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Influence of 3D printed and milled zirconia on the adhesion and viability of keratinocytes: An in vitro study

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ABSTRACT

Statement of problem. The biocompatibility and cell response of additively manufactured ceramics, important for long-term clinical success, require additional investigation.

Purpose. The purpose of this in vitro study was to compare the biocompatibility and cell response of human gingival keratinocytes with 3-dimensionally (3D) printed and conventionally milled zirconia.

Material and methods. Cylindrical specimens of 3D printed and milled zirconia were prepared, and immortalized human gingival keratinocytes (IHGKs) were cultured on specimens of these materials. Cells cultured only in growth medium were the control. Cell adhesion, viability, and morphology were assessed by using water-soluble tetrazolium (WST-1) assays and scanning electron microscopy (SEM) of the disks after culturing. Eluates from the zirconia specimens were collected and tested to assess potential cytotoxicity over time and surface roughness measured by laser scanning microscopy. For cell adhesion, an independent *t* test for 2-samples with unequal variances (Welch *t* test) was performed. For the cytotoxicity tests, differences between groups were analyzed using the post hoc test for multiple comparisons with the Bonferroni correction (α=.05).

Results. After 24 hours, no significant difference in keratinocyte adhesion was found between 3D printed and milled zirconia (P>.05). Cell viability assays showed that, while both materials exhibited lower viability compared with the control, 3D printed zirconia displayed significantly reduced cell viability after 96 hours compared with milled zirconia (P<.001). Average surface roughness (Ra) was significantly higher (P=.001) for printed (0.26 ±0.04 μm) than milled (0.08 ±0.02 μm) zirconia. SEM images confirmed good cellular adhesion and spreading on milled zirconia, with similar attachment on 3D printed zirconia.

Conclusions. Both 3D printed and milled zirconia demonstrated good biocompatibility with human gingival keratinocytes. However, under extended direct surface contact, cells on 3D printed zirconia showed lower cell viability compared with milled zirconia. While 3D printed zirconia is promising for dental applications, further refinement of its surface properties and biocompatibility may be needed. (J Prosthet Dent 2025;134:229.e1-e7)

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Clinical Implications

Both 3D printed and milled zirconia demonstrated good biocompatibility with human gingival keratinocytes.

Patient demand for ceramic restorations is increasing steadily, and zirconia has emerged as a suitable choice to meet this growing preference. Clinical success rates of zirconia crowns have been reported to be comparable with those of traditional metal-ceramic restorations.[1,2] Zirconia ceramics have been recognized for their exceptional material properties, including high strength and fracture toughness, chemical stability, low thermal conductivity, bioinert behavior, tooth-colored esthetics, and minimal bacterial surface adherence. Among all restorative ceramics, 3 mol% yttria-stabilized tetragonal zirconia polycrystal (3Y-TZP) has the highest flexural strength.[1] Consequently, 3Y-TZP has been widely used in zirconia-based implants, abutments, and various ceramic prosthetic restorations, including frameworks. Until recently, the production of zirconia in dentistry was primarily associated with subtractive manufacturing.[3] However, subtractive technologies have limitations, including material waste, the potential creation of microcracks, and space limitations imposed by the size of the milling cutter and the axis of the computer numerical control (CNC) machine.[4,5]

Recently, additive manufacturing, also referred to as 3-dimensional (3D) printing, has emerged as a promising alternative to milling, with advantages that include enabling the creation of complex geometries and reducing material waste.[6,7] The initial attempts to 3D print ceramics date back to 1990.[8,9] Vat photopolymerization is the current state of the art for additive manufacturing of dense, precise ceramic parts and involves techniques that include stereolithography (SLA) and digital light processing (DLP), where a ceramic slurry, containing ceramic powder and a liquid photosensitive binder system, is selectively polymerized to form a green body ceramic.[10–12] To obtain a homogeneous and dense ceramic, postprocessing of the fabricated green parts is necessary, involving 2 key steps: debinding and sintering. In debinding, organic material, the photopolymer matrix, is removed. In the sintering step, ceramic particles are fused together to create a dense final product.

Zirconia restorations are in close contact with oral soft tissues, including the marginal keratinized gingiva.[13–20] Potential adverse effects may include disrupted wound healing immediately after insertion or a gradual retraction of the soft tissues surrounding the prosthesis. Polished surfaces have been reported to be more biocompatible than those of glazed or layered ceramics.[21–30] However, areas such as fixed partial denture connectors are often not accessible for polishing. Therefore, an investigation is needed to determine whether 3D printed zirconia has a similar influence on soft tissue cells as milled zirconia.

In the present study, gingival keratinocytes were analyzed as these cells represent the initial barrier and are often in direct contact with dental restorative materials. The purpose of the study was to compare the biocompatibility and cell response of human gingival keratinocytes to 3D printed zirconia and conventionally milled zirconia. The null hypothesis was that no difference would be found in the growth of keratinocytes on milled zirconia compared with 3D printed zirconia.

MATERIAL AND METHODS

One 3D printed (Zirconia+) and 1 milled (Zirconia-) zirconia were investigated. From each material, Ø40×1.5-mm disks were prepared. Zirconia+ specimens were based on lithography-based ceramic manufacturing (LCM) technology and were fabricated by the manufacturer using a 3D printer (CeraFab S65 Medical; Lithoz) with a specimen orientation of 0 degrees and the surface parallel to the build platform. The proprietary slurry (LithaCon 3Y 210; Lithoz) used in this process contained a 48 vol% zirconia powder, monomers, and photoinitiators. The specimens were cleaned with compressed air, excess unpolymerized material was removed with a cleaning fluid (LithaSol 20; Lithoz), and they were then thermally posttreated. Thermal posttreatment resulted in shrinkage by an approximate factor of 1.30, and the specimens reached the final dimensions. The specimens were debound and sintered at 1450 °C in a furnace (HTCT 08/16; Nabertherm).

The zirconia disks were milled from a blank (Katana Zirconia HTML Plus; Kuraray Noritake) by a milling unit (inLab MC X5; Dentsply Sirona) and sintered to 1500 °C with a 135-minute dwell time in a furnace (inLab Profire; Dentsply Sirona). Both the Zirconia+ and Zirconia- specimens contained a 3 mol% yttria content.

Immortalized human gingival keratinocytes (IHGKs) were obtained from the epithelium of the dental papilla of a patient undergoing oral surgery. The study had been approved by the Ethics committee of the Medical Faculty of the University of Göttingen (Nr. 22/1/05). IHGKs were isolated according to standard protocols.[31,32] The cells were first cocultured with feeder cells (human gingival fibroblasts) and immortalized by transfecting a human telomerase reverse transcriptase (hTERT) gene according to Schminke et al and SV40.[33] Finally, the cells were maintained in a standard medium, (Keratinocyte Growth Medium 2 (KGM2); PromoCell) supplemented with 1% Pen-Strep solution (Sigma–Aldrich) in a

humidified atmosphere with 5% CO_2, at 37 °C. The medium was changed every third day. For the experiments, IHGKs in the eighth to tenth passage were used. For each experiment, 3 biological replicates were used in each experiment, and 3 independent experiments were conducted.

The disks of each material, prepared as described, were handled by only 1 clinician (R.B.). They were disinfected by washing for 60 seconds with 80% ethanol before testing. For the experiments with eluates, disks of each material were then immersed in KGM2 and incubated at 37 °C for 24 hours. The International Organization for Standardization (ISO) 10993–12 guidelines were used to determine the required amount of liquid to be inserted per specimen.[34] Accordingly, each specimen was immersed in 2.5 mL of medium per well in a 12-well plate. After 24 hours, the eluates were collected, and specimens further immersed in 2.5 mL of KGM2 and incubated at 37 °C for 3 additional days.

A total of 4×10^4 IHGKs/mL were seeded over each disk (n=6) in 12-well plates, suspended in 1 mL of cell culture medium in each well. For the control group, no disk was used. To promote cell adherence, the plates were incubated in a humidified atmosphere containing 5% CO_2, at 37 °C for 45 minutes, followed by the addition of 1 mL of KGM2 medium. After 24 hours, the disks were transferred, maintaining the same orientation, to new 12-well plates where other control groups had been cultured from the previous day. Each well was then supplemented with 2 mL of medium.

To measure the optical density (OD) of the remaining cells in the original plates not adherent to the disks, a water-soluble tetrazolium assay-1 (WST-1) (Roche Diagnostics) was performed as per the manufacturer's protocol, allowing for the calculation of adhesion efficiency. The medium was replaced with 1 mL of KGM2 with WST in a 1:10 dilution. After a 2-hour incubation, 300 µL from each well was transferred into 3 wells of a 96-well plate, 100 µL in each well. The OD of the specimens was assessed with a multidetection microplate reader (Infinite M200; Tecan Group). The data were analyzed using a software program (Magellan; TECAN). Absorbance measurements were made at 450 nm, with a wavelength correction at 650 nm. The disks were further incubated, half of them for 48 hours and half for 96 hours. The WST-1 assays were performed in the same way for the cytotoxicity test of 48 and 96 hours.

For the tests with eluates, 1.5×10^4 IHGKs/ mL were suspended in 1 mL of keratinocyte culture medium in each well of 24-well plates. After 24 hours, the medium was substituted with a 0.5 mL eluate of the respective material, obtained as described; this was considered day 0. For each timepoint (first, second, third, and sixth day), 3 biological replicates were used. For the plates of day 6, the medium was replaced at day 3 with fresh eluate obtained from the additional 3-day incubation of the specimens. The assays (WST-1; Roche Diagnostics) were performed on the first, second, third, and sixth day by using the same protocol.

The cell morphology and adhesion to the surface were analyzed using a high-vacuum field emission scanning electron microscope (FE-SEM) (ZEISS GEMINI, SUPRA 55VP; Carl Zeiss). The specimens were prepared as previously described, with each specimen placed in a 12-well plate, and 4×10^4 IHGKs seeded on each. After 48 hours of culture, specimens were fixed overnight with 2% glutaraldehyde at 4 °C and dehydrated in a 5-step ethanol gradient (50%, 75%, 85%, 90%, and 100%; Carl Roth), with each step lasting 45 minutes. The specimens were air dried for 2 hours at room temperature and then sputter-coated with a gold and palladium alloy for 3×60 seconds (Agar Sputter Coater) at 5 kV. The surfaces were examined under different magnifications (×100 to ×1000), and images were digitally recorded using the FE-SEM.

The surface roughness of ceramic specimens was measured with a laser scanning microscope (VKX-3050, VH-ZST; Keyence) at ×20 magnification. Four specimens were measured for both types of ceramic, and 4 surface measurements were made for each. The 4 values of each specimen were combined, and 1 total median value was calculated.

Statistical analyses were conducted using a software program (IBM SPSS Statistics, v26.0; IBM Corp) (α=.05). Normal distribution was tested with the Shapiro-Wilk test. For testing the group of gingival keratinocyte adhesion to the materials and surface roughness Sa, an independent 2-sample *t* test was used. For the cytotoxicity tests, homogeneity of variance across groups was verified with the Levene test. Differences among groups were analyzed for each observation period using a post hoc test for multiple comparisons with the Bonferroni correction.

RESULTS

The viability of IHGKs in the presence of eluates from Zirconia+ and Zirconia- was evaluated using the WST-1 assay. Gingival keratinocytes in pure medium, without any material eluates, served as controls. On the first and second day, a decrease in cell viability was observed for both tested groups (Fig. 1), which was significant just for the Zirconia+ group compared with the control (*P*=.027 for the first day and *P*=.025 for the second day), while the Zirconia- group showed no significant difference from the control (*P*=.110 and *P*=.098 respectively). For all groups, there was a constant increase in cell number up to the third day. By the third and sixth days, both the

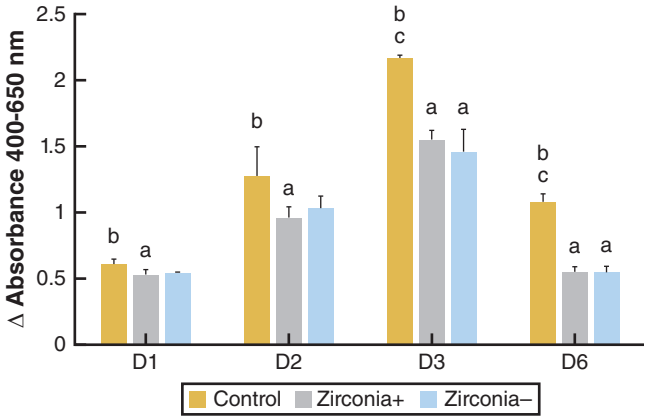

Figure 1. Tetrazolium salt reduction by IHGKs in presence of eluates of zirconium oxide (ZrO_2) printed (+) or milled (-) at day 1 (D1), day 2 (D2), day 3 (D3) and day 6 (D6). Gingival keratinocytes in pure culture medium not exposed to eluates served as controls. For each timepoint and group, 3 biological and 3 technical replicates used. Results presented as mean ± standard deviation values. Statistically significant differences between groups ($P<.05$) indicated by superscript letters, with "a" indicating difference between material versus control, "b" material versus printed Zirconia (+), "c" material versus milled Zirconia (-). IHGK, immortalized human gingival keratinocyte.

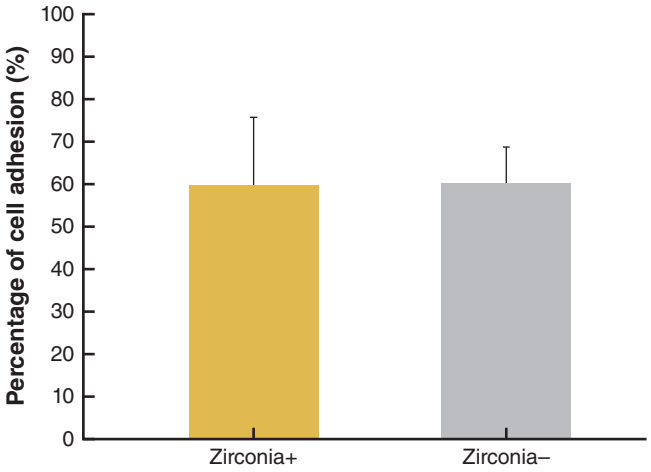

Figure 2. Gingival keratinocyte adhesion to printed (Zirconia +) or milled (Zirconia -) zirconia after 24 h. For each group 6 biological and 3 technical replicates used.

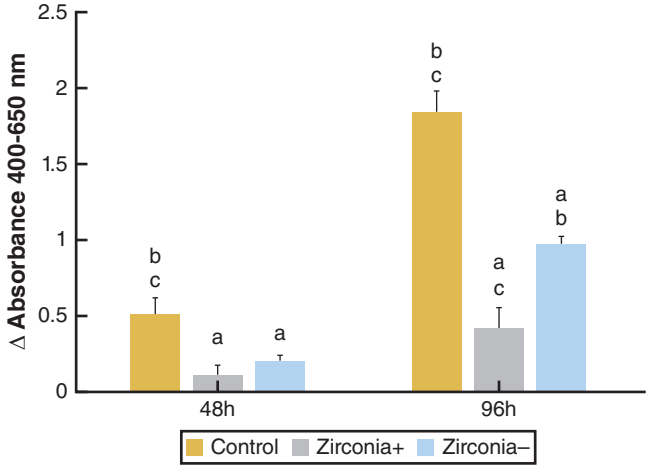

Figure 3. Tetrazolium salt reduction by IHGKs in culture over disks of printed (+) or milled (-) zirconia. Gingival keratinocytes in pure culture medium served as controls. For each group, 3 biological and 3 technical replicates used. Results presented as mean ±standard deviation values. Statistically significant differences between groups ($P<.05$) indicated by superscript letters, "a" indicating difference between material versus control, "b" material versus printed zirconia (Zirconia +), "c" material versus milled zirconia (Zirconia -). IHGK, immortalized human gingival keratinocyte.

Zirconia+ and Zirconia- groups exhibited significantly lower cell viability compared with the control ($P<.001$). Despite these differences, both materials generally maintained cell viability, with no marked cytotoxicity over the course of the study.

The results of the keratinocyte adhesion to zirconia after 24 hours showed no significant difference between Zirconia+ and Zirconia- surfaces. Zirconia+ presented a mean OD value of 0.129 ±0.051, while Zirconia- showed a mean value of 0.1275 ±0.0270). Statistical analysis using the Welch *t* test revealed a *t* statistic of 0.102 and $P=.919$, indicating that the difference in adhesion between the 2 groups was not statistically significant. In Figure 2, the adhesion was calculated from these OD values and presented as a percentage of the seeded cells. This resulted in 59.7% ±15.7% adhesion on Zirconia+ and 60.2% ±8.4% on Zirconia-.

The viability of IHGKs grown on zirconia disks was evaluated after 48 and 96 hours using the WST-1 assay. At both timepoints (Fig. 3), cell viability was significantly lower for the keratinocytes cultured on Zirconia+ and Zirconia- compared with the control group ($P<.001$). While the Zirconia- group exhibited higher cell viability than the Zirconia+ zirconia group at both 48 and 96 hours, the difference between these 2 groups was statistically significant only after 96 hours ($P<.001$). These trends suggest that both materials moderately impacted cell growth, but neither demonstrated overt cytotoxic effects over the course of the experiment.

The SEM images showed good cellular adhesion and spreading on the surface of milled zirconia (Fig. 4). The surface texture of milled zirconia was smooth, with no evidence of porosities or inhomogeneities. As expected, the Zirconia+ surface exhibited a more irregular texture with slight surface roughness and visible microstructures. Although cells adhered to Zirconia+, the spreading was less uniform compared with Zirconia-, and many cell-free areas were observed, indicating a potentially less favorable environment for cell adhesion and proliferation. The independent 2-sample *t* test revealed a significant difference between the groups ($P<.001$): the roughness values Sa of the Zirconia- group

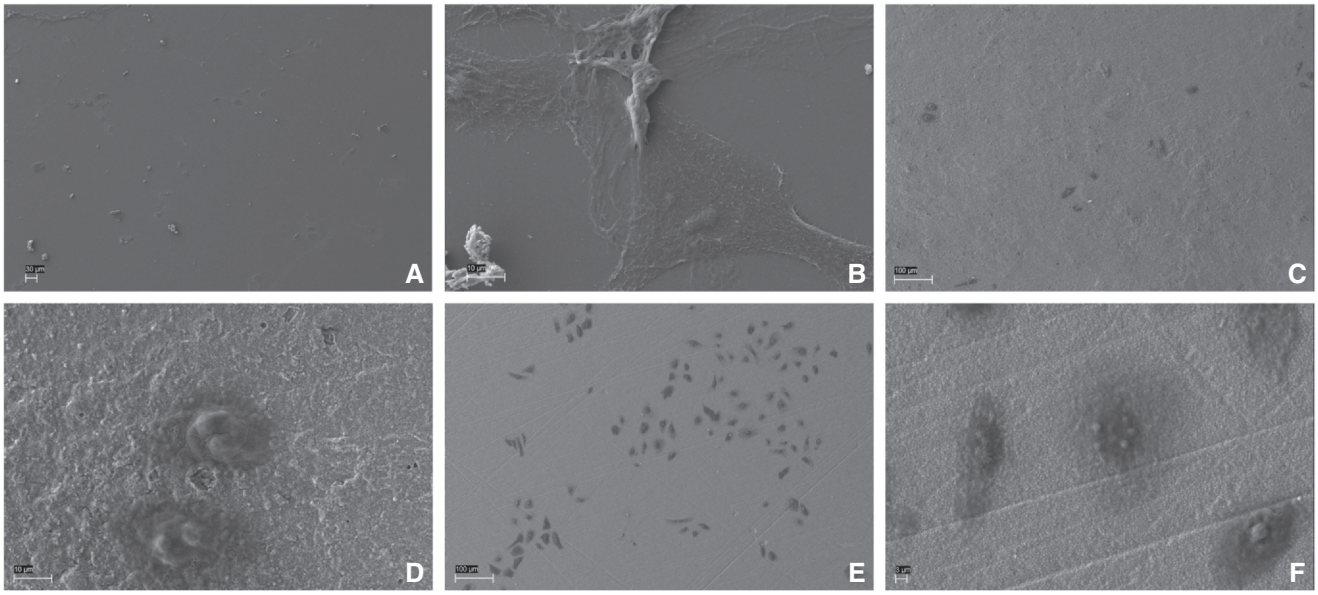

Figure 4. Scanning electron microscope images of surface textures of different material probes. Gingival keratinocytes cultured on different surfaces. A, D Keratinocytes on glass plate (control). B, E, ZrO_2 printed. C, F ZrO_2 milled. A, B, C, original magnification ×100. D, E, F, original magnification ×1000.

Table 1. Median roughness measurement Ra (μm) and standard deviation for ceramic type. Four specimens measured at 4 points each

	Ra (μm)
Subtractive zirconia	0.08 ±0.02
Additive zirconia	0.26 ±0.04

were significantly (P<.001) lower than of the Zirconia+ group (Table 1).

DISCUSSION

The purpose of this study was to compare the biocompatibility and cell response of human gingival keratinocytes with 3D printed and conventionally milled zirconia. From the results obtained, the null hypothesis that no difference would be found in the growth of keratinocytes on milled zirconia compared with 3D printed zirconia was rejected.

Printing ceramics offers greater material efficiency and design flexibility compared with milling, being able to produce complex geometries with custom designs. The ceramic is printed from a proprietary slurry containing ceramic powder and a liquid photosensitive binder to form a ceramic green body. Given the lack of knowledge regarding the precise composition of the slurry, toxic residues may remain despite the removal of the binder through debinding. Dental materials typically interact with oral epithelial cells, specifically keratinocytes, which line the oral cavity and form the stratified squamous epithelium.[13,14] Using keratinocytes as marker cells for biocompatibility testing is justified by these cells being first barrier in the oral environment and being in closest contact with the surface of the restorations.[35,36]

In the current study, the proliferation of gingival keratinocytes in the presence of zirconia eluates was lower than that of untreated controls at all tested time points. Probst et al[37] reported similar results when testing milled zirconia on keratinocytes. Furthermore, throughout the current study, no significant difference in cell proliferation was found at any time point between zirconia produced through additive or subtractive manufacturing methods. The results indicate that both manufacturing processes produce zirconia materials with comparable biocompatibility, as they similarly influenced cell proliferation without releasing detrimental substances or particles that could have affected cellular responses.[38] Despite the reduced proliferation compared with controls, both materials appear to be safe for clinical applications.

Similar adhesion of keratinocytes was observed on the surface of both 3D printed and milled zirconia after 24 h, without any significant differences (P>.05), suggesting that both materials provide a comparable surface environment for gingival keratinocyte attachment. Previous studies have also demonstrated strong cell adhesion on zirconia surfaces, in testing with osteoblast-like cells,[15] oral epithelial cells,[16] and human gingival fibroblasts.[17,18] These findings were consistent with the biocompatibility of zirconia, highlighting its ability to support initial cell attachment and tissue integration and making it a favorable material for dental and implant applications. The comparable cell adhesion obtained in the present study indicates that key surface

characteristics such as chemistry and wettability are sufficiently suitable in both materials for promoting initial cell adherence. This similarity also implies that despite variations in the manufacturing process, both 3D printed and milled zirconia can effectively support the initial interactions necessary for stable and functional clinical restorations. Proper attachment of oral tissues, like keratinocytes, to the zirconia surface promotes stability, reduces the risk of complications, and ensures the long-term success of the dental restoration.[19,20] However, other factors, including the interaction of the material's surface with surrounding tissues, may differ, as indicated by the viability results from the direct cultivation on disks.

While no difference in cell proliferation was observed after 2 days of culture on the surfaces of either material, a statistically significant difference ($P<.05$) emerged by the third day, demonstrating an advantage in keratinocyte proliferation on the Zirconia- surface. This might be attributed to the lower roughness of the polished surface of Zirconia-. A well-polished or glazed zirconia surface has been reported to promote the adhesion and proliferation of gingival fibroblasts compared with rough surfaces.[39,40] The current results support these findings for keratinocytes. Often, however, polishing is not adequate because of limited accessibility of certain parts of the restoration – or is not performed after adjustments have been made to the ceramic. These considerations must be considered even more in situations involving restorations placed near surgical sites. In such situations, the surrounding tissues are in a vulnerable state, with increased sensitivity to foreign materials associated with increased inflammation and tissue regeneration processes.[41,42] Ensuring that the restorative material, whether Zirconia+ or Zirconia-, exhibits optimal biocompatibility is essential to prevent adverse tissue responses, delayed healing, or increased risk of complications such as infection or gingival recession.[43] Consequently, the choice of material in these delicate environments demands thorough evaluation of its surface properties and interaction with soft tissues to minimize negative biological impact.

The restoration design is significant for both tooth- and implant-supported fixed partial dentures, as well as the implants themselves, especially in ensuring that all surfaces are polishable without creating niches that could harbor bacteria. Polished surfaces minimize bacterial adherence,[30] reducing the risk of inflammation and peri-implantitis. Maintaining a surface roughness threshold below 0.2 µm has been reported to effectively prevent bacterial adhesion and improve cell adherence, as well as proliferation and migration.[25–29] The average surface roughness of the zirconia disks used in this investigation was 0.08 ±0.02 µm for the milled specimens and 0.26 ±0.04 µm for the printed specimens.

The WST-1 colorimetric assay was used in the present study to quantitatively measure cell proliferation and viability. The WST test has been favored by the ISO standard[44] for cytotoxicity evaluation because of its quantitative nature, offering advantages over qualitative methods. The assay relies on detecting the formation of water-soluble formazan, which results from dehydrogenase activity in metabolically active mitochondria.[45] The amount of formazan, measured by absorbance, is proportional to the number of viable cells, providing an indirect measure of cytotoxicity.

The authors are unaware of a previous study that examined differences in the attachment and proliferation of keratinocytes on zirconia surfaces produced through additive or subtractive manufacturing. Limitations of the study included that it was solely focused on a single layer 2D-model with 1 cell type, keratinocytes. The simplified model did not account for interactions with surrounding cells, the extracellular matrix, or the influence of various signaling molecules that could impact the overall biological response. In addition, aging[46] was not modeled in this in vitro study. Ra was selected as the most appropriate parameter for the evaluation and comparison of materials. However, further modifications to the specimens may be necessary for conducting further investigations into the clinical application of the material. This could involve the consideration of additional parameters, such as different build angles and the impact of clinical polishing.

CONCLUSIONS

Based on the findings of this in vitro study, the following conclusions were drawn:

1. Both Zirconia+ and Zirconia- demonstrated good biocompatibility with human gingiva keratinocytes in a single layer 2D model.
2. Ra was significantly higher after printing compared with milling.
3. After prolonged exposure with direct surface contact, Zirconia+ showed reduced cell viability response compared with Zirconia-. This finding confirms the importance of a smooth polished surface.
4. While Zirconia+ holds promise for dental applications, further investigation of its surface properties in view of printing direction and biocompatibility may be necessary to match the performance of Zirconia- in clinical settings.

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