Physical and biological implications of accelerated aging on stereolithographic additive-manufactured zirconia for dental implant abutment

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Abstract

Purpose: This study aimed to comparatively investigate the effects of accelerated aging on the physical and biological features of zirconia manufactured by digital light processing (DLP) and conventional subtractive manufacturing (SM) with similar composition.

Methods: Both the DLP- and SM-fabricated zirconia samples (7 mm × 7.5 mm × 1.5 mm) were grouped according to aging (134 °C, 0.2 MPa, 100% humidity) times, including 0 h, 5 h, and 10 h. Phase assemblage and surface topography of zirconia manufactured by different technologies were evaluated before and after aging. The biological effects of zirconia on human gingival fibroblast (HGF) cell events, including cell viability, proliferation, morphology and adhesion, were also evaluated by live/dead viability assay, cck-8 assay, scanning electron microscopy and confocal laser scanning microscopy respectively.

Results: The DLP-fabricated zirconia showed a higher initial cubic phase content and rate of phase transformation than the SM-fabricated zirconia. Among the different aging time-based groups, the 5 h-aged group exhibited significantly lower sub-micron scale surface roughness compared with the other groups. Aging did not significantly alter cellular behavior in any zirconia type, except for minor changes in adhesive cell numbers recorded in an aging time/culturing time-dependent manner. In addition to small differences in cell alignment patterns and overall cell morphology, the two zirconia types presented comparable biological performance before and after aging.

Conclusion: Although the microstructure and surface characteristics of DLP-fabricated zirconia can be affected by autoclave aging, this newly manufactured zirconia is likely to maintain desirable long-term biocompatibility as an implant abutment material.

Keywords: Yttria-stabilized zirconia, Digital light processing, Stereolithography, Aging, Implant abutment

1. Introduction

Yttria-stabilized tetragonal zirconia polycrystal (Y-TZP) is currently a scientific and clinical research focus in the field of dental materials, owing to its excellent combination of mechanical features, aesthetics and biocompatibility[1]. Generally, zirconia-derived dental prostheses are fabricated using subtractive manufacturing (SM) technology, which applies computer aided design/computer-aided manufacturing (CAD/CAM) systems[2]. However, some problems with this technology have been recorded in recent years; for instance, it not only causes significant waste of materials and tools, but also has limitations in the fabrication of parts with complex geometries[3]. Consequently, additive manufacturing technologies have attracted increasing attention. Stereolithographic methods, including stereolithography and digital light processing (DLP), are promising for producing small and complex dental parts, which require both high accuracy and surface quality. In addition, these techniques significantly reduce the production steps and consumption of energy and raw materials[3,4].

Generally, zirconia crystals are found in three structural entities, including the tetragonal and cubic phases at temperatures of >1170 °C and >2370 °C, respectively. The tetragonal phase is stabilized at ambient by yttria addition to generate a Y-TZP ceramic[1,5]. Currently, 3 mol% yttria stabilized Y-TZP (3Y-TZP) is the most commonly used zirconia ceramic in dentistry because of its excellent mechanical properties. However, this material is susceptible to low-temperature degradation (LTD) under moist conditions[1,6]. LTD involves a nucleation and growth process, which generally starts at the surface of polycrystalline ceramics and induces the tetragonal to monoclinic transformation over a temperature range of ~30 °C to 300 °C[6,7]. Furthermore, LTD may affect the biomechanical and surface physicochemical properties and the service life and quality of 3Y-TZPs, particularly for those applied in biomedical settings, which are exposed to complex environments in vivo, such as hip implants and dental restorations[8].

DOI: https://doi.org/10.2186/jpr.JPR_D_21_00240

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Received 24 September 2021, Accepted 22 November 2021, Available online 17 December 2021

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Based on a previous systematic review, LTD does not significantly impact the surface roughness of 3Y-TZP in global analyses. However, two subgroup analyses showed that autoclave duration and sample preparation methods contribute to surface roughness alteration in aged 3Y-TZP ceramic products[9]. Moreover, according to Rocha et al., LTD may also cause slight changes in the polarity and surface energy of zirconia[10]. Other studies have demonstrated that intrinsic properties (i.e., chemical composition and grain size) may influence the propensity of 3Y-TZP to LTD, thus inducing different surface physicochemical responses[5,11,12]. In addition, it is currently admitted that the surface characteristics of a biomaterial are closely related to its biocompatibility[13,14].

In recent studies applying the DLP technique, Lu et al. obtained stereolithography-additive-manufactured zirconia with favorable microstructural characteristics and mechanical behavior[15,16]. Similar to the SM-fabricated zirconia, the DLP-manufactured zirconia can also be processed into implant abutments, which are often individually designed and have close contact with gingival tissues. It has been demonstrated that optimal soft tissue healing at the implant-abutment interface is fundamental to the clinical success of dental implants[17]. However, the biocompatibility of DLP-fabricated zirconia products has not been assessed, particularly with respect to aging.

The DLP technique, which is completely different from the SM technology, involves light-curing of resins in the ceramic slurry for layer-by-layer printing of three-dimensional structures, followed by post-treatments for organic resin removal and the generation of dense materials, including debonding and sintering steps[3,15]. As the aging resistance of this newly DLP-manufactured zirconia is inconclusive, determining its physical and biological properties before and after aging is critically important.

Therefore, this study aimed to assess the effects of aging on the surface morphology, microstructural characteristics and biocompatibility of DLP-manufactured zirconia. In addition, these properties were compared with those of SM-fabricated Y-TZP containing similar components.

2. Materials and Methods

2.1. Sample preparation

Both DLP- and SM-produced 3Y-TZPs were used for sample preparation. Detailed information about these two types of Y-TZP is presented in Table 1.

A final specimen dimension of 7 mm × 7.5 mm × 1.5 mm was designed for all tests in this study. The samples were fabricated as previously described[15,16]. A slurry containing a homogeneous mixture of zirconia powder (58 vol.%) and photocurable monomers was used for the fabrication of the DLP-based Y-TZP. The green bodies were printed on a DLP stereolithography system (CeraLab-P60, QuickDemos Company, Jiangsu, China) horizontally. The single-layer thickness was limited to 25 μm and the light intensity was set to 90 mW/cm². The above steps were followed by treatment with an indispensable debinding process (<500 °C). In sequence, the semi-manufactured products were sintered at 1510 °C for 2 h with heating and cooling rates of 300 °C/h to generate the final Y-TZP substrates. For the SM-fabricated Y-TZP, samples were milled from commercially available CAD/CAM blocks of zirconia (Zenostar T, Ivoclar Vivadent, Liechtenstein) using a Wieland Zenostar mini CAD/CAM instrument (Ivoclar Vivadent). Then the green bodies were sintered using the same sintering protocols as those used for the DLP-based Y-TZP. All the sintered Y-TZP specimens were observed using an optical microscope (100×, BX51, Olympus Co., Japan) to ensure that there were no evident cracks, pits, or pores, etc. on the specimen. Subsequently, the samples were ultrasonically washed in absolute ethyl alcohol for 30 min prior to all tests.

2.2. Aging of Y-TZP samples

All specimens were divided into three groups (n = 124) according to the aging time (0 h, 5 h, and 10 h). The samples were artificially aged, as suggested by Chevalier et al., with hydrothermal degradation performed using an autoclave (HAST-40, Kunshan Bositong, China) at 134 °C with 100% humidity under a pressure of 0.2 MPa[12]. As significant differences in surface characteristics were found between zirconia specimens treated with various autoclave times (≤5 h and >5 h)[9], and according to the protocol used in previous literature[13], the aging times in this study were set at 5 h and 10 h. Subsequently, all samples were assigned to the following groups: I) no aging (DLP0, SM0); II) 5 h of aging (DLP5, SM5); and III) 10 h of aging (DLP10, SM10).

2.3. Phase assemblage and surface roughness analysis

The percentages of the monoclinic, tetragonal, and cubic phases (n = 2) were detected by X-ray diffraction (XRD, EMPIREAN, PANalytical B.V., the Netherlands) using Cu-Kα radiation patterns (40 kV and 60 mA). The scan and time ranges were 20–90° and 3 s, respectively; the step size was 0.03°. Results were obtained by Rietveld analysis using Topas 4.0. Specifically, tetragonal and cubic phase amounts were derived from data reported by Scott at al[18,19], and Garvie RC at al[20]. The monoclinic peak intensity ratio (X_m) was derived using the method described by Garvie and Nicholson[20]:

$$X_m = \frac{I_m(\overline{1}11) + I_m(111)}{I_m(\overline{1}11) + I_m(111) + I_r(101)}$$

where $I_m(\overline{1}11)$ and $I_m(111)$ represent the integrated intensities of monoclinic peaks at approximately 28° and 31°, respectively; $I_r(101)$ is the integrated intensity of tetragonal/cubic peaks at approximately 30°. The volume fraction of the monoclinic phase (V_m) was obtained...
using the formula proposed by Toraya et al.[21]:

\[ V_m = \frac{1.311X_m}{1 + 0.311X_m} \]

Surface analyses were performed by atomic force microscopy (AFM, SPM-9600, Shimadzu, Japan) in five representative samples for each material and treatment. Five different scattered regions per specimen were measured for three surface roughness indices, including Ra (arithmetic mean of absolute values for peaks and valleys obtained in a medium plane), Rz (mean distance separating the five highest peaks and five main valleys) and Rq (standard deviation of height distribution)[13]. As zirconia nucleation was previously detected by small surface upheavals with a height of less than 10 nm and a diameter close to 1 μm, scan areas of 5 μm × 5 μm and 500 nm × 500 nm for sub-micron and nanometer resolutions, respectively, were used to analyze the surface properties of Y-TZP in the present study[6,22]. Commercial AFM tips (radius of tip curvature <10 nm; NSG01/Au, TipsNano, Estonia) were used in the tapping mode at 1 Hz.

2.4. Cell culture

Primary human gingival fibroblast (HGF) isolation was performed from gingival biopsies of individuals with no periodontal disease. This study was approved by the ethics committee of the State Key Laboratory of Oral Diseases, Sichuan University (WCHSIRB-D-2020-183), and all participants provided signed informed consent. HGFs were cultured using the outgrowth technique[13,17], and then the cells were cultured in a flask supplemented with Dulbecco’s modified Eagle medium (DMEM basicI, GIBCO, Thermo Scientific, USA), which included 10% fetal bovine serum (GIBCO, USA), 100 U/mL of penicillin/streptomycin, and 2 mM glutamine. The flask was kept in an incubator with an air atmosphere of 95% humidity, 5% CO2 and 37°C. Cells from passages 4–10 were used for all tests.

2.5. Cell viability and proliferation assessment

To evaluate HGF viability, a live/dead viability/cytotoxicity kit (BioVision, USA) was used according to the manufacturer’s instructions. After transferring the zirconia samples to the 48 well culture plates, the HGFs were seeded on the surface of each specimen at a density of 2.5 × 10^4 cells per well and were grown for 24 h (n = 5). This was followed by two phosphate buffer saline (PBS) rinses and incubation (15 min, 37 °C) with the live/dead working solution. Confocal laser scanning microscopy (CLSM, FV3000, Olympus Co., Japan) was used for analysis with the following parameters: Z-axis pattern; step size, 0.3 μm; excitation wavelengths, 408 nm and 488 nm; magnification, × 100. The assay was repeated three times.

To assess HGF proliferation on various Y-TZPs, CCK-8 (Dojindo, Japan) assay was performed. Zirconia samples were placed in 48 well plates (n = 5), which were seeded with HGFs at 2.5 × 10^4 cells per well. Cells seeded into empty wells constituted the control group, three times, and data are presented as percentages of control cells. The assays were repeated three times, and data are presented as percentages of control cells.

2.6. Cell morphology

Cell morphology and specimen surface characteristics were evaluated by scanning electron microscopy (SEM; JSM-IT500LA, JEOL, Japan) and CLSM. For SEM observation, HGFs were seeded in 48 well plates at 2.5 × 10^4 cells per well (n = 2). After 24 h of incubation, cell fixation was performed with 2.5% glutaraldehyde for 5 h at 4 °C, followed by sequential dehydration using ethanol at 30, 50, 75, 85, 90 and 100 v/v % in water. The samples were finally placed in hexamethyldisilazane solution for 10 min and exposed to air drying under a hood for 20 min. The specimens were gold-coated using a vacuum evaporator (DII-290305CTR, JEOL, Japan) and observed by SEM (accelerating voltage, 20 kV; probe current, 0.3 mA) at a magnification of × 250. The observation tests were repeated three times.

For CLSM detection, HGFs were seeded and cultured in the same manner as for the SEM observation (n = 2). At a given culture time point, cell staining was performed for filamentous actin with rhodamine phalloidin (Sigma-Aldrich, USA) and nuclei were stained with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich), as indicated by the manufacturer. In brief, the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) at 4 °C for 30 min and permeabilized with 0.5% Triton X-100 (Biofroxx, Germany) at ambient temperature for 5 min. Then, the cells were stained by successive incubations with rhodamine phalloidin and DAPI working solutions at 37 °C for 30 min and 15 min, respectively. All specimens were gently rinsed for 5–10 min twice between the two steps above. The stained sample surfaces were imaged under a CLSM at 600x magnification with 408 nm and 488 nm excitation wavelengths. The assay was repeated three times.

2.7. Cell adhesion assay

Cell counts at 1, 4 and 24 h were measured to evaluate cell adhesion on different samples during the initial seeding period (n = 5). HGF incubation was performed on various specimens in 48 well culture plates at 2.5 × 10^4 cells per well. Before observation under the CLSM, cells remaining on various specimens were fixed with 4% paraformaldehyde (Sigma-Aldrich) at 4 °C for 30 min; DAPI counter-staining was conducted at 37 °C for 5 min. To count the attached cells, three high-power fields were examined per sample. Images were acquired at 100x magnification with 408-nm excitation and processed using ImageJ software. The adhesion rates were calculated using the theoretical density of cells seeded on the zirconia samples to divide the actual density of the observed attached cells. The tests were repeated three times.

2.8. Statistical analysis

All data were assessed for normality and homogeneity of variance before further analysis. Multi-way analysis of variance (ANOVA, CCK-8 and cell adhesion assays) and two-way ANOVA (live/dead viability assay) were performed for data analysis, with post-hoc Tukey’s test. The AFM data were examined using two-way ANOVA and Bonferroni multiple comparison tests. Data are presented as the mean ± standard deviation (SD) unless otherwise stated. SPSS (version 24.0; SPSS Inc., USA) and Prism 6.0 (Graph-Pad, USA) were used for data analysis. Statistical significance was set at p < 0.05.
3. Results

3.1. Phase assemblage and surface roughness features

X-ray diffractograms were similar for the two Y-TZP types before and after aging. Prior to LTD, both the tetragonal and cubic phases could be identified in the zirconia studied, with single cubic peaks partly fused to the tetragonal doublets to form larger doublets (i.e., $2\theta = 50^\circ$). After 5 h of aging, the monoclinic phase was detected for both Y-TZP types, and the monoclinic peaks were also combined with the tetragonal and cubic peaks to generate large triplets (i.e., $2\theta = 35^\circ$). After aging for 10 h, a seemingly single and large peak reflecting a triplet comprising the tetragonal doublet and the single cubic peak (i.e., $2\theta = 50^\circ$) represented most of the monoclinic phase volume (Fig. 1).

Rietveld analysis showed that both DLP0 and SM0 contained mixed cubic and tetragonal phases, with approximately 17% and 10% of the cubic phase detected, respectively. In addition, both Y-TZP types showed significant phase transformation after 5 h of aging, with approximately 22% of the monoclinic phase in DLP5 and SM5. At 10 h, the aging process continued, with the monoclinic phase in DLP10 and SM10 at approximately 29.53% and 25.51%, respectively (Table 2).

In two-way ANOVA of AFM results, "material type" ($p < 0.05$), "aging time" ($p < 0.05$) and "interaction of material and aging time" ($p < 0.05$) had significant effects at a sub-micron resolution, whereas no such significant effects were detected at a nanometer resolution.

Specifically, at the sub-micron scale, the SM-fabricated zirconia showed a higher surface roughness than the DLP-fabricated product. In addition, SM5 and DLP5 presented lower surface roughness ($R_a; R_q; R_z$) compared with the non-aged and 10-h groups, respectively. At the nanometer scale, zirconia products fabricated by the SM and DLP technologies showed similar surface roughness, which was not significantly altered by aging (Figs. 2-3).

3.2. Cell viability and proliferation

According to the two-way ANOVA of live/dead viability assay results, negative effects for the parameters "material type" and "aging time" were observed. In the multi-way ANOVA of CCK-8 assay data, the parameter "culture time" had a significant effect on the results ($p < 0.05$), whereas no statistically significant effects of the parameters "material type" and "aging time" were observed.

Both DLP and SM groups showed low percentages of dead cells, with a negative difference after 24 h of culture. In addition, HGFs attached to DLP-fabricated and SM-fabricated Y-TZP products showed similar cell proliferation ability, and presented statistically significant increases after 48 h and 72 h of culture compared with the control groups. Aging caused no change in viability or proliferation in HGFs seeded on both zirconia types (Fig. 4).

3.3. Cell morphology

HGFs were confluent and evenly spread on all experimental specimens, regardless of the material type or aging time. SEM and CLSM observations indicated that HGFs cultured on both Y-TZP types presented a spindle or elongated phenotype with multiple extensions of filopodia, whereas the cells on the SM-fabricated zirconia seemed to show a better flat elongated shape, and those on the DLP-fabricated zirconia were mostly spindle-shaped. As shown in the SEM photomicrographs, HGFs grown on SM-fabricated Y-TZP surfaces were well arranged in multiple rows, whereas a completely random distribution of cells was observed on DLP-fabricated Y-TZP surfaces, which may be attributed to the varying surface characteristics of the two types of materials caused by different manufacturing methods (Fig. 5).

3.4. Cell adhesion ability

Multi-way ANOVA of cell adhesion ability assay data showed that the parameter "material type" had no effect on the results, whereas "culture time" and "aging time" had statistically significant effects on

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Table 2. Unit cell dimensions and phase assemblage features before and after aging as determined by X-ray diffraction analysis

| Material | Aging Time (h) | Cubic wt% | Unit Cell (Å) | Tetragonal wt% | Unit Cell (Å) | Monoclinic wt% | Unit Cell (Å) | Total |
|----------|---------------|-----------|---------------|----------------|---------------|----------------|---------------|-------|
|          |               | $a$       | $b$           | $c$            | $a$           | $b$           | $c$           |       |
| DLP      | 0             | 17.29     | 5.130         | 82.71          | 3.601         | 5.173         | 0             | 100.0 |
|          | 5             | 12.27     | 5.127         | 65.21          | 3.596         | 5.165         | 22.52         | 5.142 | 5.190 | 5.327 | 99.232 | 100.0 |
|          | 10            | 11.18     | 5.131         | 59.29          | 3.597         | 5.165         | 29.53         | 5.140 | 5.182 | 5.328 | 99.269 | 100.0 |
| SM       | 0             | 9.53      | 5.132         | 90.47          | 3.602         | 5.173         | 0             | 100.0 |
|          | 5             | 8.45      | 5.134         | 69.35          | 3.599         | 5.170         | 22.2          | 5.142 | 5.195 | 5.332 | 99.299 | 100.0 |
|          | 10            | 6.79      | 5.135         | 67.7           | 3.598         | 5.169         | 25.51         | 5.139 | 5.189 | 5.330 | 99.257 | 100.0 |

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Fig. 1. XRD patterns for the zirconia products tested. Cubic (blue arrows), tetragonal (gray arrows) and monoclinic (black arrows) peaks are indicated in the spectra.
adhesive cell amounts.

Specifically, DLP- and SM-fabricated Y-TZP products exhibited comparable results at the three time points examined. After culture for 4 h, the number of attached cells in the 5-h aging group was lower than that in the non-aged and 10-h aging groups, regardless of material type (Figs. 6-7).

4. Discussion

Zirconia exhibits a higher melting point, higher thermal shock sensitivity and lower sinterability than other types of additive-manufactured materials. Therefore, it is difficult to acquire fully consolidated and defect-free parts using additive manufacturing methods that produce directly sintered bodies[3]. By adjusting the composition of the ceramic slurry (CeraMatrix, QuickDemos Company, Jiangsu, China), accurately controlling the thickness of each layer in the process of three-dimensional printing, and using the area light source for preliminary curing, the compact structure of the zirconia embryo is extensively formed, and its flexural strength can be close to that of zirconia products made using SM technology[15]. As a promising biomaterial extensively applicable for dental implant restoration, the DLP-fabricated zirconia should be physically and biologically resistant to aging. However, most existing studies assessing additive-manufactured zirconia products are related to their mechanical properties, fabrication accuracy, and trueness[23,24]. Consequently, scarce information is available about the aging resistance of zirconia manufactured using the DLP technology. Here, the effects of aging on the microstructural, surface, and biological features of the DLP-fabricated zirconia were evaluated.

Effective soft tissue sealing around the implant can protect the alveolar bone from bacterial invasion, which is critical for the optimal long-term function of dental implants[13,17]. Nonetheless, the orientation of soft tissue attachment to the dental implant is quite different from that of a natural tooth. Around teeth, periodontal fi-
**Fig. 3.** Surface roughness of the zirconia products studied at the sub-micron scale (a) and nano-scale (b). Asterisks over black dotted lines indicate statistical significance among various groups (p < 0.05).

**Fig. 4.** (a) CLSM micrographs depicting Live/Dead viability assay results. Live (green) and dead (red) cells are presented (×100). A few dead cells are indicated by white arrows. The doughnut charts showed the ratio of live cells (green) and dead cells (red). (b) CCK-8 assay data, quantitatively evaluating HGF proliferation on the zirconia at 1, 2, and 3 days. No significant differences were found among the different experimental groups. Scale bar = 200 μm.
bers are radially located; considering dental implants, connective tissue fibers are parallel to the surface of the material, causing sub-optimal attachment and vulnerability to mechanical or microbial destruction[25,26]. Specifically, connective tissue attachment to dental implants is analogous to scar tissue containing elevated amounts of fibroblasts and collagen fibers (representing approximately 1/3 and 2/3 in volume, respectively, with collagen fibers mainly secreted by HGFs for gingival wound healing and tissue regeneration[27]). Therefore, the amounts and bioactivity of HGFs resting on implant abutment materials are vital for the generation of peri-implant soft tissue seals. In addition, zirconia abutments are mostly used in the esthetic area of the anterior maxilla, where the soft tissue is thin and more prone to gingival recession[13,28]. This requires biocompatibility of zirconia abutment and further investigation of the biological effects of DLP-manufactured zirconia on HGFs.

Both the cubic and tetragonal phases were detected in DLP0 and SM0, which was confirmed by a previous study demonstrating that 3Y-TZP ceramics sintered at >1450 °C show a dual microstructure generated by cubic and tetragonal grains[29]. Crystalline phase analysis demonstrated that both Y-TZP types contained different percentages of cubic and tetragonal phases prior to aging. Although the material information provided by manufacturers indicates that Y-TZP fabricated by the SM and DLP technologies have similar compositions, they may have minor differences in the specific contents of various components. In addition, differences in the manufacturing methods and the distribution of oxide dopants may also contribute to the observed discrepancy of phase composition. After aging for 5 h, a significant increase in the monoclinic phase (approximately 22%) was observed for both Y-TZP types, which was concomitant with the cubic and tetragonal phase reductions. In addition, the Y-TZP studied complies with the ISO standard 13356:2008, requiring that Y-TZP should not contain more than 25% of the monoclinic phase after 5 h of autoclave-induced aging at 134 °C and 2-bar pressure[30]. As the aging time increased to 10 h, DLP10 and SM10 corresponded to 29.53% and 25.51% in the monoclinic phase, respectively. The different aging resistance levels of the DLP and SM fabricated Y-TZP products may be attributed to their disparate contents of the cubic phase before aging, which was shown to have a critical effect on aging in 3Y-TZP[29,31]. Cubic grains have high amounts of yttrium, which may lead to yttrium depletion in adjacent tetragonal grains during the aging process. Furthermore, cubic grains serve as nucleation sites for tetragonal to monoclinic phase transformation[29].

AFM analysis showed that the surface performance of Y-TZP presented variable susceptibility to aging under disparate observation resolutions. At the sub-micron scale, both Y-TZP types exhibited significantly lower surface roughness after 5 h of aging and comparable

Fig. 5. Cell morphology of HGFs grown on the zirconia for 24 h. (a) SEM microphotographs showing cell alignment patterns and overall cell morphology (×250). (b) CLSM micrographs depicting the filamentous actin (green) and nuclei (blue) of HGFs (×600). Scale bar = 100 µm (a) or 30 µm (b).
surface roughness to that of non-aged Y-TZP after aging for 10 h. At
the nanometer scale, aging caused no significant surface changes
for any of the Y-TZP types. It may be cautiously inferred that the sur-
face roughness alteration of Y-TZP after aging mainly occurs at the
sub-micron scale. According to previous reports, the surface change
tendency of autoclaved zirconia remains controversial, as it can be
influenced by multiple factors, including zirconia composition, preci-
sion of detecting instruments, aging protocol, and specimen prepa-
ration methods, which might cause an evident change in aging be-
havior[9,12,32]. Yang et al. demonstrated that aging time (≤5 h and
>5 h) significantly affects the surface roughness of zirconia ceramic
products, which is probably explained by the different processes and
extent of aging within or after more than 5 h[9]. In addition, Deville et
al. indicated that the aging kinetics of zirconia is critically influenced
by the surface stress state, and the tetragonal to monoclinic transfor-
mation preferentially occurs on neighboring areas of residual stress
concentration[6,32]. Thus, it can be assumed that the effects of LTD
(i.e., grain pullout) on the zirconia surface first occur in the proximity
of grains with the highest topography (superficial layer), where an
uneven distribution of stress is most likely to co-exist with increased
susceptibility to water contact, resulting in reduced surface rough-
ness compared to non-aged zirconia[32,33]. With increasing aging
time, phase transformation is extended to the entire surface and in-
side the crystal grain via a nucleation and growth mechanism, yield-
ing Ra values comparable to those of non-aged samples[6,9,32]. Al-
though this mechanism still needs further investigation, the present
study examined different surface features of zirconia aging for the
5- and 10-h time points.

In this study, zirconia manufactured by different technologies
showed similar biological behaviors, including the effects on the vi-
ability and proliferation of HGFs after growth in direct contact. In ad-
dition, no cytotoxic effect of any Y-TZP type was detected even after
10 h of accelerated aging. The responses of HGFs indicated favorable
and stable biocompatibility of the newly fabricated DLP-zirconia, al-
though minor changes in surface roughness were found at the sub-
micron scale after aging. The present results were in agreement with
those of a previous study that found that autoclave aging could in-
duce some changes in the physicochemical properties of machined
Y-TZP, without interfering with the cellular behavior of HGFs[10].
Moreover, Pandoleon et al.[13] found that metabolic activity is overt-
ly reduced in HGFs cultured on autoclaved Y-TZP surfaces during the
first 48 h, in comparison with those seeded on non-aged specimens.
The authors considered that LTD causes elemental distortion in the
crystalline structure of zirconia, including yttrium depletion and
oxygen vacancy formation, which may lead to a transitory increase
of cell death. The discrepant results of the aforementioned research
and the present work might be attributed to multiple factors, includ-
ing differences in zirconia composition, manufacturing methods and
experimental protocols. Furthermore, in the present study, all zir-
conia specimens underwent ultrasonic washing in ethyl alcohol for
approximately 30 min before application in biological experiments,
which may also alter the cell response to the material.

HGFs grown on both zirconia types showed spindle or elon-
gated cell shape, reflecting good attachment to the specimen sur-
face, and the minor difference in Ra values at the sub-micron scale
seemed to cause no major change of cell morphology. Differences in
cell alignment patterns and overall cell morphology on disparately
manufactured Y-TZP were observed in the present study, which may
be explained by the different surface features of Y-TZP. Specifically,
the DLP-fabricated zirconia showed a flat and smooth surface, and
the cells exhibited a completely random distribution on the surfaces.
However, shallow grooves originating from the machining process
can be observed on zirconia fabricated by SM technology. The natu-
ral grooves on the SM-fabricated zirconia seemed to control the
position of cells in an organized pattern, with a better flat spindle-
shaped cell alignment[10]. This observation was in agreement with

Fig. 6. CLSM micrographs of cell nuclei stained with DAPI (blue). (a–c) Cell
adhesion to the zirconia after HGF seeding on the surface for 1, 4 and 24 h
(×100). Scale bar = 200 µm.
previous studies, which reported that surface topography may affect fibroblast adhesion and intracellular signaling via focal adhesion and integrin-related pathways[14,34]. When the machined zirconia was used as implant neck, firm connective tissue adhesion resembling that of the machined titanium was observed, with a parallel/parallel-oblique orientation to the implant surface[26]. However, without further investigation, it cannot be concluded that the seemingly well-arranged pattern of SM zirconia is better than the random alignment of the DLP zirconia. In addition, although the 5 h-aged zirconia showed a significant decline in sub-micron surface roughness, such a decrease failed to cause major changes of cell morphology or distribution patterns.

The association between specimen surface roughness and early stage cell adhesion and proliferation abilities remains controversial. Some reports have demonstrated that fibroblasts favor smooth surfaces[35–37], whereas others claim the opposite[14,38]. Moreover, there are no clear definitions for smooth and rough surfaces, as surfaces having various roughness levels in different measurement scales have been tested previously, with disparate results obtained by multiple methods, hindering the direct comparison of these conclusions. Nevertheless, it was clearly demonstrated that surface topography, particularly at the nanometer scale, influences cell behavior in peri-implant soft tissues. The vast majority of surface features observed in and on natural tissues (i.e., bone, enamel) are on the nanometer scale[39]. In addition, nanoscale topographic structures regulate gene expression in fibroblasts and epithelial cells, and minute alterations of nanometer surface features can considerably impact gingival regeneration[17,22]. In the present study, the nanoscale surface roughness levels of all zirconia products tested were similar, regardless of material type and aging time, which possibly accounts for the absence of major cell behavior differences among all groups. Additionally, nano-surface topography can dictate initial protein adsorption, thus mediating subsequent cellular adhesion[34,40]. This probably explains the above results, where HGF attachment rates were similar in all types of zirconia tested, except for small aging time/culturing time-dependent cell number differences. In addition, according to previous experiments studying the influence of sub-micron scale roughness on cell behaviors, a large change (at least sufficiently large for the cells) is usually required to cause significant responses of cells[22,38]. In the present study, only minor changes in surface roughness were detected at the sub-micron scale, which seems to be too small in comparison with the mean size of HGFs (100 μm). Therefore, no evident changes in cellular behavior were observed, as previously demonstrated[38,41].

5. Conclusion

This study demonstrated that aging can cause significant changes in the phase composition and sub-micron-scale surface roughness of the DLP-fabricated zirconia. However, the accelerated aging process did not interfere with the biocompatibility of this new type of zirconia, even when the aging time reached 10 h. Moreover, despite overt differences in manufacturing methods and specific phase compositions, zirconia products fabricated by the DLP and SM technologies showed comparable biological properties both before and after aging. The conditions in this in vitro study were not identical to those found in vivo. However, these findings may contribute to the estimation of long-term physical and biological behavior of DLP-made zirconia products, and may expedite the dental applications of this additive-manufactured ceramic. Further research is required to assess its performance in experimental animals and humans.

Acknowledgments

The current study was funded by the National Natural Science Foundation of China (No. 82071145) and the Research Fund of Interdisciplinary Innovation Project from West China Hospital of Stomatology Sichuan University (RD-03-202009). The study sponsors had no role in design of the study, collection and analysis of data, writing of the manuscript, or decision to publish.

Conflicts of interest

The authors declare no conflict of interest.

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