In Vitro Analysis of the Cytotoxicity of Indirect Restorative Materials

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This study aimed to compare the cytotoxicity of the Vita AC12, Lava Ultimate, Vita Enamic and InSync indirect restorative materials. Extracts of each material were prepared by incubation for 1, 7 and 40 days, with daily washing. Human gingival fibroblasts were exposed to the extracts, and cell viability was evaluated by sequential assessment of mitochondrial activity (XTT), membrane integrity (NRU) and cell density (CVDE). Extracts of polystyrene beads and latex fragments were used as negative and positive controls, respectively. Differences between groups and experimental times were evaluated by analysis of variance. At the 24 h extraction, significant differences between the control and both Vita AC-12 and InSync were observed in the XTT assay (p<0.05), and between the control and both Enamic and Lava Ultimate, in the CVDE assay (p<0.05). AC12, Lava Ultimate, and InSync presented significantly lower cell viability than Enamic and the control group, in the NRU assay (p<0.05). The Vita Enamic and Lava Ultimate hybrid ceramic-like materials presented better biocompatibility at the 24 h extraction time point than the AC12 and InSync ceramic materials. However, a simulation of the removal of toxic components by biological fluids, conducted by using longer extraction times and daily washing, led to the absence of cytotoxicity in all the tested restorative materials. These findings can be viewed as positive for the clinical indication of these restorative materials, considering their contact with adjacent soft tissues for extended periods of time.

Introduction

For decades, full-crown restorations have been used to preserve the masticatory function and aesthetics of destroyed natural teeth. Metal-ceramic crowns have been the restoration of choice (1) for many professionals, owing to their high strength, precise adjustment, marginal integrity and extended lifetime. However, in vitro assays have shown that dental casting alloys may present cytotoxicity, mostly through the release of components into the cell culture media (2). This toxicity, together with the need for more resistant materials, led to the development of novel non-metal aesthetic materials. These indirect aesthetic materials, used for the reconstruction of extensively destroyed teeth, can be classified into dental ceramics (glass-matrix ceramics and polycrystalline ceramics) and ceramic-like materials (resin-matrix ceramics) (3).

Among the dental ceramics, zirconia was introduced with the purpose of meeting the requirements of aesthetics and resistance, and its use has been widely disseminated in conjunction with computer-aided design/computer-assisted manufacturing (CAD/CAM) systems (4). Resin-matrix ceramics or indirect composites for CAD/CAM applications can be classified according to their microstructure into dispersed fillers (zirconia-silica nanofillers and organic matrix) and polymer-infiltrated-ceramic-networks (PICNs), based on the infiltration of a pre-sintered glass-ceramic scaffold with a polymeric material (5). PICNs constitute an innovative class of CAD/CAM materials offering promising perspectives in prosthodontics (6) since these materials present better fracture toughness and flexural strength than classical composites (7).

One such dispersed filler material is Lava Ultimate. Just like a composite resin, this material is not brittle and therefore resists fracturing. Since it is also a glass ceramic, it has excellent polish retention, as well as long-lasting aesthetics (5). Vita Enamic is another PICN, based on a nano-hybrid composite resin reinforced with glass fibers. According to the manufacturer, the dominant ceramic is enhanced by a polymer network, creating a material that exhibits the positive characteristics of both ceramics and composite resins for use as dental prostheses on natural teeth, and, more recently, on implants (8).

From a biological point of view, ceramics are good choices for restorative materials. A previous study (9) identified no release of potentially toxic elements from various ceramics in experiments using cell cultures. Also, biocompatibility studies conducted in animals have demonstrated similar soft tissue integration results for alumina, zirconia, and titanium (10). On the other hand, the in vitro cellular responses to several types of dental ceramics in use have not always been favorable (11). The CAD/CAM-milled, all-ceramic materials used in implant abutments...
come in close contact with oral soft tissues such as the keratinized marginal gingiva, which may raise concerns about the safety and biocompatibility of these products (4). According to Tassin et al. (12), significant cytotoxicity may be observed for conventional composites, while PICNs such as Vita Enamic proved to be very biocompatible during in vitro assays.

Consequently, the high variability of the physicochemical characteristics of indirect aesthetic materials may lead to controversy regarding their biocompatibility, reinforcing the relevance of in vitro standardized testing of novel ceramic materials. In this context, InSync is a recently launched layering ceramic system that, according to the manufacturer, is indicated for zirconia and lithium disilicate restorations, and can be employed for both conventional and implant-supported prostheses (13), for which there are no studies in the scientific literature assessing its biocompatibility or in vitro cytotoxicity.

Therefore, the aim of this study was to evaluate and compare the cytotoxicity of the representatives of different classes of aesthetic materials, namely: Lava Ultimate (UDMA composite dispersed filler), Vita Enamic (UDMA + TEGDMA glass-fiber nano-hybrid PICN), Vita AC12 (inlay CAD/CAM ceramic) and InSync (layering ceramic), employing a standardized multiparametric assay with a human primary gingival fibroblast model.

Material and Methods
This study was approved by the local Institutional Research Ethics Committee (CAAE 50319115.0.0000.5243).

Sample Preparation
The following materials were tested in this study: Vita Enamic (VITA Zahnfabrik, Bad Säckingen, Germany), Lava Ultimate (3M ESPE, St. Paul, MN, USA), Vita AC-12 (VITA Zahnfabrik, Bad Säckingen, Germany), and InSync (Chemical, Vaduz, Liechtenstein) (Table 1).

The materials were ground, powdered, and only then weighed, to increase their contact surface with the culture medium, as described for irregularly shaped solid devices in ISO 10993–5:2009 (14). A sample of 0.2 g of each material was immersed into 1 mL of DMEM-high glucose culture medium added with 10% Fetal Bovine Serum (FBS; Gibco-Invitrogen, Grand Island, NY, USA) at 37 °C, in a humidified atmosphere of 95% air and 5% CO2, for the time periods of either 24 h, 7 days or 40 days. The conditioned culture medium underwent daily renewal with fresh medium to simulate the effects of gingival crevicular fluid washing the material in the patient’s mouth.

Cell Culture and Exposure
Primary cells were collected from a male and female. The participants were patients of the Dentistry Clinic at the Fluminense Federal University, who met the following criteria: subjects who were indicated for surgery that allowed the collection of a gingival fragment without affecting the original surgical plan, who had no chronic disease, made no continuous use of drugs and had no gingival bleeding. Human gingival fibroblast (HGF) cultures were isolated from gingival fragments according to a previously established protocol (15).

Cells at the second passage were cultured in a DMEM-high glucose medium (Cultilab, Campinas, SP, Brazil) supplemented with 10% FBS (Gibco-Invitrogen, Grand Island, NY, USA) and two antibiotics, namely 10,000 IU/mL penicillin and 10 mg/mL streptomycin. Cells were then seeded in 96-well culture plates at an initial density of 3 × 104 cells per well, followed by incubation for 24 h at 37°C under 5% CO2.

Subsequently, the cell cultures were exposed to the test samples by replacing 180 µL of the medium in each well with 180 µL of one of the test material extracts and then incubated for 24 h. Extracts of latex fragments, with well-known toxicity, were employed as a positive control.

Table 1. Description of the materials tested in the study

| Product and manufacturer | Composition | Application |
|--------------------------|-------------|-------------|
| Vita Enamic (VITA Zahnfabrik) | Silicon dioxide (SiO₂) 58-63%; aluminum oxide (Al₂O₃) 20-23%; sodium oxide (Na₂O) 9-11%; potassium oxide (K₂O) 4-6%; boron trioxide (B₂O₃) 0.5-2%; zirconium dioxide (ZrO₂) < 1%; calcium oxide (CaO) < 1% (UDMA + TEGDMA) | CAD/CAM (Cerec) |
| Lava Ultimate (3M ESPE) | Composite resin material 20% (UDMA) with 80 wt% silica and zirconia nanoparticles and zirconia/silica nanoclusters | CAD/CAM |
| Vita AC-12 (VITA Zahnfabrik) | Silicon dioxide (SiO₂) 15-17%; aluminum oxide (Al₂O₃) 14-17%; boron trioxide (B₂O₃) 12-15%; titanium dioxide (TiO₂); 3-5%; lanthanum oxide (La₂O₃) 39-48%; ceric oxide (CeO₂) 2-5%; calcium oxide (CaO) 2-4% | CAD/CAM (CELAY System) |
| InSync (Chemichl) | Silicon dioxide (SiO₂) 55-75%; aluminum oxide (Al₂O₃) 6-20%; boron trioxide (B₂O₃) 10%; potassium oxide (K₂O) 3-12%; sodium oxide (Na₂O) 3-12%; lanthanum oxide (La₂O₃) 0.05-4%; ceric oxide (CeO₂) 0.1-2%; calcium oxide (CaO) 0-3% | Build-up technique |
whereas extracts of biocompatible high-density polystyrene beads were used as a negative control. The blank, unexposed group (cells plus culture medium) was exposed only to the culture media (DMEM-high glucose). Each condition was tested in three replicates and three different assays.

**Multiparametric in Vitro Assay**

After 24 h exposure of cells to the experimental groups and controls, cell viability was assessed using a multi-parameteric assay kit (In-Cytotox, Xenometrix, Allschwil, Switzerland), which evaluates three different cell parameters sequentially in the same cell culture (16), as described below.

**Mitochondrial Activity (XTT)**

The multiparametric assessment began with the XTT assay. XTT (2,3-bis [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxy aniline salt) is a tetrazolium salt that is converted to formazan through a succinate dehydrogenase system in the mitochondrial respiratory chain of viable cells. After addition of the reagent, the conversion of water-soluble yellow tetrazolium XTT salt into orange formazan was monitored by measuring the optical density (O.D.) at 480 nm on a UV/Vis microplate reader (Synergy II, BioTek Instruments, Winooski, VT, USA).

**Membrane Integrity (Neutral Red Uptake, NRU)**

The cells subjected to the XTT assay were washed twice with PBS and underwent the NRU assay, which determines the viable cell estimation through cellular membrane integrity. The NRU test is a survival/cell viability assay based on the ability of the cell to incorporate and retain the neutral red dye in the cell’s lysosomes, which accumulates on its internal membrane. The cells were fixed after 3 h of incubation with the dye. NR dye was then extracted, and the optical density of the supernatant measured at 540nm, which is directly related to the proportion of viable cells (18).

**Cellular Density (Crystal Violet Dye Exclusion, CVDE)**

After conducting the NRU assay, the fixed cells were washed twice with PBS and evaluated by the CVDE assay, which quantifies nuclear DNA as a measure of cell density. After washing/removal of excess dye, optical density at 540nm was directly proportional to the number of adherent cells in each well.

**Statistical Analysis**

Analysis of variance (one-way ANOVA) was used to test the interactions between three sources of variation (test material, assay type and time) and the proportions of viable cells for each test material, compared with those of the control groups. Statistical significance was set at $\alpha=0.05$. Tukey’s post-test was performed to compare groups at a same given time point. All statistical analyses were performed with GraphPad Prism 6 (Graphpad Software, Inc., San Diego, CA, USA).

**Results**

The assay was internally validated by the behavior of the positive and negative controls, which promoted the expected levels of cell death and survival with all parameters assessed (Fig. 1). Regarding the test materials, significant differences ($p<0.05$) were observed between...
the blank group and both Vita AC-12 and InSync at the
24 h time point in the XTT assay (Fig. 1A). Vita AC12, Lava
Ultimate, and InSync presented significantly lower cell
viability (p<0.05) than Enamic and the blank group at the
24 h time point, in the NRU assay (Fig. 1B).
In the CVDE assay, Enamic and Lava Ultimate presented
significantly lower cell viability when compared to the
negative control (p<0.05) at the 24 h time point (Fig. 1C).
All of the reduced levels of cell viability were considered
borderline regarding the cutoff value of 70% defined by
international standards (14). Furthermore, cellular viability
increased after 24 h and was never lower than 75% at the 7
and 40 day time points for all the tested ceramic materials.

Discussion
The indirect restorative materials tested in the present
study were designed to be used in close contact with
gingival tissue and, therefore, to be biocompatible with
gingival fibroblasts. Human gingival fibroblasts were
chosen for the cytotoxicity assays as the in vitro model
that best simulates this tissue response. In comparison
with immortalized cell lines, HGFs provide results which
are closer to those obtainable with cells of normal
phenotype behavior, thus constituting good candidates
for in vitro biocompatibility tests of trans-gingival implant
components (17).
Several techniques can be used to evaluate the in
vitro cytotoxicity of dental materials. Those include
advanced methodologies that present high sensitivity
and specificity for the determination of adverse effects,
including the use of flow cytometry, the determination
of apoptosis, or assessments the release of inflammatory
markers. Nevertheless, for a simple initial assessment
of the cytotoxicity of restorative materials, one of the
advantages of the present methodology is that it allowed
the evaluation of three different classic cell viability
parameters sequentially, in the same assay, and for the same
group of treated cells. The relevance of this difference is
evidenced when one considers that the results obtained
with a single method may often be misleading due to
bias or methodological limitations (16). When employing
HGF cells in the multiparametric assessment, differences
were observed in the first parameter of cytotoxicity
(mitochondrial activity) after 24 h exposure to the two
classes tested, where the ceramic materials (Insync and
AC12) presented lower cell viability than the hybrid
materials. It is noteworthy that Tassin et al. (12) found
improved biocompatibility for PICNs, including Enamic,
when employing a similar methodology (24 h exposure and
assessment of mitochondrial activity). This method has been
standardized by ISO for the evaluation of dental materials
and is widely used in the scientific literature.
Among the tested ceramic materials, VITA AC 12 is an
alumina-based material, suitable for indirect restorations
fabricated with a CAD-CAM system. Alumina has broad
clinical use and is usually recommended for anterior and
posterior crowns, as well as for previous single-retainer
resin-bonded fixed partial dentures (18). The reduced
cell survival (61%) observed in the present study after
exposure to this material corroborates previous literature
reports that alumina composite materials exhibit in vitro
cytotoxic effects (19).
The present study also provides the first literature
report assessing the cytotoxicity of InSync. This ceramic
material is a layering system indicated for zirconia and
lithium disilicate restorations placed in close contact with
gingival fibroblasts. In the present study, InSync presented
a significant reduction in cell viability, as measured by both
XTT and NRU tests with 24 h extraction.
Vita Enamic (a PICN) presented good in vitro biocompatibility
(Fig. 1A, 1B). This result could be explained by the innovative polymerization mode of the material’s HT-
HP (high-temperature and high-pressure) monomers, and
its high degree of conversion. In fact, the level of conversion
of monomers has been found to influence cellular response
(20), corroborating the findings of Gupta et al. (21), who
reported that the polymerization of resins influences their
cytotoxicity. Unlike most photopolymerizable resins, PICNs
do not contain Bis-GMA, a monomer that has shown higher
in vitro cytotoxicity to HGFs than other methacrylate
monomers (21). In addition, a study comparing CAD/CAM
restorative materials found that both resin-infiltrated
ceramic (Enamic) and composite resin reinforced with
nano-ceramics (Lava Ultimate) showed only minor suffering
from grinding-induced chipping damage (22). This finding
represents another biological advantage of these ceramic-
like materials in clinical practice since these materials can
be placed in intimate contact with gingival tissue, as in the
case of implant-supported prostheses. Furthermore, it has
been established that human gingival fibroblasts attach
better to electro-polished than to etched or sandblasted
surfaces (23).
Mihali et al. (24) showed that Lava Ultimate, the second
hybrid material tested in this study, reduced bone resorption
around the implant and did not show occlusal wear after a 3-
month period of intraoral evaluation. In the present study,
although a decrease in membrane integrity (NRU assay)
was observed for Lava Ultimate at the 24 h extraction, it
did not induce cytotoxic levels (cell survival above 70%)
in the two other assays (MTT and CVDE).
However, if these results initially seem to corroborate
the idea of a significant difference in cytotoxicity between
these classes of materials (dental ceramics and ceramic-
like materials), interesting changes in our methodology
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entanto, uma simulação de remoção de componentes tóxicos por fluidos biológicos, realizada com o uso de tempos de extração mais prolongados e lavagem diária, levou à ausência de citotoxicidade em todos os materiais restauradores testados. Esses achados podem ser vistos como positivos para a indicação clínica desses materiais restauradores, considerando seu contato com tecidos macios adjacentes por longos períodos de tempo.

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