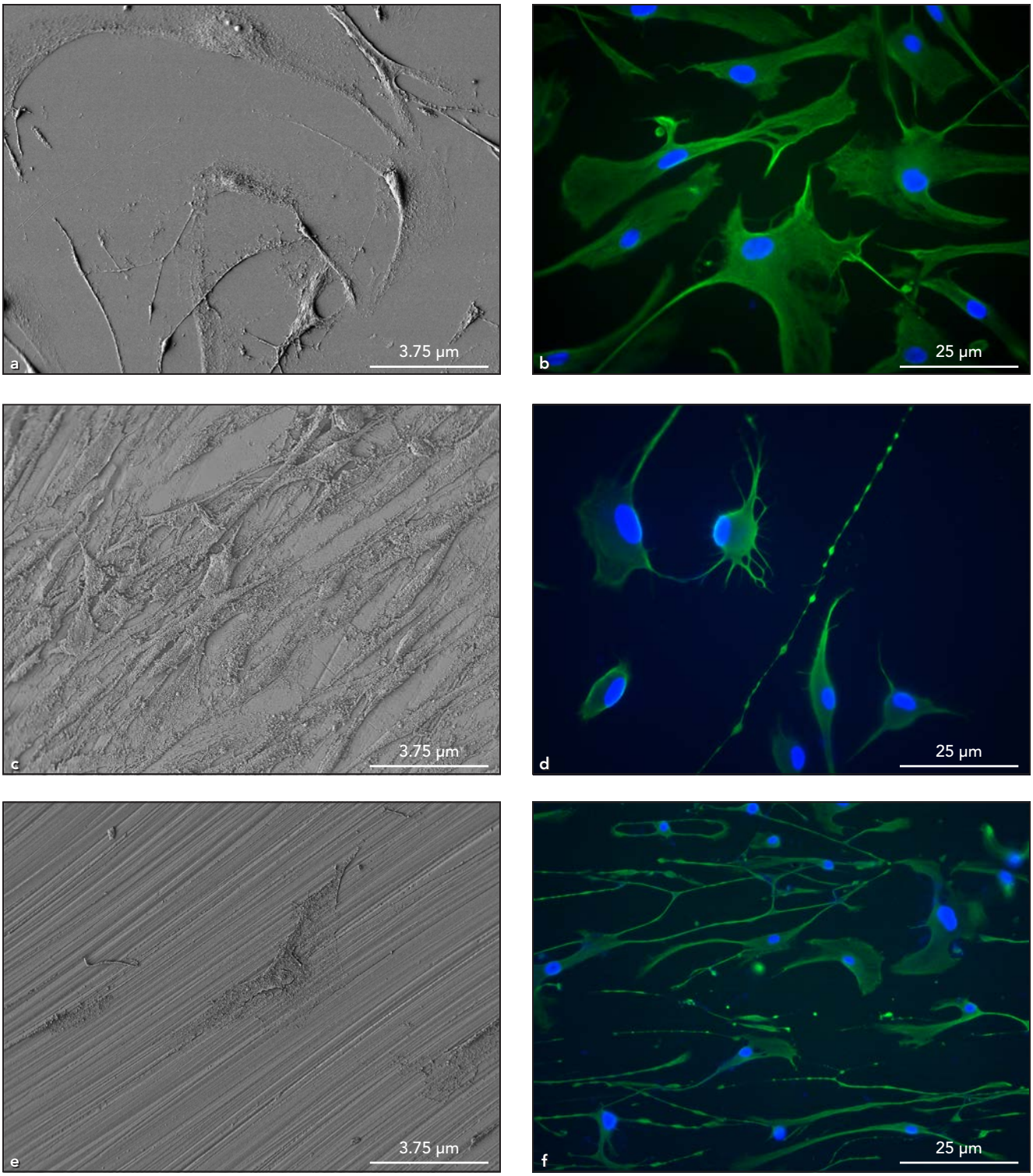

Fig 1 *Morphology of gingival fibroblasts. (left) Scanning electron micrographs (scale bar = 3.75 µm) and (right) epifluorescence (scale bar = 25 µm) for vimentin on human gingival fibroblasts cultured on* (a and b) *polystyrene,* (c and d) *pressed lithium disilicate, and* (e and f) *zirconia after 48 hours of cell culture. Nuclei stained with DAPI (4′,6-diamidino-2-phenylindole) appear in blue.*

Table 2 Relative mRNA Expression of VIM and COLIII in Fibroblast Cells Cultured on Pol, Zir, and LD Surfaces

	24 h	48 h	96 h
VIM expression			
Pol	$0.75 \pm 0.03^{C,b}$	$1.85 \pm 0.03^{B,b}$	$2.72 \pm 0.18^{A,b}$
Zir	$1.35 \pm 0.29^{C,a}$	$2.63 \pm 0.22^{B,a}$	$3.44 \pm 0.31^{A,a}$
LD	$1.78 \pm 0.17^{C,a}$	$2.72 \pm 0.24^{B,a}$	$3.31 \pm 0.23^{A,a}$
COLIII expression			
Pol	$1.02 \pm 0.27^{B,b}$	$1.35 \pm 0.22^{B,b}$	$5.95 \pm 0.98^{A,a}$
Zir	$3.59 \pm 0.18^{B,a}$	$5.02 \pm 0.18^{A,a}$	$7.11 \pm 2.18^{A,a}$
LD	$3.53 \pm 0.42^{B,a}$	$5.57 \pm 1.15^{A,a}$	$6.89 \pm 0.28^{A,a}$

mRNA = messenger ribonucleic acid; VIM = vimentin; COLIII = type III collagen; Pol = polystyrene; Zir = zirconia; LD = pressed lithium disilicate. Values are presented as mean ± SD. Different uppercase letters represent significant differences at different time points. Different lowercase letters indicate significant differences among the groups at each experimental time point.

their studies, Pae et al[13] and Tetè et al[11] reported that surface landscape, such as grooves, may influence cell spreading and growth, especially in the initial stages of cell proliferation.

The effect of the grooved surface on the orientation of fibroblasts merits further discussion. Studies on the response of fibroblasts, epithelial cells, and bone cells to different surface topographies have shown that while the response is dependent on cell type, overall, the cell elongates in the direction of the groove and travels with guidance from the grooves.[18–20] This phenomenon is known as contact guidance,[20] though the exact mechanism of and triggering factors responsible for contact guidance remain to be established. Several studies proposed that contact guidance is a result of preferential protein adsorption on sharp surface discontinuities, such as the ridge edges on microgrooved surfaces.[21] Others have suggested that contact guidance is caused by mechanical forces on the cell's filopodia, which cause them to reshape actin filaments and adjust to substrate landscape.[22]

Furthermore, the morphologic findings of the present study revealed that the fibroblasts on the analyzed surfaces showed spindles, occasionally cytoplasmic extensions (filopodia and lamellipodia), and particulate matter. The phenomena of interaction between cells and substrates are characterized by mechanisms in which cells are able to respond to stimuli from the outside environment that influence their adhesion, proliferation and differentiation.[13]

A unique morphologic feature of the fibroblasts using indirect immunofluorescence assays was the presence of a network of dendritic extensions observed on the pressed LD surface. This uniqueness is generally observed in histotypical cultures, which are cells organized into three-dimensional structures (ie, when cell growth occurs three-dimensionally), qualifying them for specific functions of motility and surface adhesion.[23]

For successful tissue integration of prosthetic ceramic materials, cells must uniformly colonize the surface, a process that should also involve the deposition of extracellular matrix elements (especially collagen) and other components, such as fibronectin and proteoglycans,[24] in response to different growth surfaces and physical and chemical stimuli. Therefore, the present study evaluated the synthesis of COLIII in cells grown on different surfaces. COLIII is the most abundant type in loose connective tissue,[25] constituting 43% of collagen, and plays an important role on tissue elasticity and adhesion.[25] Most of the collagen is present in the form of type I and III fibers, at a ratio of 3:1. These fibers are present in the periodontal tissue and can be found at the periphery of the fibers, which are inserted into the alveolar bone.[26]

The results have shown that the surfaces did not alter the COLIII and VIM gene expression. These findings corroborate those by Att et al,[17] who demonstrated in an in vitro study that surface landscape features are of utmost importance for collagen synthesis.

Tetè et al[11] also examined type I collagen synthesis in vitro at 24 hours and 72 hours on both Zir and LD surfaces, finding high levels of this protein within 24 hours on the Zir surface. At 72 hours, the levels were higher on the LD surface, although this did not reach statistical significance.

Conclusions

The results of the present study indicate that the polished LD surface showed favorable biologic behavior, thus making it a suitable candidate for use in highly esthetic sites in peri-implant regions.

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