SEM evaluation of human gingival fibroblasts growth onto CAD/CAM zirconia and veneering ceramic for zirconia

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Summary

Aim. **To evaluate the growth of Human Gingival Fibroblasts (HGFs) cultured onto sample discs of CAD/CAM zirconia and veneering ceramic for zirconia by means of Scanning Electron Microscope (SEM) analysis at different experimental times.**
Methods. **A total of 26 experimental discs, divided into 2 groups, were used: Group A) CAD/CAM zirconia (3Y-TZP) discs (n=13); Group B) veneering ceramic for zirconia discs (n=13). HGFs were obtained from human gingival biopsies, isolated and placed in culture plates. Subsequently, cells were seeded on experimental discs at 7,5x10^3/cm^2 concentration and cultured for a total of 7 days. Discs were processed for SEM observation at 3h, 24h, 72h and 7 days.**
Results. **In Group A, after 3h, HGFs were adherent to the surface and showed a flattened profile. The disc surface covered by HGFs resulted to be wider in Group A than in Group B samples. At SEM observation, after 24h and 72h, differences in cell attachment were slightly noticeable between the groups, with an evident flattening of HGFs on both surfaces. All differences between Group A and group B became less significant after 7 days of culture in vitro.**
Conclusions. **SEM analysis of HGFs showed differences in terms of cell adhesion and proliferation, especially in the early hours of culture. Results showed a better adhesion and cell growth in Group A than in Group B, especially up to 72h in vitro. Differences decreased after 7 days, probably because of the rougher surface of CAD/CAM zirconia, promoting better cell adhesion, compared to the smoother surface of veneering ceramic.**

Key words: CAD/CAM zirconia, veneering ceramic, human gingival fibroblasts, scanning electron microscope, zirconia cell attachment, ceramic cell attachment.

Introduction

In the last decades, the introduction of metal-free restorations has led to the development of innovative ceramic materials showing excellent optical properties and better mechanical characteristics compared to the early dental ceramics; besides, the introduction of Computer Aided Design/Computer Aided Manufacturing (CAD/CAM) technologies has allowed high precision and predictability of the restorative results. The most noticeable advantages of the metal-free materials are: translucency, esthetic natural appearance, chromatic stability, low plaque retention and fluids absorption, high hardness, wear resistance, low thermal conductivity and chemical inertness (1).
Many *in vitro* and clinical studies reported optimum biocompatibility for high strength polycristalline ceramics (e.g. alumina, zirconia), showing favorable biological responses in soft tissues. In particular, the use of zirconia has become more and more widespread in the clinical practice, for the fabrication of single crowns, fixed dental prostheses and implant abutments (2-5).
Prolonged contact between these prostheses and oral soft tissues makes the biocompatibility and the integration of these materials critical for long-term success (6, 7). Several all-ceramic materials and surface modification methods were proposed in order to improve biocompatibility and soft tissue integration of fixed dental restorations (1, 3).

Some *in vitro* and *in vivo* studies on animal models showed that the interaction between gingival fibroblast cells and zirconia surface depends on a number of variables related to the surface microtopography, the chemical composition and the cell phenotype characteristics (8). It was shown that the surface roughness might alter cellular activity *in vitro* (9). This could be accounted not only for the chemical and biological properties but also for the structure of the surface, as it is known that fibroblasts show greater affinity for smooth or finely grooved surfaces than for rough ones (10, 11).
The aim of the present investigation was to evaluate the growth and cell attachment of Human Gingival Fibroblasts (HGFs) onto samples of CAD/CAM zirconia and veneering ceramic for zirconia at different experimental times by means of Scanning Electron Microscope (SEM) morphologic and qualitative analysis.

Methods

A total of 26 experimental discs were prepared for this in vitro study. Thirteen discs of CAD/CAM yttria-stabilized tetragonal zirconia (3Y-TZP) (IPS e.max Zir CAD, Ivoclar Vivadent AG, Liechtenstein, ISO standard 13356. 1997) were fabricated in a milling center without receiving any surface treatment (Group A), while other 13 discs of veneering ceramic for zirconia were obtained by die-casting in a laboratory and then polished and glazed (Group B). All the samples had a mean surface of 2,8 cm^2. The discs were cleaned and disinfected by ultrasonic treatment in Alconox®-water solution for 5 min; then, they were rinsed with sterile purified water (cell-culture grade) and ultrasonically treated again for 5 min in isopropyl alcohol. The samples for HGFs culture were then transferred aseptically to sterile 12-well cell-culture trays and submerged in isopropyl alcohol for 20 min, rinsed twice with sterile purified water and dried for a minimum of 8 h at 60° C under aseptic conditions. These procedures of disinfection were congruent with previously published techniques for testing ceramic materials (12).
One disc per group was randomly selected and analyzed by Scanning Electron Microscope (SEM Zeiss EVO-50, Cambridge, UK) for surface morphology observation.
HGFs were obtained from fragments of healthy marginal gingival tissue from the retromolar area taken during surgical extraction of impacted third molars in adult subjects (aged 18 to 60). Each patient gave written informed consent for participating in this study as donor of HGFs in accordance with the Local Ethics Committee, in compliance with Italian legislation and the code of Ethical Principles for Medical Research involving Human Subjects of the World Medical Association (Declaration of Helsinki).
Before gingival tissue withdrawal, each subject underwent complete medical anamnesis for systemic and oral infections or diseases. All the selected patients had healthy systemic conditions, including the absence of any diseases that would contraindicate oral surgery. The exclusion criteria were: uncontrolled periodontal disease, severe illness, unstable diabetes, drug abuse, history of head and neck irradiation, chemotherapy. Moreover, each subject was pretreated for 1 week with professional dental hygiene and antibiotic therapy was administered pre-operatively (amoxicillin/clavulanic acid, 2 g 1 hour before extraction). The tissue fragments were immediately placed in Dulbecco's modified Eagle's medium (DMEM) for at least 1 h, rinsed 3 times in phosphate-buffered saline solution (PBS), minced into small tissue pieces and cultured in DMEM containing 10% foetal bovine serum (FBS), 10% penicillin and streptomycin and 1% fungizone. Cells were maintained at 37°C in a humidified atmosphere of 5% (v/v) CO_2. Cultured HGFs with DMEM containing 10% FBS, 1% penicillin and streptomycin and following 4-8 passages were used.
Subsequently, each experimental disc was placed in a 12-well plate and HGFs were seeded in each well at $7,5\times10^3/cm^2$ concentration and cultured for a total of 7 days. HGFs at same concentration were also seeded in an empty well as control.
A qualitative analysis was performed under SEM for all the discs at different experimental times. After 3 h, 24 h, 72 h and 7 days *in vitro*, test discs were fixed in glutaraldehyde 2% in 0.1 M phosphate buffer pH 7.2, rinsed with phosphate buffer 0,15 M, dehydrated in an increasing ethanol series and finally dried in hexamethyldisilazane. The samples were then metallized with gold in a sputtering device and observed by SEM at 100x, 800x and 1600x magnification. Cell morphology was assessed on micrographs randomly taken indifferent areas of each experimental discs. The trial was conducted in triplicate for each experimental point.

Results

SEM observation of CAD/CAM zirconia discs showed a surface with regular roughness related to the milling procedure of manufacturing, while discs of veneering ceramic showed a smooth surface with only little defects, probably due to the glazing treatment (Figs. 1-3).
SEM analysis of HGFs on the different experimental surfaces showed differences in terms of cell adhesion and growth.
Group A revealed HGFs almost completely adherent to the surface with a flattened profile and rather elongated morphology, already after 3 h *in vitro* (Fig. 4). Moreover, at the same time, the area covered by HGFs resulted higher in Group A than in Group B. HGFs in Group B discs appeared round shaped, flattened on the surface with round nuclei. After 24 h of culture, both Group A and Group B samples showed HGFs with definitive morphology, long cytoplasmic elongations, even if a reduced area was covered by HGFs in Group B discs (Fig. 5).
After 72 h *in vitro* culture, SEM observation showed an evident flattening of HGFs on both surfaces and differences in surface coverage could be not noticed between the groups at that experimental time (Fig. 6). After 7 days of culture, both surfaces appeared entirely covered by HGFs and no differences between the groups could be evidenced (Fig. 7).

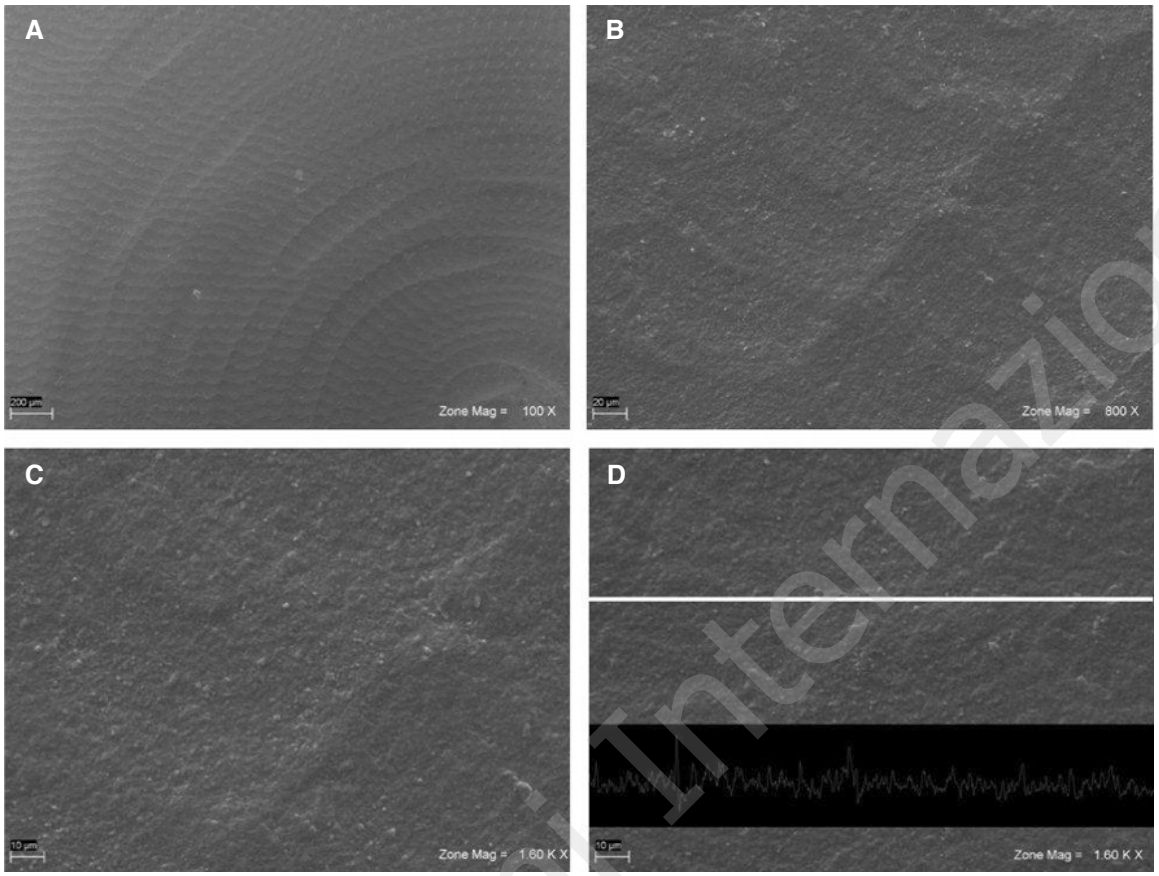

Figure 1. Scanning electron micrographs analysis of CAD/CAM zirconia disc sample: A) 100x magnification; B) 800x magnification; C) 1.60Kx magnification; D) Graphic representation of the surface roughness.

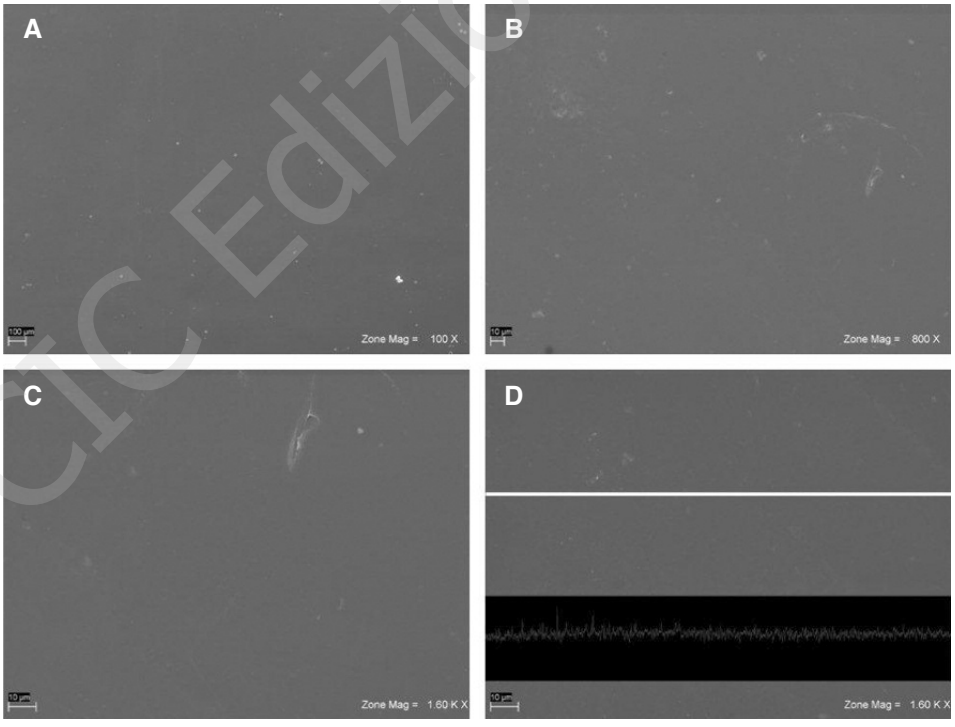

Figure 2. Scanning electron micrographs analysis of veneering ceramic for zirconia disc sample: A) 100x magnification; B) 800x magnification; C) 1.60Kx magnification; D) Graphic representation of the surface roughness.

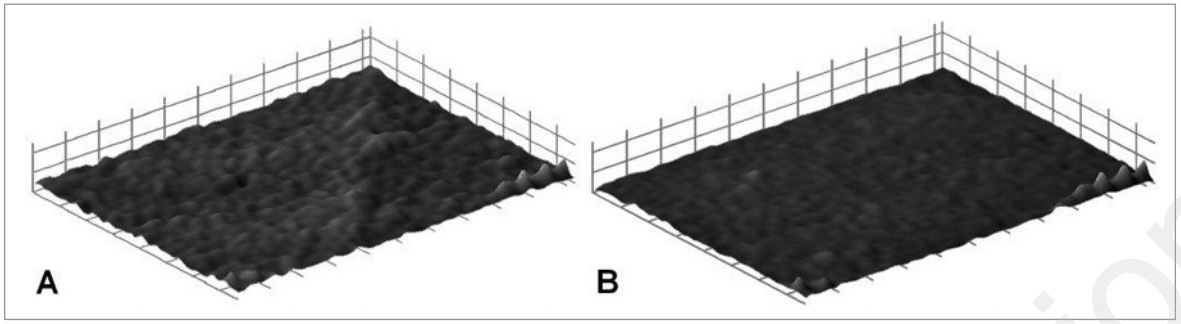

Figure 3. Graphic 3D reconstruction of the surface morphology: A) CAD/CAM zirconia disc sample; B) veneering ceramic for zirconia disc sample.

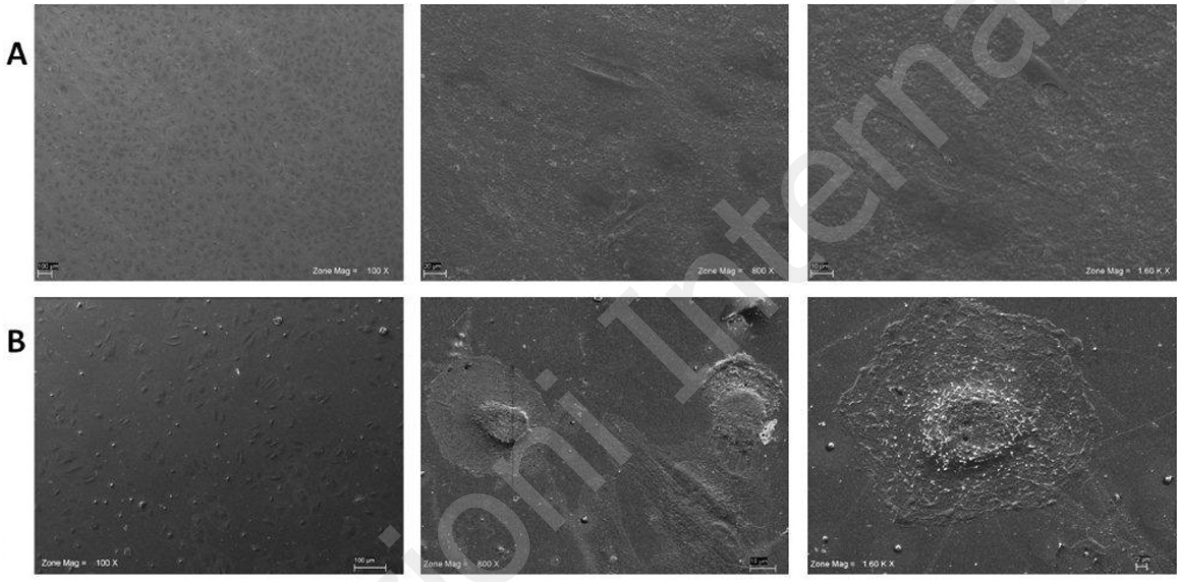

Figure 4. Scanning electron micrographs of HGFs cultured for 3 h on the different experimental discs at 100x, 800x, and 1600x magnification: A) CAD/CAM zirconia disc sample; B) veneering ceramic for zirconia disc sample.

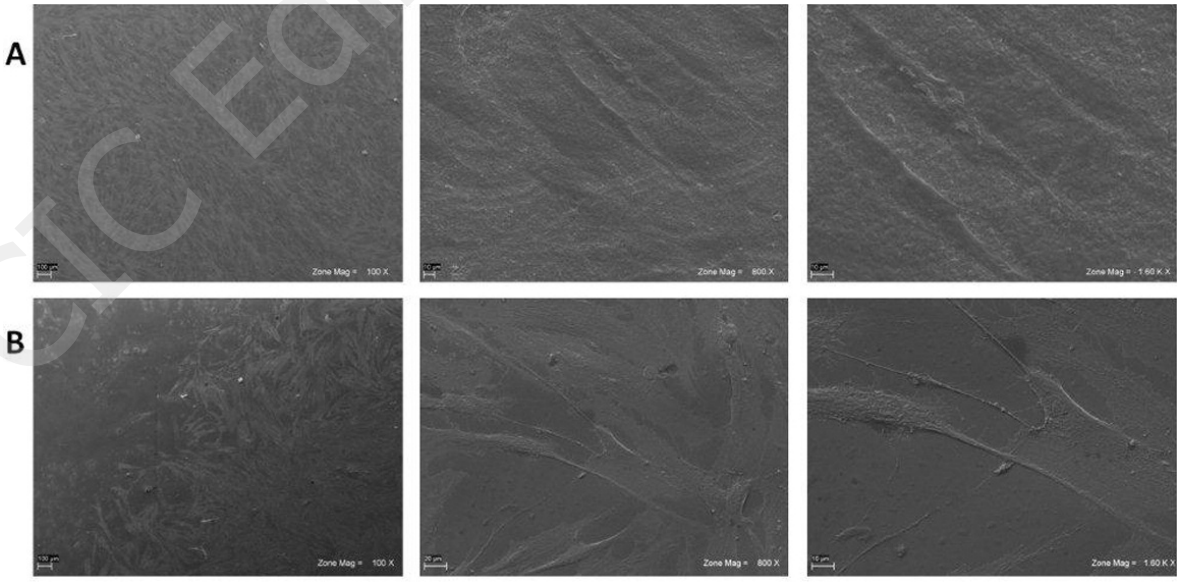

Figure 5. Scanning electron micrographs of HGFs cultured for 24 h on the different experimental discs at 100x, 800x, and 1600x magnification: A) CAD/CAM zirconia disc sample; B) veneering ceramic for zirconia disc sample.

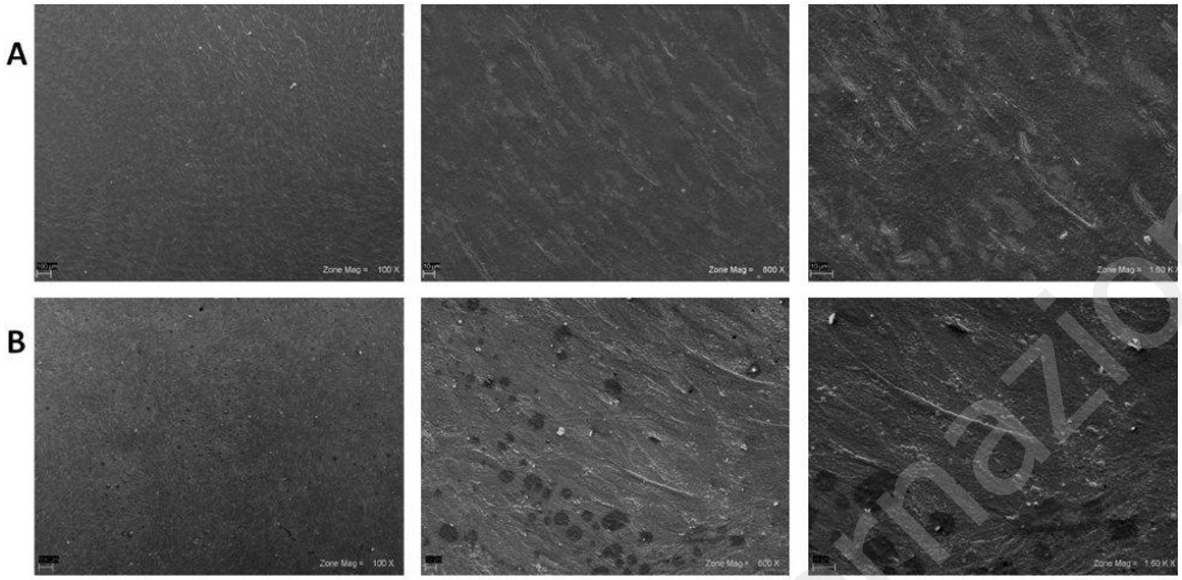

Figure 6. Scanning electron micrographs of HGFs cultured for 72 h on the different experimental discs at 100x, 800x, and 1600x magnification: A) CAD/CAM zirconia disc sample; B) veneering ceramic for zirconia disc sample.

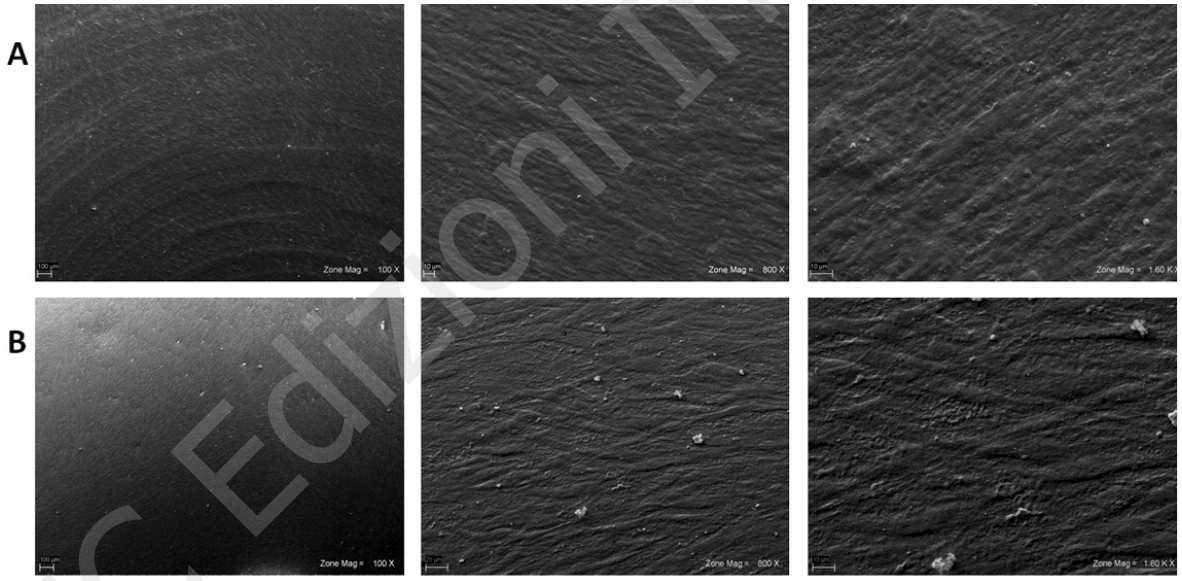

Figure 7. Scanning electron micrographs of HGFs cultured for 7 days on the different experimental discs at 100x, 800x, and 1600x magnification: A) CAD/CAM zirconia disc sample; B) veneering ceramic for zirconia disc sample.

Discussion and conclusion

Despite the complexity of cells and dental ceramic interaction *in vivo*, which is governed by a number of chemical and physical processes, *in vitro* studies may be very useful for a comprehensive material selection to achieve optimal soft tissue integration. Among the variables related to the structure and composition of biomaterials, one of the main topics is surface topography, as it reflects *in vitro* cell behavior in term of cell morphology, proliferation and adhesion.

Zirconia has been used in the last years as an excellent biomaterial for orthopedic and oral applications. The addition of yttria as an allotropic transformation cathalytic stabilizer was also investigated, resulting in a stabilized zirconia ceramic (3Y-TZP), exhibiting well demonstrated medium/long term mechanical performances when used for the fabrication of crown/bridge frameworks (4, 5, 13-15). Due to a fairly high incidence of mechanical complications, like the veneer ceramic chipping (16), in the last years the dental research has been increasingly focused on the possibility of using

zirconia as a monolithic material, for anatomically-shaped restorations, without the less fracture-resistant feldspathic veneering ceramic.

The CAD/CAM zirconia discs appeared to be rougher than the veneering ceramic ones. The evidenced fewer and slower HGFs attachment to veneering ceramic, compared to that to zirconia surface, could be explained considering that a rough surface could favor fibroblasts adhesion and induce more rapidly the typical flattened phenotype. In fact, HGFs showed a better adhesion and growth on CAD/CAM zirconia rather than on ceramic veneer discs surface up to 24 h of *in vitro* culture. These findings suggested that a different surface roughness can affect HGFs adhesion and growth onto the different samples. After 24 h of culture, differences in cell attachment between Group A and Group B discs became less evident, thus suggesting that, after an initial phase of adaptation, HGFs proliferated significantly faster on the smooth veneering ceramic respect to the rougher surface of CAD/CAM zirconia, as reported by Yamano et al. (17).

For successful tissue integration to prosthetic ceramic materials, cells have to uniformly colonize the surface. Since after 24h of culture both CAD/CAM zirconia and veneering ceramic materials favored an effective HGFs proliferation process, the results of the present *in vitro* investigation allowed to hypothesize that both the tested surfaces could be suitable to support *in vivo* soft tissue integration. Further *in vitro* and clinical studies will be needed to support the present findings.

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References

1. Zarone F, Russo S, Sorrentino R. From porcelain-fused-to-metal to zirconia: clinical and experimental considerations. Dent Mater 2011; 27:83-96.
2. Manicone PF, Rossi Iommetti P, Raffaelli L. An overview of zirconia ceramics: basic properties and clinical applications. J Dent 2007; 35:819-826.
3. Denry I, Kelly JR. State of the art of zirconia for dental applications. Dent Mater 2008; 24:299-307.
4. Sorrentino R, Galasso L, Tetè S, De Simone G, Zarone F. Clinical evaluation of 209 all-ceramic single crowns cemented on natrural and implant-supported abutments with different luting agents: a 6-year retrospective study. Clin Implant Dent Relat Res 2012; 14:184-197.
5. Sorrentino R, De Simone G, Tetè S, Russo S, Zarone F. Five-year prospective clinical study of posterior three-unit zirconia-based fixed dental prostheses. Clin Oral Investig 2012; 16:977-985.
6. Messer RL, Lockwood PE, Wataha JC, Lewis JB, Norris S, Bouillaguet S. In vitro cytotoxicity of traditional versus contemporary dental ceramics. J Prosthet Dent 2003; 90:452-458.
7. Li J, Liu Y, Hermansson L, Söremark R. Evaluation of biocompatibility of various ceramic powders with human fibroblasts in vitro. Clin Mater 1993; 12:197-201.
8. Piconi C, Maccauro G. Zirconia as a ceramic biomaterial. Biomaterials 1999; 20:1-225.
9. Brunette DM. The effect of implant surface topography on the behavior of cells. Int J Oral Maxillofac Implants 1988; 3:231-246.
10. Chehroudi B, Gould TR, Brunette DM. A light and electron microscopic study of the effects of surface topography on the behavior of cells attached to titanium-coated percutaneous implants. J Biomed Mater Res 1991; 25:387-405.
11. Könönen M, Hormia M, Kivilahti J, Hautaniemi J, Thesleff I. Effect of surface processing on the attachment, orientation, and proliferation of human gingival fibroblasts on titanium. J Biomed Mater Res 1992; 26:1325-1341.
12. Messer RL, Lockwood PE, Wataha JC, Lewis JB, Norris S, Bouillaguet S. In vitro cytotoxicity of traditional versus contemporary dental ceramics. J Prosthet Dent 2003; 90:452-458.
13. Luthardt RG, Holzhüter M, Sandkuhl O, Herold V, Schnapp JD, Kuhlisch E, et al. Reliability and properties of ground Y-TZP-zirconia ceramics. J Dent Res 2002; 81:487-491.
14. Ambré MJ, Aschan F, von Steyern PV. Fracture strength of yttria-stabilized zirconium-dioxide (Y-TZP) fixed dental prostheses (FDPs) with different abutment core thicknesses and connector dimensions. J Prosthodont 2013 Jan 4. doi: 10.1111/jopr.12003. [Epub ahead of print].
15. Takaba M, Tanaka S, Ishiura Y, Baba K. Implant-supported fixed dental prostheses with CAD/CAM-fabricated porcelain crown and zirconia-based framework. J Prosthodont 2013 Jan 4. doi: 10.1111/jopr.12001.
16. Kimmich M, Stappert CF. Intraoral treatment of veneering porcelain chipping of fixed dental restorations: a review and clinical application. J Am Dent Assoc 2013; 144:31-44.
17. Yamano S, Ma AK, Shanti RM, Kim SW, Wada K, Sukotjo C. The influence of different implant materials on human gingival fibroblast morphology, proliferation, and gene expression. Int J Oral Maxillofac Implants 2011; 26:1247-1255.