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Evaluation of the biological behaviour of various dental implant abutment materials on attachment and viability of human gingival fibroblasts

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ABSTRACT

Objective. This study aimed to investigate the biological effects of yttria-stabilized zirconia (Y-TZP) compared to other dental implant abutment materials, i.e. lithium disilicate (LS2) and titanium alloy (Ti), as well as the effects of aging of Y-TZP on viability/proliferation and attachment properties of Human Gingival Fibroblasts (HGFs).

Methods. Cylindrical specimens of each material were prepared as per manufacturer's instructions. Y-TZP specimens were divided into three groups: 1. no aging (Zr0), 2. aging for 5 h, 134 °C, 2 bars, 100% humidity (Zr5), 3. aging for 10 h under the same conditions (Zr10). Surface roughness was evaluated by optical profilometry; cell metabolic activity/viability by MTT assay, morphological changes by Scanning Electron Microscopy (SEM) and ratio of live/dead cells by confocal microscopy.

Results. Results showed statistically significant reduction of HGF metabolic activity/viability in contact with Y-TZP after aging. Nevertheless, non-aged zirconia showed no significant differences compared with LS2, Ti and control cultures. In contrast, significant stimulation of cell metabolic activity/viability was observed in HGFs exposed to LS2 eluates. Differential morphological patterns were observed for HGF in contact with different materials/treatments, with obviously increased number of dead cells and sparser distribution of HGFs cultured on Zr10 specimens. These effects were not correlated with surface topography, since Y-TZP aging did not alter surface micro-roughness.

Significance. These findings indicate that Y-TZP shows comparable biological properties to Ti and LS2 as implant abutment material. Nevertheless, Y-TZP aging might influence gingival cell attachment and proliferation properties, providing an alert to a potentially negative effect on the long-term maintenance of gingival architecture.

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1. Introduction

Ceramics have gained outstanding popularity in recent years due to increased aesthetic demands, together with biocompatibility concerns related to the use of dental alloys [1]. Zirconia is a high-strength biomaterial satisfying patients' demands for superior esthetics and metal-free prostheses with long-term durability [2]. In addition, over the last twenty years, implantology has developed faster than any other area of modern dentistry in conjunction with the innovative application of Computer Aided Design/Computer-Aided Manufacturing (CAD/CAM) technology [3]. Nowadays, zirconia abutments comprise the most widely applicable abutments marketed by implant manufacturers for aesthetic implant-supported restorations. These abutments are available in both prefabricated and customized forms and may be prepared either by dental technicians or by utilizing CAD/CAM systems.

The clinical success rates of dental implants relate to several factors promoting implant fixture osseointegration and the successful healing of soft tissues at the implant-abutment interface.

The primary disadvantage of zirconia implant abutments stems from their intrinsic vulnerability to low temperature degradation (LTD), caused by aging phenomena occurring during clinical service *in situ* [4]. This drawback primarily concerns zirconia frameworks, or their parts not covered by porcelain veneering, but also affects zirconia implant abutments that are partially exposed to the oral environment.

Pure zirconia is a polymorphic material occurring naturally in three crystalline forms: monoclinic (m) at room temperature (RT), tetragonal (t) above 1170 °C and cubic (c) above 2370 °C. The transformation from the higher energy state of the t phase to the lower energy state of the m phase occurring at RT during LTD phenomena is accompanied by a volume expansion of 3–4% permitting water penetration into the bulk of the material [5]. By alloying pure zirconia with different stabilizers added to the starting powder, the metastable t phase can be maintained at RT to a greater extent [6].

The most widely investigated stabilizers for forming alloys with zirconia to be used as a dental biomaterial are CaO [7], MgO [8] and Y_2O_3 [9]. However, only yttria-stabilized zirconia (ZrO_2-Y_2O_3) complies with the ISO standard 13356:2008 [10], (Implants for surgery — Ceramic materials based on yttria-stabilized tetragonal zirconia, Y-TZP), since this standard requires that the monoclinic phase should constitute less than 25% after autoclave-induced aging of the materials for 5 h at 134 °C and 2-bar pressure. This requirement is based on an aging study by Chevalier et al. which argues that the aging of zirconia brought about by exposure to 1 h of autoclave treatment at 134 °C under 2 bar pressure results in a t to m transformation corresponding to approx. 3–4 years of clinical service of the material [11]. Using Y_2O_3 with the addition of 2–3 mol % to produce a material containing only the tetragonal phase was first suggested by Rieth et al. [12] and Gupta et al. [13].

The utilization of zirconia as a reliable material for prosthetic restorations requires an awareness on this t to m transformation process and its effects on the long-term mechanical and biological properties in clinical practice. When this transformation is initiated by externally applied stresses, it produces an expansion and shape transformation within individual grains; this absorbs energy and increases damage resistance [5].

As an abutment material, the biocompatibility of zirconia regarding soft connective and epithelial tissue is essential. The long-term preservation of an acceptable esthetic outcome is closely associated with maintaining the gingival architecture and attachment on zirconia surfaces after *in vivo* aging. In a systematic review [14] of different types of abutments for fixed implant reconstructions, soft tissues in contact with ceramic abutments (zirconia, alumina) exhibited a higher incidence of soft tissue recession. The reason behind this effect remains unclear, although, in the study, the higher degree of recession was attributed to the more frequent use of ceramic abutments in the esthetic zone of the anterior maxilla where there may be a greater risk of recession than in the molar region, where soft tissue is usually thicker. Moreover, in this study no distinction was made between alumina and zirconia ceramic abutments. Similar effects have not been previously reported for conventional lithium disilicate (LS2)- and titanium alloy (Ti-6Al-4V)-based (Ti) dental implant abutment materials. Consequently, there remains a need for more in-depth evaluation of the long-term biological behavior of zirconia abutments in contact with the gingival tissues.

Based on the above, the present study aimed to investigate the biological effects of Y-TZP in comparison with LS2- and Ti-based dental implant abutment materials on cell attachment and viability/proliferation of human gingival fibroblasts (HGFs) and to correlate results with material surface micro-roughness properties. Additional objective of this study was to investigate the effects of *in vitro* accelerated aging of Y-TZP on the aforementioned biological properties of HGFs. The research (null) hypotheses were that no differences exist among various implant abutment materials in biological performance on the cell level and that *in vitro* accelerated aging of Y-TZP, corresponding to several years of clinical service, would have no impact in its biological behaviour on HGFs.

2. Materials and methods

2.1. Materials

For the experimental procedures of this study, disc specimens of the following materials were used: 1. yttrium stabilized zirconia; Zr (IPS e-max ZirCAD, Ivoclar-Vivadent AG, Lichtenstein). 2. lithium disilicate ceramic; LS2 (IPS e-max CAD, Ivoclar-Vivadent AG, Lichtenstein). 3. titanium alloy; Ti (Ti 6AL 4V- ELI Titanium Alloy for implants, grade V, Zapp Medical Alloys GmbH). The zirconia specimens were divided into the following experimental groups based on the type of treatment: 1. no aging procedure was applied (Zr0), 2. aging was performed for 5 h (Zr5), 3. aging was performed for 10 h (Zr10), as described in 2.2.2.

2.2. Methods

2.2.1. Manufacture of material specimens

A total of 189 zirconia specimens (63 for each experimental group), 63 LS2 specimens and 63 Ti specimens were prepared for the experimental needs of this study. The final dimensions of all specimens (after the sintering of the ceramic materials) were 8 mm in diameter and 2 mm in height and the total surface area of each specimen was calculated to be approx. 1.5 cm^2. The ceramic blocks were first cut by means of a CAD-CAM device (Milling machine YENA model D40). Following this, the cylinders were cut into disc-shaped specimens using a 12.7 × 0.381 × 1.27 cm diamond wafering blade (UKAM #2 3-4 889ME) with a diamond depth of 4 mm. Sintering was performed using a Vita Zircomat T furnace for zirconia and an ep500 Ivoclar furnace for the lithium disilicate discs, according to cycles/temperatures recommended by the manufacturers.

2.2.2. Aging of Y-TZP specimens

For induction of accelerated aging of the zirconia specimens, the method proposed by Chevalier et al. [11] was utilized, with the aging times set at 5 and 10 h. To achieve the conditions for aging required by the specific protocol, an autoclave, KavoKlave 2100, was used (KavoDental, Biberach/Riss, Germany). This device provides the ability of creating a 100% humidity environment under a pressure of 2 bars and a temperature of 134 °C. After the *in vitro* aging process, the specimens were dried using hot air and stored until used in a desiccator at RT.

2.2.3. Evaluation of surface roughness of material specimens

Evaluation of surface roughness was performed with a 3D optical profilometer [Bruker, ontour GT, Tucson, AZ, USA] at 5 regions per specimen (n = 5) of each group (Ti, LS2, Y-TZP for the first objective and Zr0, Zr5, Zr10 for the second objective). Vertical scanning of surface was performed three times beginning from 5 different scattered spots and combined with interferometry mode [VSI] to compare values and evaluate surface roughness alterations. Vision 64 software was used at a magnification [×10] of captured surface images.

The surface roughness parameters measured were the amplitude parameters of the arithmetic average of the absolute values of the surface height deviations measured from the best fitting plane (Sa); the standard deviation of the height distribution (Sq); the 10-point height over the surface, representing the average difference between the 5 highest peaks and 5 lowest valleys (Sz). A mean Sa, Sq, and Sz was determined for each scaled side of each specimen to ascertain the overall roughness of the surface; the smaller the value, the smoother the specimen.

2.2.4. Establishment of primary human gingival fibroblast (HGF) cultures

HGF cultures were established from human biopsies of gingival tissues taken during routine third molar extraction. The study was approved by the ethical committee of the Institutional Review Board (11/23.11.17). Cultures were established using the outgrowth method, as previously published [15]. Briefly, small pieces of gingival tissues produced by mincing were transferred into tissue culture flasks (together with a small amount of Dulbecco's modified Eagle's medium, DMEM, Invitrogen, Carlsbad, CA) and were then allowed to adhere to the bottom of the flasks for 30 min. Subsequently, 5 ml DMEM supplemented with 10% fetal bovine serum (FBS, Invitrogen) and antibiotics (100 U/ml medium of penicillin, 100 mg/ml streptomycin, Invitrogen) were added to the flasks. The cultures produced were kept at 37 °C in an incubator in an air atmosphere with 95% humidity and 5% CO_2. When a substantial fibroblast outgrowth (80% confluence) had been obtained, the cells were detached by trypsinization (using 0.25% trypsin/1 mM EDTA) and then subcultured under standard conditions. The cells of passages 2–6 were used for the experiments.

2.2.5. Direct cytotoxicity assay

In order to evaluate the potential cytotoxicity, the material specimens were transferred to 48-well culture plates and HGFs were seeded on top of each specimen at a density of 10^4 cells/well. The HGFs were incubated in direct contact with the materials for 24, 48 and 72 h. Subsequently, an MTT assay was performed in order to determine the mitochondrial dehydrogenase activity. In short, at the end of each incubation period, a solution of 0.5 mg/ml MTT in complete culture medium was added to each well and the cells were then incubated for 4 h at 37 °C and 5% CO2. Subsequently, the culture supernatant was discarded and the insoluble formazan dissolved using DMSO for 1 h at 37 °C. The absorbance was measured against blank (DMSO) at a wavelength of 545 nm and a reference filter of 630 nm using a micro plate reader (Epock, Biotek, Biotek instruments, Inc, Vermont, U.S.A). As a control, cells directly seeded into the bottom of the wells were employed. The experiments were run in six replicates and repeated three times. The results were calculated as the averaged absorbance of all replicates and then expressed in the form of a % percentage of the values for the control (cells).

2.2.6. Indirect cytotoxicity assay

For indirect cytotoxicity testing, eluates of zirconia, lithium disilicate and titanium alloy were prepared by incubating specimens of each experimental group in DMEM medium supplemented with antibiotics for 72 h at 37 °C. The ratio between the surface area of the specimens and volume of extraction medium was 1.5 cm^2 /ml, in accordance with the ISO 10993/12 requirements [16]. In order to test the cytotoxicity, HGFs were seeded in 96-well plates at 10^4 cells/well and then allowed to attach for 24 h. Subsequently, the culture medium was replaced by 200 μL per well of each of eluates from the following experimental groups: Y-TZP, LS2 and Ti for the first objective and zirconia aged for 0, 5 and 10 h (Zr0, Zr5, Zr10) for the second objective. All the eluates from each material were enriched with 10% FBS immediately prior to being exposed to the cells. The cells were incubated for 24, 48 or 72 h at 37 °C in a humidified 5% CO_2 atmosphere. This was followed by an MTT assay performed as described for the direct cytotoxicity test, using eluate-free cells as a control for the % percentage expression.

2.2.7. *Scanning electron microscopy (SEM) analysis*

Specimens from each experimental group (Y-TZP, LS2 and Ti for the first objective and Zr0, Zr5, Zr10 for the second objective) were placed in 48-well plates and seeded with 2×10^4 cells/well. After 72 h, the cells were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate containing 0.1 M sucrose, at pH 7.4 and dehydrated using a graded series of ethanol concentrations. Finally, the specimens were exposed to Hexamethyldisilazane (HMDS) for 10 min and placed under air-drying in a hood for 20 min [17]. After carbon coating of the specimens with an average thickness of 200 Å using a vacuum evaporator JEOL-4x, cell morphology was evaluated by a Scanning Electron Microscope (J.S.M. 840A; JEOL, Tokyo, Japan). All analyses were performed with a 20-kV accelerating voltage and 0.4 mA probe. The images were taken in magnifications × 250 and × 500.

2.2.8. *Live/dead staining and confocal laser scanning microscopy (CLSM)*

Quantification of the live/dead cells in contact with the cell-seeded specimens of each experimental group (Y-TZP, LS2 and Ti for the first objective and Zr0, Zr5, Zr10 for the second objective) after a cultivation time of 72 h was performed by means of immunofluorescent labelling with Calcein AM/Ethidium homodimer (EthD-1) staining respectively, and visualization by CLSM (Nikon Eclipse C1; EZ C1 3.20 software). Co-expression was confirmed in Z-stacked serially captured images and analyzed by pixel analysis using the Image J color pixel counter plug in.

2.2.9. *Statistics*

All experiments were performed in 3–6 replicates and repeated 3 times. Statistical analysis of biological experiments was performed by using two-way analysis of variance (ANOVA) and multiple comparisons between groups was made by Tukey's post-hoc test. Statistical analysis of the surface roughness experimental data was performed by using two-way analysis of variance (ANOVA) and multiple comparisons between groups was made by Bonferroni post-hoc test. Normal distribution was confirmed by Kolmogorov-Smirnov normality tests. Data were expressed as means (standard deviation; SD). All analyses were performed using Prism 6.0 Software (GraphPad, CA, USA) ($p < 0.05$).

3. Results

3.1. *Evaluation of surface roughness microtopography of material specimens*

Titanium presented the lowest surface roughness among the materials tested (Sa: 0.17 ± 0.07; Sq: 0.23 ± 0.11; Sz: 4.08 ± 1.04 μm), difference which was statistically significant ($p < 0.001$) compared to LS2 (Sa: 1.60 ± 0.09; Sq: 2.07 ± 0.12; Sz: 23.55 ± 4.52 μm), as well as compared to Y-TZP (Sa: 1.35 ± 0.13; Sq: 1.76 ± 0.21; Sz: 15.50 ± 1.03 μm). Moreover, LS2 presented statistically significantly higher roughness compared to Y-TZP ($p < 0.05$ for all parameters Sa, Sq and Sz) (Fig. 1a–h).

Aging of zirconia did not cause any statistically significant alterations to surface roughness parameters, except from a minor but statistically significant increase of the Sz value for zirconia exposed to 5 h of aging as compared to the non-aged and aged for 10 h aged specimens ($p < 0.05$) (Fig. 1a–h).

3.2. *Evaluation of cell metabolic activity/viability after direct and indirect metabolic-based tests (MTT assay)*

Based on the two-way ANOVA analysis of the results of the direct MTT assay, statistically significant effects of the parameters "material type" ($p < 0.0001$), "time" ($p < 0.0001$) and "interaction of material and time" ($p < 0.05$) were observed. The same was the case for the parameter "aging time" of Y-TZP ($p < 0.0001$).

In particular, a minor but statistically significant increase of cell metabolic activity (as indirect marker of cell viability) was observed for HGFs grown in direct contact with Ti specimens after 72 h as compared to control cultures ($p = 0.0032$) as well as compared to HGF grown in contact with Y-TZP (0.0029) (Fig. 2A). This increase of HGF viability on Ti surfaces was statistically significant during time ($p < 0.0001$)

Regarding aging of Y-TZP, a statistically significant reduction of cell metabolic activity of HGFs grown in direct contact with Y-TZP specimens after aging for 5 h (Zr5; 48 h, $p = 0.0004$) and 10 h (Zr10; 24 and 48 h, $p = 0.0118$ and $p < 0.0001$ respectively), as compared with control cultures were observed. However, cell metabolic activity increased from 48 to 72 h in both Zr5 and Zr10, reaching similar levels to the control cultures ($p < 0.01$ for both groups) (Fig. 2B).

Based on the two-way ANOVA analysis of the results of the indirect MTT assay, statistically significant effects of the "material" ($p < 0.0001$), "aging time" of Y-TZP ($p < 0.0001$) and "time" ($p < 0.05$) were observed. In particular, a statistically significant stimulation of cell metabolic activity could be observed in HGFs exposed to eluates of LS2 after 48 h ($p = 0.0410$) and 72 h ($p < 0.0001$), as compared to control cultures, as well as compared to Ti eluates ($p = 0.0282$) (Fig. 2C). Increase of cell viability after exposure to LS2 eluates also showed statistical significance with time ($p < 0.01$).

In contrast, respective increases observed for cells exposed to Zr0 and Zr5 eluates failed to reach statistical significance compared to control cultures (Fig. 2D).

3.3. *SEM evaluation of cell morphology*

HGFs in direct contact with the experimental specimens showed differential morphological and cell adhesion patterns, as assessed by SEM analysis. A typical mosaic-shaped confluent cell layer could be formed on all types of specimen surfaces. The cells appeared to be intimately attached to all surfaces and were evenly spread.

In more detail, cells grown on Ti specimens presented a flattened phenotypic expression and were widely spread, indicating good attachment, most probably due to the smooth, polished titanium surface (Fig. 3a). Cells grown on LS2 surfaces presented a completely random distribution and a typical spindle-shape phenotype with multiple filopodia (Fig. 3b). Cells grown in contact with Y-TZP specimens presented a characteristic elongated phenotype and appeared to be well-aligned in multiple rows (Fig. 3c, d). This was also the case for the aged specimens, although in some areas a less dense

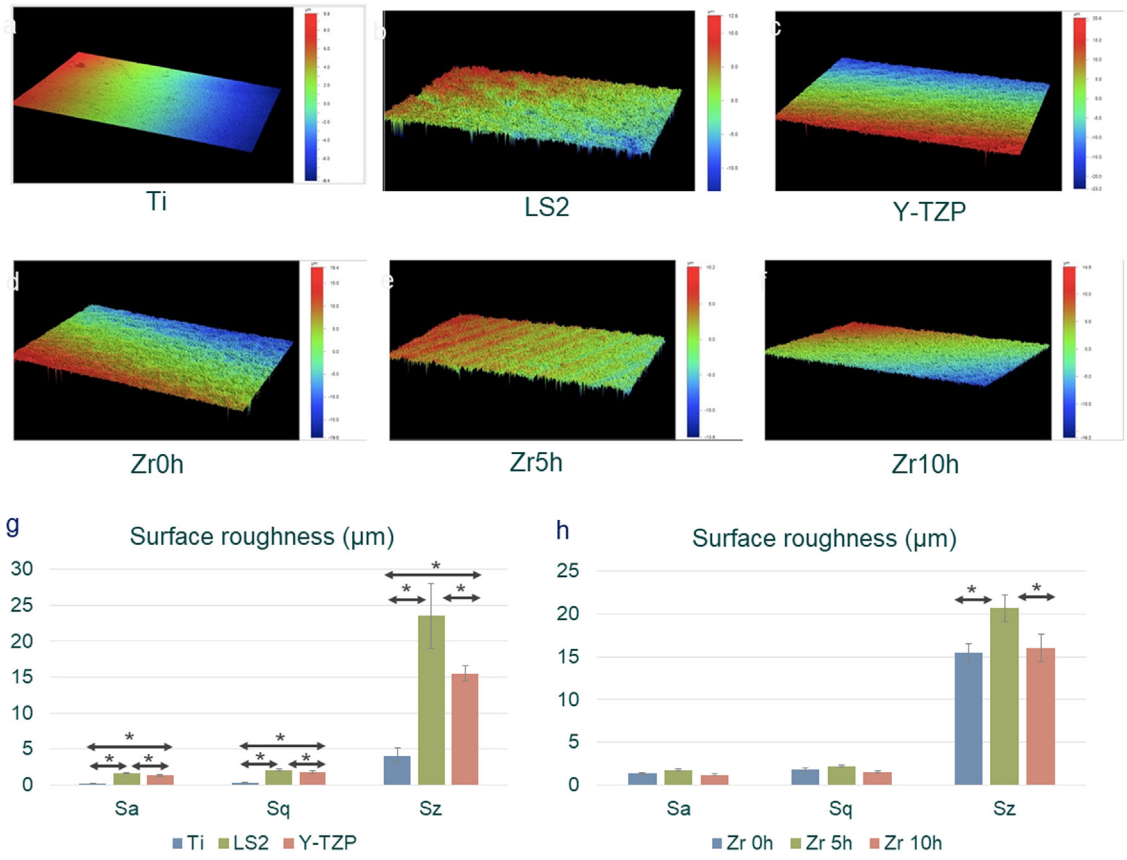


Fig. 1 – Evaluation of surface roughness of (a–c) various dental implant abutment materials (Ti, LS2, Y-TZP) and (d–f) yttrium-stabilized zirconia before and after aging (Zr0, Zr5 and Zr10) with 3D optical profilometry at 5 regions per specimen (n = 5) of each group. A mean of each of the surface roughness parameters Sa, Sq, and Sz was determined for each scaled side of each specimen to ascertain the overall roughness of the surface (g, h). Asterisks over black double arrows indicate statistically significant differences among different experimental groups ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

distribution was evident in Zr5 (Fig. 3e) and Zr10 (Fig. 3f) specimens indicating a minor effect of zirconia aging on cell attachment properties.

3.4. *Live/dead fluorescent staining and CLSM*

Live/dead fluorescent staining showed that HGF viability in direct contact with all materials under investigation exceeded 90% in all cases. A higher number of living (calcein positive cells stained green) was observed on Y-TZP- (98.2 ± 1.3%), as compared to Ti- (91.1 ± 3.2%) and LS2- (90.3 ± 5.6%) specimens, without reaching statistical significance (Fig. 4a–c).

Moreover, a significantly increased (p <0.05) number of EthD-1 positive (dead) cells, and a sparser distribution (x 100 magnification) of the cell population of HGFs cultured on Zr10 (10.9 ± 1.3%) specimens compared to Zr0 (3.1 ± 0.6%) and Zr5 (3.0 ± 1.1%) specimens was observed (Fig. 4d–f).

4. Discussion

In the present study, three materials widely used for dental implant abutment fabrication, i.e. a widely applied titanium alloy (Ti-6 A L-4 V), a lithium disilicate-based ceramic (LS2) and an yttrium stabilized zirconia (Y-TZP) material for dental application were comparatively evaluated regarding cell attachment, morphology, metabolic activity and viability of gingival cells. The long-term stability of dental implants relies on an efficient biological seal of soft tissue at the implant-abutment interface brought about through the development of sufficient connective tissue attachment to act as an effective barrier to bacterial invasion and proliferation [18]. Titanium alloy abutments have been successfully applied and have had the status of the “gold standard” for the transmucosal part of dental implants for several decades. Titanium is the dominant metal used because of its biocompatibility and stability regarding the attachment of the surrounding hard and soft tissues [19]. On the other hand, titanium abutments may sometimes be associated with aesthetic problems, particularly in the anterior region. To enhance the aesthetics of dental implants, zirconia abutments with high biocompatibility and exceptional mechanical properties have been proposed and are now used in clinical practice [20,21]. Another advantage is related to the fact that zirconia has proved less susceptible to the accumulation of plaque than metal substrates [22]. These are all factors that seem influential in soft tissue preservation

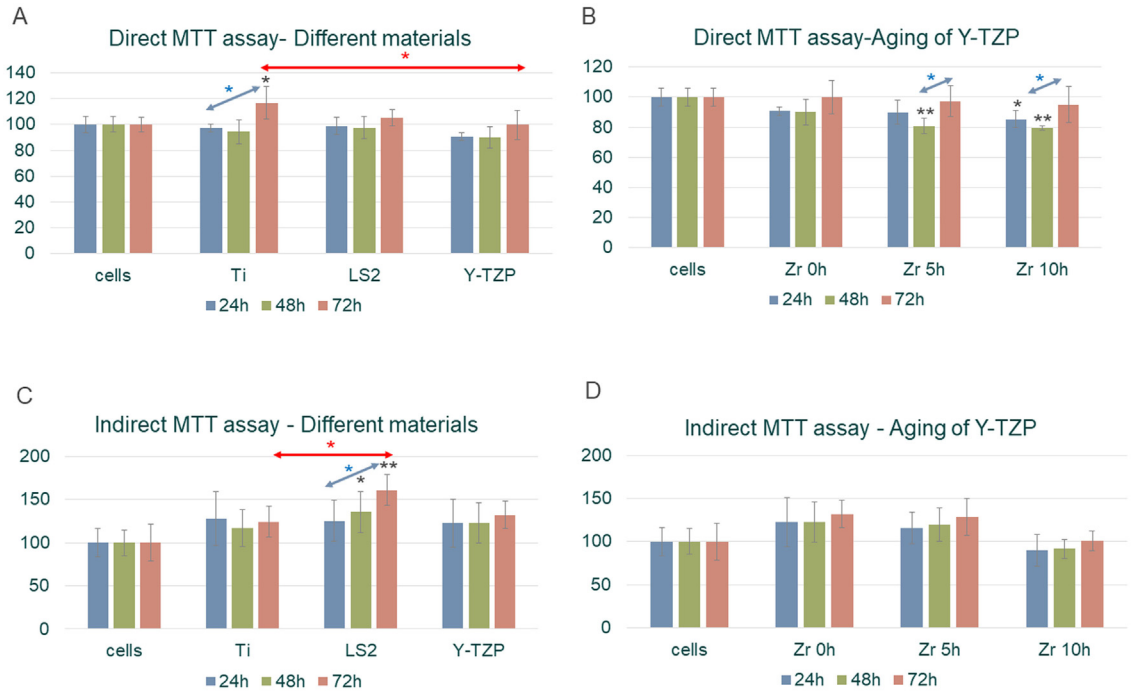


Fig. 2 – Assessment of HGF metabolic activity/viability after (A) direct contact with the abutment materials under investigation (Ti: Titanium alloy, LS2: lithium dissilicate and Y-TZP: yttrium-stabilized zirconia) (B) direct contact with Y-TZP before and after aging (Zr0: non-aged, Zr5: aged for 5 h and Zr10: aged for 10 h) (C) indirect exposure of HGF to eluates of the abutment materials under investigation (Ti: Titanium alloy, LS2: lithium dissilicate and Y-TZP: yttrium-stabilized zirconia) and (D) indirect exposure of HGF to eluates of Y-TZP before and after aging (Zr0: non-aged, Zr5: aged for 5 h and Zr10: aged for 10 h), by MTT assay (24, 48 and 72 h): *vertical axis* represents cell metabolic activity/viability expressed as % percentage of control cultures and *horizontal axis* depicts the different materials and/or aging groups. Black asterisks over each bar indicate statistically significant differences compared to control cultures (cells grown on plastic surfaces); Red asterisks over red double arrows indicate statistically significant differences among different experimental groups. Blue asterisks over blue double arrows indicate statistically significant differences during time (24–72 h) ($^{*}p < 0.05$ and $^{**}p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

and implant success rates at bone level by limiting the resorption of peri-implant tissues. Nevertheless, concerns related to aging phenomena occurring inside the Y-TZP structure under conditions of masticatory loading in the oral environment necessitate further investigation of its biological behavior at the abutment-gingival tissue interface [23,24]. Indeed, very limited knowledge exists so far on this aspect, as most existing studies on the biological behavior of zirconia are derived from the orthopedic field, where very different conditions regarding loads, standard temperature and liquid flow in a closed environment exist, as compared to the open oral cavity. In addition, studies on dental implants are documenting an overall acceptable biocompatibility of Y-TZP on the alveolar bone tissue [25].

The present study utilized primary cultures of human gingival fibroblasts (HGFs), which represent the main cell type interacting with, and being responsible for the soft tissue responses around implant abutment materials [26]. In particular, gingival fibroblasts are the most important cells participating in the transmucosal adhesion of implant-supported prostheses. Connective tissue plays a critical role in the formation of the mucosal seal around a prosthetic restoration, such as the implant abutment. The fibroblast attachment to the prosthetic substructure surface plays an important role in the formation of an efficient mucosal seal, which allows the successful maintenance of the integration of the implant and restricts any soft tissue recession in contact with ceramic abutments [27].

After implant placement and the insertion of implant abutments, the marginal gingiva is frequently irritated or injured triggering regenerative procedures. During the first phase of this process, fibroblasts migrate from the wound edges into the wound area and generate the underlying collagen matrix. The next step involves the migration of keratinocytes across this collagen matrix to create the covering epithelial keratinocyte layer mainly responsible for influencing the final aesthetics (e.g., after implant placement and implant abutment insertion) [28]. Ideally, this barrier also adheres to the surface of zirconia implant abutments to protect the implant-abutment connection and consequently the peri-implant bone against exogenous and noxious bacteria [29]. This could be especially significant when using bone-level implants where the zirconia implant abutments are closely in contact with the attached gingiva throughout their total thickness. When this peri-implant barrier is insufficient, the overall result could be the development of peri-implantitis,

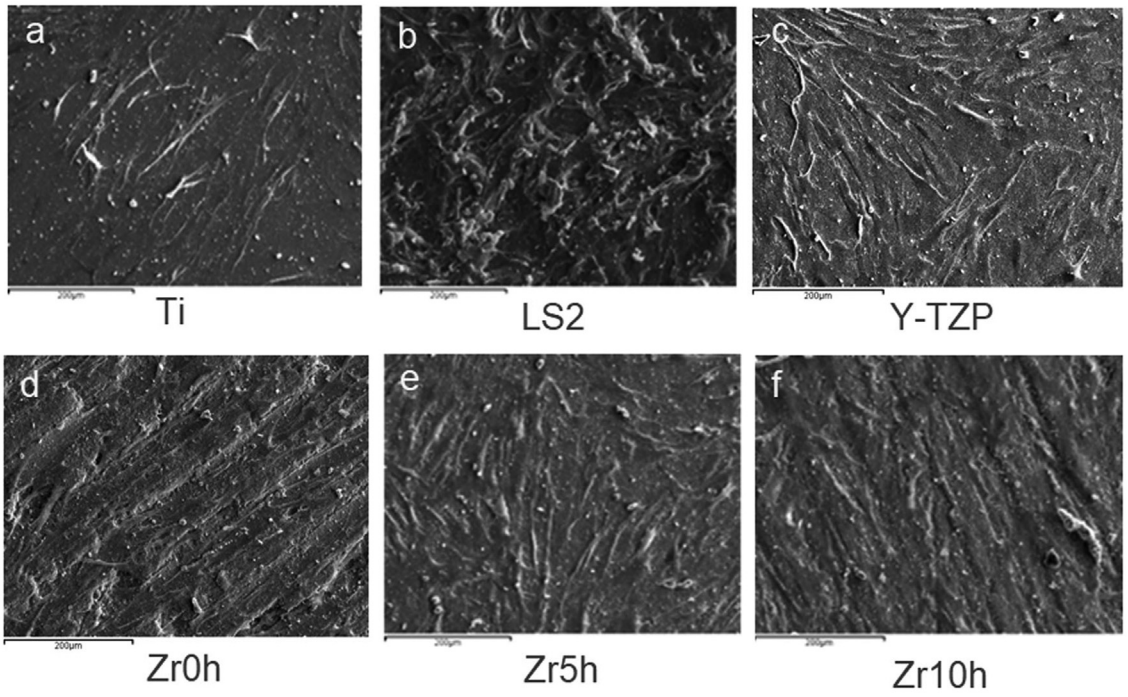
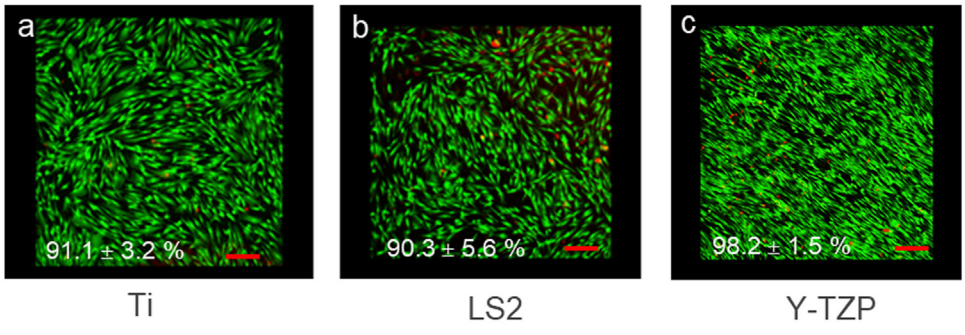


Fig. 3 – SEM microphotographs showing adhesion and spreading properties of HGF on the surface of (a–c) different abutment materials (Ti: Titanium alloy, LS2: lithium dissilicate and Y-TZP: yttrium-stabilized zirconia) and (d–f) yttrium-stabilized zirconia before and after aging (Zr0: non-aged, Zr5: aged for 5 h and Zr10: aged for 10 h).

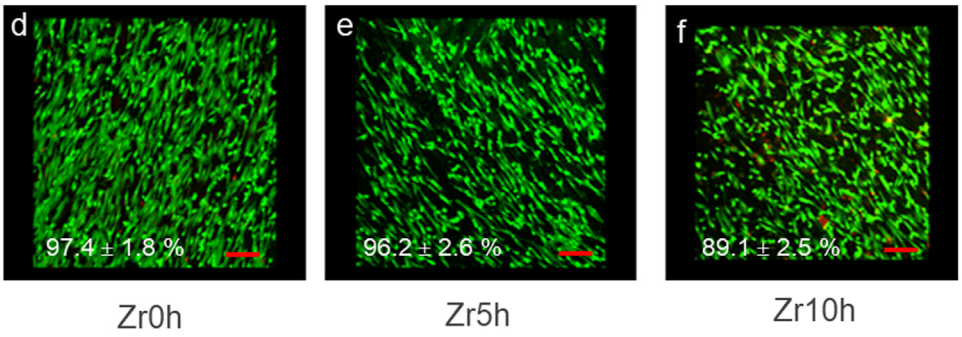
peri-implant bone loss and the possibility of an early implant failure [30].

The results of the present study indicate comparable cell behavior in terms of metabolic activity of HGFs grown in contact with all tested materials (Ti, LS2 and Y-TZP), although a significantly higher metabolic activity (as indirect marker of cell viability) was recorded for the Ti specimens by means of the MTT assay after 72 h of direct contact, therefore rejecting the first research hypothesis. In a recent study by Foster et al. [31] comparing lithium-disilicate, yttrium modified zirconium dioxide and cobalt chromium alloy surfaces regarding metabolic activity/viability of human oral epithelial cells, it was confirmed that lithium-disilicate exhibited the best properties in terms of epithelial cell attachment and proliferation, while in another study by Pae et al. [32] comparable results could be obtained for zirconia and titanium surfaces regarding attachment and growth behavior of HGFs. This is in contrast with the results of the present study showing a minor but statistically significantly higher metabolic activity/viability of HGF in contact with Ti- as compared to Y-TZP specimens after 72 h. Apart from the profoundly low surface roughness of Ti specimens that might have favored cellular response [33], other factors regulating the interactions between cells and dental materials might have influenced the obtained results. These include material chemical and physical features, such as surface topography, microstructure, reactive surface, chemical composition, impurity content and distribution, that cumulatively determine the *in vitro* cell behavior in terms of morphology, proliferation and functionality.

The results of the present study indicate absence of any major cytotoxic effects of Y-TZP in direct contact with HGFs, even following the extreme conditions of accelerated aging simulating several years of clinical function, based on the protocol by Chevalier et al [11]. used for *in vitro* aging of zirconia. The latter is currently the most accepted method for the estimation of the transformation rate of zirconia [34]. Several *in vitro* studies on the cytocompatibility of dental ceramic materials including zirconia have been conducted since the nineties employing various cell lines [35]. It can be concluded, overall, that ZrO_2 is not a cytotoxic biomaterial [36–38]. It has been also confirmed that zirconia does not exert any mutagenic and carcinogenic effects on the fibroblasts growing on it [39]. Only a few studies evaluating zirconia powders have reported adverse cellular responses, something which has not been found for the sintered material. Furthermore, Raffaelli et al. concluded that zirconia is a more biocompatible material when compared to feldspathic porcelain and that it can strongly increase the adhesive ability and proliferation rate of fibroblasts [40]. Tete et al. suggested that zirconia showed a collagen fiber orientation similar to that of a machined titanium surface and demonstrated less plaque formation, resulting in better periodontal health besides the improved esthetics [41]. Recent studies on zirconia surfaces seeded with HGFs have shown efficient cell attachment and proliferation, therefore supporting the clinical appropriateness of zirconia abutments [42,43]. However, none of these studies investigated zirconia behavior after *in vitro* aging as in present study, making direct compassions with current results not possible. Only sparse studies have reported cytotoxicity of zirconia as biomaterial, either related with slow release of zirconia and yttrium ions or mainly caused by the release of wear particles of size less than 0.22 μm [44,45]. Such particles are released



	Ti	LS2	Y-TZP
Live (% ± SD)	91.1 ± 3.2	90.3 ± 5.6	98.2 ± 1.6
Dead (% ± SD)	9.7 ± 0.6	10.5 ± 1.1	2.2 ± 0.3



	Zr0	Zr5	Zr10
Live (% ± SD)	97.4 ± 1.8	96.2 ± 2.6	89.1 ± 2.5 *
Dead (% ± SD)	3.1 ± 0.6	3.0 ± 1.1	10.9 ± 1.3 *

Fig. 4 – Confocal microscopy microphotographs (live/dead fluorescent staining with Calcein AM and EthD-1) showing levels of cell viability of HGF on the surface of (a–c) different abutment materials (Ti: Titanium alloy, LS2: lithium dissilicate and Y-TZP: yttrium-stabilized zirconia) and (d–f) yttrium-stabilized zirconia before and after aging (Zr0: non-aged, Zr5: aged for 5 h and Zr10: aged for 10 h). Scale bars represent distance of 100 μm. The tables underneath each set of microphotographs depict the results of quantification by the Image J software and the color pixel counter plug in. Asterisks indicate statistically significant differences among groups i.e. Zr10 compared to both Zr0 and Zr5 ($p < 0.05$).

in the body especially at the cases of hip joint heads, due to their friction wear. Nevertheless, such wear products have not been reported so far to be released from dental zirconia during masticatory function.

Despite absence of any major cytotoxicity effects, the results of the direct MTT assay in this study showed a statistically significant reduction of cell metabolic activity of HGFs grown in direct contact with the aged (Zr5 and Zr10) zirconia specimens during the first 48 h, as compared to the non-aged (Zr0) zirconia surfaces, therefore rejecting the second research hypothesis. These results were further confirmed by the morphological analysis by means of SEM, as well as the live/dead fluorescent staining assays. The minor changes in Y-TZP surface roughness values observed with the 3D profilometry analysis could not be correlated to this specific cell behavior recorded in the present study after aging. It can, therefore be suggested that other factors, rather than surface roughness, such as the dynamic process of phase transformations on the zirconia surface, accompanying by the well-established elemental distortion in its crystalline structure (i.e. yttrium depletion and generation of oxygen vacancies) may have caused increased cellular death [46,47]. These variations are in the nano or atomic scale. Since the adhesion sites of the cell (focal adhesions) are in the range of 5–200 nm [48], cells are more likely to be affected primarily of these transformations, rather than microscale surface alterations, such as surface micro-roughness.

Dental ceramic materials have been, in general, considered as highly biocompatible [49]. This opinion is based on some of their inherent characteristics, such as a composition devoid of toxic elements, low degradability, high chemical stability in aqueous environments, low element release and only a slight tendency toward bacterial adherence and plaque accumulation. Although the biological compatibility of ceramic materials is often taken for granted, previous studies have reported different amounts of mass loss from ceramic materials [50], as well as the cytotoxicity of some of the more recent formulations [51]. Previous studies do indeed confirm that dental ceramics cannot, in reality be considered as “biologically inert”, but may exert various low to medium

cytotoxic effects based on their composition and processing. This has been reported by Killic et al. who found a high initial reduction of mitochondrial dehydrogenase activity (MTT assay) of cells grown in direct contact with lithium-disilicate (IPS e.max) specimens [52]. However, this was not evident for alumina-based glass infiltrated ceramics, zirconia-reinforced glass-infiltrated ceramics, leucite-reinforced glass ceramics and yttria-stabilized zirconia; thus, designating those ceramic materials as non-cytotoxic. The results of the present study could firmly confirm minor but recoverable signs of cytotoxicity of HGFs grown in contact with aged YTZ specimens. It has been also shown that differential cellular attachment properties exist at different surface topographies. Previous reports [53] have indicated that aging of zirconia is accompanied with grain transformation and surface uplift, as well as increased number of surface micro-porosities and micro-cracks. This complies with our recently published data, showing that yttrium depletion from the superficial layers facilitates the t to m transformation and LTD susceptibility of Y-TZP [54]. This destabilization process, following yttrium depletion from the lattice, may have sequentially affected cell response, even if it is not accompanied by substantial changes in surface micro-roughness, as already explained. The more elongated phenotype of HGFs grown in contact with Zr specimens may indicate that the alignment itself may have significant impact on adhesion strength, while the sparser spread and higher number of dead cells in contact with Zr5 and Zr10 specimens suggests that differences in surface topography as a result of the LTD and yttrium depletion may influence biologic responses, such as cellular attachment and spreading.

In contrast to the direct MTT assay, a statistically significant stimulation of cell metabolic activity/viability could be observed in HGFs exposed to eluates of the lithium disilicate-based ceramic in the indirect tests. The latter could potentially be attributed to ion leaching, such as Zr^{4+}, Li^{+}, Mg^{2+}, Ca^{2+} from the material at concentrations capable of triggering cell responses, as previously described [55]. Specifically, the stimulating effect of Li^{+} ions on oral cell proliferation via activation of the canonical Wnt/beta-catenin signaling has been previously reported by our group [56].

5. Conclusions

The biocompatibility of implant-abutment materials is of major clinical relevance, since they come in close contact with the soft-tissues for an extended period of time. The results of the present study indicate that Y-TZP shows comparable biological properties to conventionally applied abutment materials, such as titanium alloy and lithium disilicate ceramic. Nevertheless, Y-TZP aging under experimental conditions corresponding to the clinical application of these materials might influence gingival cell attachment and proliferation, something that raises questions about a potentially negative effect on the long-term maintenance of the gingival architecture. Since this study has been designed as *in vitro* research, it is difficult to transfer the findings directly to an *in vivo* situation. But our results may facilitate the estimation of the biologic risks of all- ceramic materials and should provide guidance for the additional biological testing required to determine their risk in clinical use.

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