# Interactions of Modern Aesthetic Materials for Prosthetic Restorations with Oral Mucosa

## A pilot study

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*The specific objective of the present study is to assess the interactions between cells from a human gingival epithelial cell line and various aesthetic materials used in modern prosthetic dentistry. For this study six types of dental materials were selected: Cr-Co non-precious metal alloy, ceramics applied on Cr-Co non-precious metal alloy, zirconia, ceramics applied on zirconia, polymethyl methacrylate and pressed ceramics/ lithium disilicate. Cells from a human gingival epithelial cell line, Ca9-22 (Health Science Research Resources Bank), were cultured on the chosen surfaces for 3, 5 and 7 days. Cellular proliferation, cell attachment (using Multiplex Arrays Technology) and cytotoxicity (MTT- Assay) were evaluated at distinct predetermined intervals. Measurements performed at each distinct predetermined interval showed no significant difference for cell proliferation and cytotoxicity between the selected surfaces, however the highest levels were registered for the polymethyl methacrylate surface. Different attachment patterns were observed for epithelial cells attached on substrates, such as significantly different levels of adherence of E-Cadherin and N-Cadherin molecules; E-Cadherin adhesion levels indicate that pressed ceramics may be the dental material which, compared to the selected materials, influences the least the homeostasis of oral mucosa.*

*Keywords: aesthetic dental materials, epithelial cell proliferation and adhesion, E-Cadherin, N-Cadherin*

Various aesthetic dental materials are currently available for clinical use in prosthetic dentistry. Dentists' selection of appropriate aesthetic materials could become compulsory as long as these materials have different biophysical properties that can vary even within the same class of materials [1] as diverse manufacturing techniques are recommended for each material and different clinical applications are described, along with specific cementation or bonding protocols. Of all these aspects, oral biocompatibility of dental materials represents an important topic in modern scientific research but few comparative analysis and data regarding the newest aesthetic dental materials and their interaction with the human oral tissues are available. Ceramics, as an aesthetic dental material used in prosthodontics, can be manufactured by both traditional laboratory procedures and CAD/CAM (Computer Aided Design / Computer Aided Manufacturing) [2]. A wide range of aesthetic all ceramic restorations obtained by CAD/CAM technology is nowadays available to dental clinicians and technicians: CAD/CAM glass ceramics (feldspathic ceramics, mica-based ceramics, leucite-reinforced ceramics, milling lithium disilicate reinforced ceramics, glass infiltrated alumina and zirconia ceramics); CAD/CAM compatible polycrystalline alumina and zirconia. The high success of these materials is related to the fact that the CAD/CAM technique is, in the first place, time-friendly and predictable, which makes it increasingly popular [3]. On the other hand, due to the combination of predictable strength and reasonable aesthetics, the traditional metal-ceramic restorations are still popular, being especially recommended in the lateral areas of the dental arches [4]. Along with these materials, PPMA (polymethil metacrylate) is frequently used to fabricate temporary esthetic dental restorations with multiple purposes, including provisional single/multiple unit restorations [5], temporary implant supported restorations and conventional dentures.

As previously stated, the interaction between aesthetic materials and the oral soft tissue has been the focus of intense research in the recent years Despite this, not so many studies were conducted on this particular subject. A relevant study [6] highlights that lithium disilicate, one of the most appreciated esthetic materials, has the best biocompatibility when compared to zirconia and cobalt-chromium alloys. Titanium, zirconium dioxide and the collagen membrane were also found appropriate for application in the oral cavity [7]. This in vitro study points out that human gingival cells attach and proliferate on all tested materials even after long periods of culture, e.g. up to 70 days. Human oral keratinocytes cultured on mirror-surfaced pure titanium (Ti) and yttrium stabilized tetragonal zirconia poly-crystals (TZP) were studied [8] with the aim to identify cell attachment, their mRNAs and proteins expression of laminin $\gamma 2$ and integrin $\beta 4$, cell spreading and cell morphology. This study suggested that yttrium stabilized tetragonal zirconia poly-crystals (TZP) have the same capacity to form epithelial attachment as Ti has.

Our group had previously focused on the interaction between oral epithelial cells and various surface and topographies. In our studies, we isolated and characterized several sub-populations of oral keratinocytes: oral keratinocyte stem cells, progenitor cells and normal oral keratinocytes [9]. We also demonstrated that these cells can regenerate their tissue of origin [10] and that stem cells can maintain their phenotype in vitro [11]. At the same time, we proved that these cells can interact with various

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polymeric nanoparticles and can modify their in vitro behavior depending/ on the drug incorporated in the nanoparticles.

To the best of our knowledge, few studies have focused on the interaction between oral keratinocyte cells and aesthetic materials, such as zirconia or ceramics. Our current hypothesis is that epithelial cells have different attachment patterns following their interaction with selected substrates. Therefore, the main aim of the present study is to analyze several attachment molecules such as E-Cadherin and N-Cadherin, as well as cellular behavior in relation to cell toxicity and cell proliferation after incubating the cells on various aesthetic materials such as polymethyl methacrylate (PMMA), zirconia, ceramics or pressed ceramics.

## Experimental part

*Material and methods*

Sample preparation

Six groups of samples were obtained for the present study, corresponding to the selected materials, as follows: 1. Cr-Co non-precious metal alloy (*Simex Italia S.R.L.*); 2. Ceramics *(Novacer/Novadent/Germany*) applied on Cr-Co alloy (*Simex Italia S.R.L.*); 3. Zirconia/pre-sinterd, yttrium-stabilized ZrO2 (*Novazir ST/Novadent/Germany*); 4. Ceramics (*Novacer/Novadent*) applied on zirconia (*Novazir ST/Novadent/Germany*); 5. Polymethyl methacrylate (*Ivoclar Vivadent/Liechtenstein*); 6. Pressed Ceramics/ lithium disilicate glass-ceramic (*IPS e.maxPress/Ivoclar Vivadent/Liechtenstein).* Three experimental samples were produced for each mentioned group, resulting 18 samples in total.

The resulting disk-samples were designed to be circular in cross section, 2mm thick, with a 30mm diameter. A 2mm edge was designed for the non-precious metallic alloy (*M*), for zirconia (*Zr*) and polymethyl methacrylate (*PMMA*) samples, respectively a 3mm edge was obtained for the samples having 1mm ceramics layer applied (metal layered with ceramics/*M-C* and zirconia layered with ceramics/*Zr-C*). As an exception, the pressed ceramics samples had a slightly-concave circular shape, with a 15mm diameter and 2mm thickness.

All studied materials were obtained according to the producers' recommendations. The Cr-Co samples (M) were obtained by casting; we included this metal alloy in our study as it can be considered as a traditional, reference material in prosthetic dentistry. The zirconia samples (Zr) were manufactured by using the *CAD-CAM* technology: based on *SolidWorks* software, a *STL* file with the required dimensions was generated and imported to a *CAM* machine (Fig. 2); the zirconia samples were milled and then sintered. The PMMA experimental samples were also obtained using the CAD/CAM technology. In addition, all the samples layered with ceramics (metal-ceramics/*M-C* and zirconia-ceramics/*Zr-C*) were prepared according to a settled protocol, by a certified ceramic dental technician. The pressed ceramic (*PC*) samples were obtained following the standard ingot-press technique, as specified by the manufacturer.

Cell Culture

Cells from a human gingival epithelial cell line, *Ca9-22 (Health Science Research Resources Bank*), were cultured in *Dulbecco's modified Eagle's* medium *(Invitrogen, Carlsbad, CA, USA)* supplemented with 10% fetal bovine serum *(Invitrogen*) at 37°C in an atmosphere containing 5% carbon dioxide ($CO_2$). For each experiment, the cells were plated with a concentration of $4x10^5$ cells/disk and allowed to attach overnight (Fig. 1). Experiments were carried out on the 3rd and 7th days for each selected dental substrate. Data was collected from 3 independent experiments.

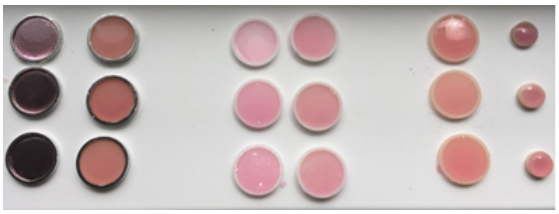

Fig. 1. Oral epithelial cells plated on dental material substrates

Viability of oral keratinocyte cells

For all substrates, cellular viability was assayed using *Trypan Blue Exclusion Test. Trypan blue* stains dead cells in blue; thus, the number of dead blue cells among the total number of cells was counted. For statistical purposes the assay was performed five times.

Cell adhesion molecules

E-Cadherin and N-Cadherin molecules were detected using a multiplex approach. The method of detection strictly followed the manufacturer's instructions. Thus, the cells were subjected to enzymatic dissociation (0.25% trypsin) followed by centrifugation. The cells were lysed using the cell lysis buffer provided by the manufacturer. Supernatant was mixed with primary antibodies solution at 1:4 dilution and incubated at room temperature for one hour. Following subsequent washing steps (using phosphate buffer saline) the plate was analyzed using a *Luminex 200* equipment. Results were compared using *xPONENT* software.

## Results and discussions

*Cell number*

On the 3rd day the cell proliferation analysis showed no significant difference between the selected substrates. The highest cell count was observed on cells plated on PMMA and the lowest on cells plated on metal. Results for cell proliferation following the 3-day incubation: M - 20000 ± 2000 cells; M+C - 21000 ± 9900; Z - 21300 ± 1110; Z+C - 22100 ± 2400; PMMA - 23200 ± 2312; PC - 21900 ± 1876 following 3 independent experiments (Fig. 2). As expected, cell number doubled after 7 days of incubation compared to the data obtained after the 3rd-day. However, no statistical differences were observed between the samples. As in the case of the 3-day experiment, the highest cell count was again observed on Z+C and the lowest on metal (M). Results for cell proliferation following the 7-day incubation (Fig. 3): M - 38900 ± 1000 cells; M+C - 39200 ± 1210; Z - 39500 ± 1200; Z+C - 41000 ± 2300; PMMA - 38300 ± 2092; PC - 40900 ± 1987, following 3 independent experiments.

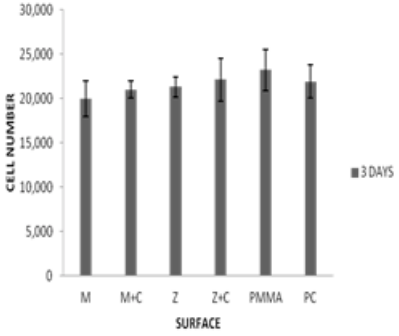


Fig. 2. Cell proliferation after 3-day incubation

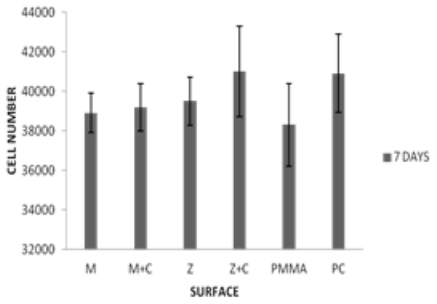


Fig. 3. Cell proliferation after 7-day incubation

*Adhesion Molecules*

E-Cadherin data shows that epithelial cells grown on PC (Pressed Ceramics) had the lowest concentration of adhesion molecules and that this concentration was statistically significant when compared to the cells grown on other surfaces. At the same time, PMMA had the highest concentration of E-Cadherin. Results for cell proliferation after 3-day incubation (fig. 4): M - 431 ± 13 ng/mL; M+C - 398 ± 23; Z - 470 ± 21; Z+C - 403 ± 18; PMMA - 520 ± 16; PC - 370 ± 19, following 3 independent experiments. The results obtained after the 7-day incubation were consistent with the ones obtained following the 3-day analysis. The lowest adhesion potential was observed for PC (the difference was statistically significant), while the highest adhesion potential was found for PMMA. These observations lead to the conclusion that pressed ceramics (PC) may be the dental material that influences the least the homeostasis of oral mucosa. Results for cell proliferation following the 7 day-incubation (fig. 5): M - 453 ± 13 ng/mL; M+C - 405 ± 16; Z - 483 ± 20; Z+C - 410 ± 18; PMMA - 550 ± 19; PC - 387 ± 11, following 3 independent experiments.

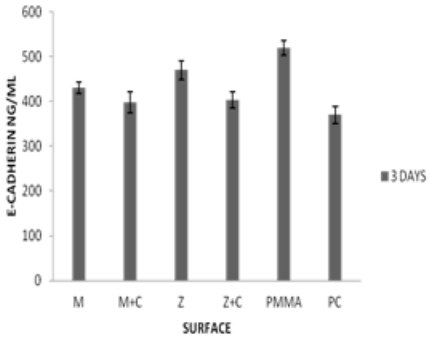


Fig. 4. E-Cadherin levels after 3-day incubation

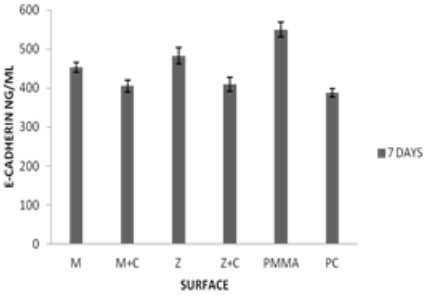


Fig. 5. E-Cadherin levels after 7-day incubation

After 3 days of incubation the cells showed the same N-Cadherin levels for all analyzed substrates. Results for cell proliferation following the 3-day incubation (fig. 6): M - 100 ± 13 ng/mL; M+C - 110 ± 23; Z - 90 ± 21; Z+C - 93 ± 18; PMMA - 120 ± 16; PC - 117 ± 19, following 3 independent experiments. The data obtained following the 7 days of incubation is similar with the one resulting from the 3-day assay. Results obtained for N-Cadherin following both the 3-day and 7-day experiments suggest that none of the analyzed substrates influence normal cellular homeostasis and may not induce pathological changes in the analyzed cells. Results for cell proliferation after the 7-day incubation (fig. 7): M - 121 ± 13 ng/mL; M+C - 105 ± 16; Z - 113 ± 20; Z+C - 110 ± 18; PMMA - 140 ± 19; PC - 128 ± 11, following 3 independent experiments.

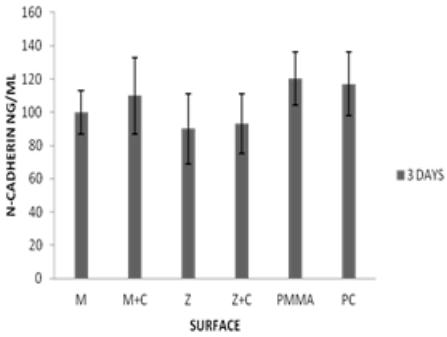


Fig. 6. N-Cadherin levels after 3-day incubation

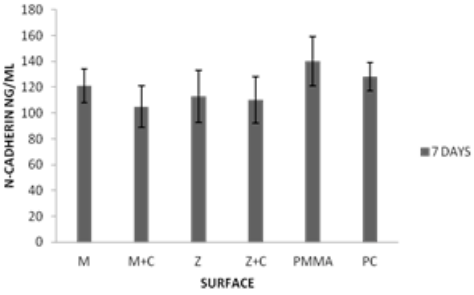


Fig. 7. N-Cadherin levels after 7–day incubation

Biocompatibility is becoming a rapidly evolving field of research that has profound implications for dental practice [12-14]; understanding the biologic responses to a dental material and measuring biocompatibility imply a complex, interdisciplinary process. The aspects regarding biocompatibility of modern aesthetic materials should concern scientific researchers as well as dental practitioners, as long as little evidence based on real-world data are available.

Recent results [6], similar to the ones obtained in our present study, confirm that lithium disilicate, one of the most promising dental materials in digital dentistry [1], is associated with a very good biological response. This in vitro study [6] evaluates the proliferation rate of cultured human epithelial cells on three commonly used restorative materials: lithium disilicate ceramic, yttrium zirconium dioxide and cobalt-chrome alloy. The results indicate that the examined restorative materials are equally suitable for subgingival restorations, while lithium disilicate exhibited the best biocompatibility. The clinical significance of the resulting data resides in the conclusion that the analyzed materials are appropriate for being used in restorative procedures in direct contact with the sulcular epithelial tissue.

However, a previous study [15] indicates that lithium disilicate becomes more cytotoxic after polishing and exhibits more severe cytotoxicity in vitro than dental alloys and composites. Other in vitro tests [16] assert that CAD/CAM polished zirconia should better integrate in vivo, compared to CAD/CAM lithium disilicate and feldspathic ceramic. Our preliminary results indicate that epithelial cells grown on pressed ceramics (PC) had the lowest concentration of adhesion molecules and that this concentration was statistically significant when compared to the concentration in the cells grown on other selected surfaces included in our study.

Regarding zirconia, a recent study that analyzes the effect of acid etching on surface characteristics, flexural strength and osteoblast cell response of glass-infiltrated zirconia [17] shows that cell proliferation rate on the 3rd day on ZGS-E15 (glass-infiltrated, sandblasted and 5 min acid-etched zirconia) and ZGS-E25 (glass-infiltrated, sandblasted and 25 min acid-etched zirconia) groups was

significantly higher than that of other groups: untreated zirconia (Z); glass-infiltrated zirconia (ZG); glass-infiltrated and sandblasted zirconia (ZGS); glass-infiltrated, sandblasted and 5 min acid-etched zirconia (ZGS-E5). The results of this study point out that the osteoblast cell response is enhanced by the surface roughness caused by acid etching following glass infiltration; the strength of zirconia was improved by glass infiltration but slightly reduced by severe acid etching. In this context, our previous study [18] that evaluates the influence of mechanical and chemical treatments on zirconia by means of chemical composition and microstructure (*Scanning Electron Microscope with Energy Dispersive Spectrometry -SEM-EDS*), phasic composition (*X-Ray Diffraction – XRD*) and roughness (surface profilometry) highlights the fact that a slight decrease of roughness parameters was observed after the etching of zirconia (*Novazir ST/Novadent/ Germany*) with hydrofluoric acid 9% for 90s, respectively with hydrofluoric acid 40% for 90s. In addition, higher roughness values were obtained for the zirconia samples that had been airborne abraded with $Al_2O_3$ abrasive particles / 75μm and 250μm.

In terms of testing the ceramic materials applied on different infrastructures corresponding to fixed prosthetic restorations, in vivo and in vitro studies showed good biological response to the layering ceramics [15, 19- 21]. As an example, all three ceramics selected in a relevant study [17], respectively: *GC Initial LF Dentine/ GCCorporation/Tokyo/Japan; IPS-d-Sign Dentine/Ivoclar/ Schaan, Liechtenstein; IPS Classic Margin/Ivoclar/Schaan/ Liechtenstein*, were almost equivalent as regards their in vitro biological behavior and presented increased rate of cell proliferation after the 3rd day of cultivation period. In our study, after 3 days of incubation, even if cell proliferation analysis showed no significant difference between the selected substrates, the highest cell count was observed on the cells plated on PMMA while the lowest was registered on cells plated on metal; furthermore, the metal-ceramic samples showed lower cell level compared to the zirconia-ceramic samples. After 7 days of incubation, the highest cell count was observed on zirconia-ceramics while the lowest was registered on metal.

A recent in vivo study [22] highlights the fact that the presence of all-ceramic restorations did not induce inflammatory reactions in periodontal healthy patients; additionally, no differences between gingival reactions to lithium disilicate and zirconia restorations could be proved.

All the aspects presented above generally correspond to our results, yet, based on E-Cadherin adhesion levels obtained in this present study, pressed ceramics may be the dental material that, compared to the selected materials, influences the least the homeostasis of oral mucosa.

Epithelial-Cadherin is a transmembrane glycoprotein that mediates cell-cell adhesion, which is essential for colony formation. E-Cadherin is also a surface marker used to identify induced pluripotent stem cells. Moreover, high levels of E-Cadherin were also shown to be associated with the undifferentiated state characteristic to embryonic stem cells. In the present paper, we highlight the fact that the pressed ceramics, out of all the studied substrates, has the lowest levels of this adhesion molecule and, at the same time that these levels are statistically significant when compared to the other surfaces. Data resulting after 3-day incubation was consistent with the one obtained after the 7 days of incubation. These results suggest that pressed ceramics may be the first choice as an aesthetic material, out of the studied ones. As expected, the highest concentration of E-Cadherin was found in PMMA group. This material is prone to gingival attachment together with subsequent accumulation of dental plaque and oral bacteria, probably due to its high porosity [23, 24]. Summing up, the lowest adhesion potential was observed for pressed ceramics (with statistical significance) while the PMMA had the highest adhesion potential.

Another important indicator for normal cellular behavior is cell number following incubation on different surfaces. Our data showed that, after 3 days of incubation, the highest cell number was observed on cells grown in contact with PMMA and the lowest cell number was observed for cells plated on metal. The results showed no statistical difference between sample groups. Same pattern was observed following 7 days of incubation with the cell count doubling for all analyzed substrates. The results suggest that PMMA may over-stimulate cellular growth; nevertheless, more long-term studies are required in order to further clarify present results.

N-Cadherin or Cadherin-2 is a transmembrane protein present in virtually all tissues with the main role of mediating cell-to-cell adhesion. This molecule is normally involved in human development multiple cell types such as neurons or cardiomyocytes. However, abnormal levels or altered functions of this protein have been linked to pathologies such as abnormal organ growth or cancer development. Present results show that N-Cadherin levels were the same for all analyzed samples, both on the third and seventh days. The findings suggest that none of the dental materials used in the experiments induces pathological changes in the analyzed cells and that these substrates do not influence normal cellular homeostasis. However, present results have to be addressed with caution since the present experiments were performed for a short period of time.

Among the limitations of this study we include: the present study analyzed only short-term behavior of cells incubated on selected substrates, it employed only a limited number of adhesion molecules and used normal human oral epithelial cells isolated from oral mucosa. Future studies are necessary in order to expand present findings to other sub-populations of oral keratinocytes cells, such as stem cells and transit amplifying cells. Long-term studies are also required in order to fully validate short-term results.

## Conclusions

Following the results obtained during this study, several relevant conclusions can be outlined, as follows: the modern esthetic dental materials analyzed in this paper are biocompatible with oral epithelia; moreover, pressed ceramics has the lowest adhesion potential and influences the least the homeostasis of oral mucosa, when compared to other surfaces, such as zirconia, layered ceramics, PMMA or Cr-Co alloys.

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