In vitro cytotoxicity of all-ceramic substructural materials after aging

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Received 25 February 2012; Final revision received 9 March 2012
Available online 26 October 2012

Abstract Background/purpose: Several all-ceramic materials have been developed to meet the most challenging requirements in restorative dentistry. However, only limited reports have focused on the issue of biocompatibility of all-ceramics. The purpose of this study was to evaluate the effects of different types of all-ceramic substructural materials to determine their biological performance under experimental conditions.

Materials and methods: We tested six all-ceramic systems [glass-infiltrated alumina-reinforced ceramic (In-Ceram Alumina, Turkom Cera), glass-infiltrated zirconia-toughened alumina ceramic (In-Ceram Zirconia), low-fusing ceramic (Finesse), yttria-stabilized tetragonal zirconia ceramic (Zirkonzahn), and lithium disilicate glass ceramic (IPS e.max)] using a tetrazolium assay prior to and 3 days after aging, to determine their ability to alter cellular mitochondrial dehydrogenase activity. Mann–Whitney U test and Wilcoxon t test were used for statistical analysis (α = 0.05).

Results: According to the results of the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide tests, the toxic effects of Finesse and Zirkonzahn were statistically insignificant (P > 0.05) compared with the negative control group. In contrast, In-Ceram Alumina, In-Ceram Zirconia, Turkom Cera, and IPS e.max demonstrated statistically significant toxic effects (P < 0.05) compared to the negative control group. When effects of aging on cytotoxic properties were evaluated, In-Ceram Zirconia and Turkom Cera showed increased cytotoxic effects on the 1st day following the aging process, whereas IPS e.max and Zirkonzahn displayed cytotoxic effects on the 2nd day and Day 7, respectively. The cytotoxic effect of Zirkonzahn and IPS e.max was decreased on the 1st day and at the 2nd week, respectively.

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This study was presented at the International Association for Dental Research (IADR) annual meeting, Barcelona, Spain, July 2010.

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http://dx.doi.org/10.1016/j.jds.2012.09.004
Conclusion: Several types of all-ceramic substructures did not cause the same *in vitro* responses. Finesse and Zirkonzahn did not carry high biologic risk. However, our results suggest that In-Ceram Alumina, In-Ceram Zirconia, Turkom Cera, and IPS e.max should not be considered as entirely biocompatible materials.

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Introduction

In recent years, dental ceramics have become increasingly important materials in tooth restoration.1,2 Since patients desire tooth-colored restorations, more types of all-ceramics have inevitably been developed as more modern solutions to meet restorative needs. Several all-ceramic materials and processing techniques have been introduced to meet the most challenging requirements in restorative dentistry.3,4 The prolonged contact of all-ceramics with the gingiva and oral mucosa makes the biocompatibility of these materials crucial in respect to their long-term safety.5,6 However, only limited information is available concerning molecules associated with dental ceramics that might promote gingival and periodontal inflammation.7,8 Recent *in vitro* tests involving aging techniques have been applied to alloys and composites in order to estimate long-term biologic responses to these materials.9,10 However, only a few reports have focused on the issue of loss of mass and biocompatibility of ceramics.7–12

The purpose of this study was to evaluate how different types of all-ceramic substructural materials vary with respect to their response to exposure to gingival fibroblasts, both initially and after aging over time. Our results may facilitate estimation of the biologic risks of all-ceramic materials, and should provide guidance for additional *in vitro* and biological testing required to determine their risk in clinical use. Our first research hypothesis was that all tested all-ceramic substructural materials would not show any evidence of cytotoxicity in the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test during different time intervals. The second hypothesis was that aging process would not affect the proliferation of cells exposed to six different all-ceramic substructural materials.

Materials and methods

We studied the cytotoxicity of all-ceramic substructures used to fabricate dental restorations over different time intervals using L 929 mouse skin fibroblasts and the MTT test. After an initial test, the specimens were aged 96 hours under sterile conditions and then tested again at different time intervals.

Sample preparation

Modern all-ceramic materials have sufficient physical properties to be used in clinical conditions, such as for posterior crowns and fixed partial dentures.13–15 The most commonly used systems can be classified according to laboratory processing procedure (pressable, slipcasting, milling, or sintering) and chemical composition (feldspar: high leucite and low leucite; glass ceramic: lithium disilicate and mica; and core reinforced: alumina, magnesia, and zirconia).16 Based on the sintering temperature, dental ceramics are traditionally classified as high-, medium-, low- and ultralow-fusing ceramics. In general, the high-fusing feldspatic ceramics are more corrosion resistant than ceramics with lower sintering temperatures. Although, high- and medium-fused ceramics exhibit better corrosion resistance than low- and ultralow-fused ceramics, they are reported to create more wear of the antagonist.16 Some low-fusing ceramics (Finesse, Dentsply International Inc., Ceramco, NJ, USA) (Fs) have demonstrated less wear on the enamel than conventional feldspatic ceramics.17 Different all-ceramic materials are selected for testing their cytotoxicity and are described as follows.

The IPS e.max glass ceramic (IPS e.max; Ivoclar Vivadent AG, Schaan, Liechtenstein) (Li-E) is composed primarily of a modified lithium disilicate glass ceramic that forms the primary components of IPS Empress 2 (Ivoclar Vivadent AG).2 In comparison with IPS Empress 2, Li-E material exhibits substantially improved physical properties and greater translucency,18–20 and hence can be used to form a core or an entire crown.21

The mechanical properties of high-performance alumina- and zirconia-based ceramics make them attractive as potential materials for all-ceramic restorations in high stress-bearing areas.22,23 In-Ceram Alumina (Vita-Zahnfabrik, Bad Sackingen, Germany) (In-A) is an alumina-reinforced ceramic that has been used as a core material for crowns and anterior three-unit fixed partial dentures since the early 1990s.16,23 In-Ceram Zirconia (Vita-Zahnfabrik) (In-Z) system combines glass-infiltrated alumina with 35% partially stabilized zirconia for core or an entire crown.21

Similar to the In-A system, this ceramic uses a slip-casting technique to create the framework.22,24 Turkom-Cera all-ceramic material (Turkom-Ceramic SDN-BHD, Kuala Lumpur, Malaysia) (Tur-C) is a ceramic system that incorporates a crystal-hardened or glass-infiltrated high alumina core. A new all-ceramic alumina-core material, Tur-C, is being introduced in an attempt to provide a high-quality, high-strength, cost-effective coping that will result in improved clinical success.22

The Zirkonzahn (ICE Zirkon; Zirkonzahn GmbH, Bruneck, Italy) (Zz) is an yttria-stabilized tetragonal zirconia, and has been used for posterior and anterior fixed partial dentures with the introduction of computer-aided design/computer-aided manufacturing (CAD/CAM) technology.26 Zz blank is a partially sintered material, and its use involves designing an enlarged framework and milling the framework from the partially sintered zirconia blank.
Zirconia blanks are processed by milling with the aid of CAD/CAM systems. Depending on the system, partially or fully sintered blanks are used. The framework structure has a linear shrinkage of 20–25% during sintering until it reaches the desired final dimensions.\textsuperscript{26–29}

All-ceramic materials were selected to represent common types of clinically used ceramics (materials tested for cytotoxicity are listed in Table 1). For Zz specimens, presintered zirconium oxide blocks (ICE Zirkon; Zirkonzahn GmbH) were milled using the copy milling technique. Then they were sintered in a sintering furnace (Zirkonofen 600/VZ, Zirkonzahn GmbH) at 1500°C for 16 hours. All other ceramic specimens were prepared following the manufacturers’ instructions. All specimens \((n = 12, \text{specimen discs; 6 mm in diameter and 3 mm thick})\) were polished using rubber wheels (medium- and fine-grit silicone polisher; Brasseler USA, Savannah, GA, USA) and diamond polishing pastes Tru-luster diamond polishing paste (2–5 μm); Brasseler USA to obtain a mirror-like surface. The materials were then cleaned using a laboratory soap on a soft toothbrush (Oral-B no. 35, soft bristles; Oral-B Laboratories, Belmont, CA, USA) and disinfected using ultrasonic treatment and isopropyl alcohol in preparation for biologic testing. These procedures were described in detail by Wataha.\textsuperscript{7} Following disinfection, specimens were maintained under sterile conditions prior to testing to determine cellular response.

Cells

The cells used for the experiments were L 929 mouse skin fibroblasts (L 929; Sigma, St Louis, MO, USA). They were grown as monolayer cultures in 25 T-flasks (Costar, Cambridge, MA, USA); subcultured three times a week at 37°C, in an atmosphere of 5% CO\(_2\) in air and 100% relative humidity; and maintained at a third passage. The culture medium consisted of Dulbecco’s Modified Eagle Medium with 10% fetal calf serum, gentamycin (10 μg/mL), penicillin (100 units/mL), streptomycin (100 μg/mL), and glutamine (2 mol/L) (all from Gibco BRL, Gaithersburg, MD, USA). Adherent cells at a logarithmic phase were detached with a mixture of 0.025% trypsin (Sigma, St Louis, MO, USA) and 0.02% ethylene diamine tetra acetic acid (EDTA) (Sigma), incubated for 2–5 minutes at 37°C, and used for cell inoculation.

Aging

We used the chemical aging solution bovine serum albumin (BSA; Sigma), which had been determined to be the most effective “accelerating” solution in previous studies,\textsuperscript{5,7,28} for the aging procedure. The aging procedure was used to simulate long-term use of dental materials accurately and presumably encourages an accelerated release of components from the material such that subsequent corrosion was similar to that observed after 8–10 months in vivo. The specimens were rinsed in sterile water and then were submerged into 3% BSA solution for 96 hours. After BSA treatment, the specimens were rinsed once more by dipping into sterile water and then tested for cellular response, as described in the “Cellular response” section.
Cellular response

Cell-culture tests were carried out using a direct-contact format according to ISO 10993 specifications. The surface area to volume ratio of the specimen in the cell-culture medium was 65 mm²/mL (within the ISO-recommended range of 50–600 mm²/mL) and durations of contact with cells were 1 day, 2 days, 1 week, and 2 weeks. The extracts were used to assess cytotoxicity. Each material was tested three times. Wells containing 100 μL medium without cells and reagents were used as negative controls, and dimethyl sulfoxide was used as a positive control.

We assessed cellular response using the MTT method for mitochondrial succinate dehydrogenase (SDH) activity. SDH activity reflected a combination of cellular viability and cell number around the ceramic specimens, and has commonly been used for biologic assessments. The ceramic specimens were removed from the culture wells following exposure for 1 day, 2 days, 1 week, and 2 weeks. Cells were then detached by 0.25% trypsin with 1 mM EDTA for 5 minutes at 37°C, following which they were resuspended in medium at 1 × 10⁵ cells/μL. After verification of cellular viability by trypan blue dye exclusion assay, 100 μL of cell suspension were distributed into each well of 96-well microtiter plates (Costar), and each plate was incubated for 24 hours. Wells containing 100 μL medium without cells and reagents were used as negative controls. After treatment during the stated incubation times, 10 μL of MTT solution (5 mg/mL sodium succinate buffer; Sigma) was added to each well, and the microplates were further incubated at 37°C for 4 hours. During this time, active mitochondria converted the yellow, soluble MTT into intracellular blue, insoluble MTT-formazan. Cells were then fixed with Tris-buffered formaline (4%, pH 7.2), which was followed by a water rinse. Finally, the MTT-formazan was dissolved in dimethyl sulfoxide (Sigma), and the optical density of the resulting solution was read at 450 nm using a plate reader (Bio-tek EL 312; Bio-tek Instruments, Winooski, VT, USA). Results are presented as percentage of cell viability determined as 100 – (A of experimental well/A of negative control well) × 100. Each experiment was repeated three times, as represented in the data.

Statistical analysis

Statistical evaluation of the study results was performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA) and using the Mann–Whitney U test and Wilcoxon t test. A P value of <0.05 was considered to be statistically significant.

Results

Results of the viability of cells in contact with the test materials are given in Fig. 1 and Fig. 2. The results show that In-A decreased the cell proliferation on the 1st day compared with the negative control group (100%) (P < 0.05, 69%), but no difference in cell proliferation was detected on the 2nd day (82.8%), or at the 1st and 2nd weeks (84.6% and 90.2%, respectively), based on the negative control group (P > 0.05) (Fig. 1). Tur-C, In-Z, and Li-E had lower percentages of cell proliferation in comparison to the negative control group on the 1st and 2nd days, and at the 1st and 2nd weeks (P < 0.05) (Fig. 1). There was no statistically significant difference between Fs and the negative control group with respect to the percentage of cellular proliferation on the 1st and 2nd days (94.5% and 87%, respectively) and at the 1st and 2nd weeks (102% and 102.5%, respectively) (P > 0.05) (Fig. 1). Comparing the proliferation measurement of cells in contact with Zz on the 1st and 2nd days (108.5% and 103%, respectively) and at the 1st and 2nd weeks (140% and 126%, respectively) with that of the negative control group, the cellular proliferation was found to be higher than the negative control group at the 1st and 2nd weeks (P < 0.05) (Fig. 1).

When the cell proliferation measurements following the aging process of In-A (85.2%, 76%, 82.8%, and 90.2%, respectively) were compared with those of the negative control group (100%), it was clear that this material had...
a statistically significant cytotoxic effect on gingival fibroblast cells on the 2nd day, compared with the negative control group ($P < 0.05$). Yet it had no statistically significant effect on the 1st day, or at the 1st or 2nd week, when compared with the control group ($P > 0.05$) (Fig. 2). Comparing the proliferation percentages of cells in contact with Tur-C and In-Z with the negative control group (100%), no differences were found at the 2nd week ($P > 0.05$, 88% and 83%, respectively). At the other time intervals, a decreased cell proliferation was observed ($P < 0.05$) (Fig. 2). When Fs was compared to the negative control group (100%) with respect to cell proliferation percentages (88%, 91%, 94.5%, and 97%, respectively), no differences were detected ($P > 0.05$) (Fig. 2). Comparison between Zz and the negative control group (100%) with respect to cell proliferation measures on the 1st and 2nd days (132% and 96%, respectively), at the 1st and 2nd weeks (105% and 132%, respectively) suggested that Zz increased cell proliferation more than the negative control group ($P < 0.05$) on the 1st day and at the 2nd week. No difference was observed on the 2nd day or at the 1st week ($P > 0.05$) (Fig. 2). The results of this study showed that Li-E decreased cell proliferation on the 1st and 2nd days (59% and 46%, respectively), and at the 1st and 2nd weeks (50% and 73.5%, respectively) ($P < 0.05$), when compared with the negative control group (100%) (Fig. 2).

Cell proliferation measurements of the six different all-ceramic substructural materials prior to and after the aging process on the 1st and 2nd days, and at the 1st and 2nd weeks are shown in Figs. 3–6. Comparison of the effects of aging with respect to the cytotoxicity values of all-ceramic substructural materials showed that the In-Z and Tur-C decreased cell proliferation statistically on the 1st day (Fig. 3). Fig. 4 and Fig. 5 show that Li-E and Zz decreased cell proliferation statistically on the 2nd day and at the 1st week, respectively, and consequently increased the cytotoxic effect ($P < 0.05$). On the other hand, Zz and Li-E showed increased cell proliferation on the 1st day and at the 2nd week, respectively, and consequently decreased the cytotoxic effect ($P < 0.05$) (Fig. 3 and Fig. 6). The results of this study revealed that the aging process also increased cell proliferation at other time intervals; however, this increase was not statistically significant ($P > 0.05$).

**Discussion**

The results of this study clearly suggest that all-ceramic materials are not equal with respect to their *in vitro* biological effects, initial fabricated states, or aging (Figs. 1–6). Therefore, the results do not support the first or second research hypothesis. We found that Fs and Zz all-ceramic extractions did not show any evidence of cytotoxicity in the MTT test. In a previous study, the cytotoxicity of dental ceramics, including Fs low-fusing ceramic, was evaluated using the Millipore filter method, the agar overlay method, and the MTT assay, and no cytotoxicity was detected. In the report by Josset et al., who studied the reaction of human osteoblasts cultured with

![Figure 2](image2.png) *Cellular proliferation percentages of all-ceramic substructure materials on the 1st and 2nd days, and at the 1st and 2nd weeks after the aging process. Values are median, maxima, minima, and 25% and 75% percentiles; the data are expressed as percentages of negative control group (100%). *Significant difference ($P < 0.05$). Fs = Finesse; In-A = In-Ceram Alumina; In-Z = In-Ceram Zirconia; Li-E = IPS e.max; Tur-C = Turkom-Cera; Zz = Zirkonzahn.*

![Figure 3](image3.png) *Cellular proliferation percentages of six all-ceramic substructure materials on the 1st day prior to and after the aging process. Within each set of columns, different letters represent the difference between groups ($P < 0.05$). Fs = Finesse; In-A = In-Ceram Alumina; In-Z = In-Ceram Zirconia; Li-E = IPS e.max; Tur-C = Turkom-Cera; Zz = Zirkonzahn.*
zirconia, cytotoxicity was estimated with DNA synthesis and cell proliferation, and no cytotoxicity was observed for the zirconia. Thus, our results were consistent with former reports.\textsuperscript{10,11}

The proper assessment of cytotoxicity requires precise and accurate \textit{in vitro} laboratory tests.\textsuperscript{29,30} The cleavage of MTT has several desirable properties for assaying cell survival and proliferation. MTT was cleaved by all living, metabolically active cells that we tested. The main advantage of the colorimetric assay is the speed with which samples can be processed. The assay can be read a few minutes after the addition of dimethyl sulfoxide, and the color is stable for a few hours at room temperature. The results are also apparent visually, which is very useful if rapid qualitative results are required.\textsuperscript{31}

In this study, we evaluated biologic effects of dental ceramics because they are commonly used in clinical practice for all-ceramic restorations. Generally, physical and mechanical properties are addressed in the evaluation of potential dental materials, and biological properties are often neglected. However, it should be emphasized that the biocompatibility evaluation of newly developed materials is necessary prior to their use in clinical practice.\textsuperscript{6}

Brackett et al\textsuperscript{21} investigated the effect of aging on cytotoxic properties of all-ceramics, including five different lithium disilicates. Three lithium silicates were produced by pressing while the others were produced with the CAD–CAM system, using MTT analysis \textit{in vitro}. According their results, 50–70\% of cellular mitochondrial activity is suppressed at the first stage in all lithium silicate materials. Researchers preserved the samples in sterile artificial saliva to allow the release of components from the materials and to imitate clinical practice conditions. This aging process decreased the cytotoxicity of the lithium silicate. As our study shows, these materials suppressed mitochondrial activity, but nonetheless, after a few weeks of the aging process, the initial cytotoxicity of these materials decreased, showing that they were clinically suitable for long term.\textsuperscript{21} We found that the cell proliferation percentage of Li-E was lower than that of the negative control group at all time intervals prior to the aging process. This result may be related to the composition of the Li-E material. Li-E contained ZnO, which is a known and potent suppressor of cell activity. Following the aging process using 3\% BSA over 96 hours, the cell proliferation measurement decreased on the 2nd day, whereas it increased at the 2nd week. Both studies had similar results with respect to methods and outcomes, showing that low-weight components and ions released from ceramic materials in a biological medium changed the metabolic activity of the target cell.

Messer et al\textsuperscript{5} evaluated the cytotoxicity of two feldspathic ceramics (Vita Omega and Duceragold), two lithium disilicate all-ceramics (Stylepress and IPS Empress 2), and one leucide all-ceramic (IPS Empress 1) in a cell culture generated using the mouse fibroblast Balb/c 3T3 via the MTT method; they reported that IPS Empress 1, Stylepress, and IPS Empress 2 groups inhibited cell proliferation significantly, and that lithium disilicate-based ceramic was
the most toxic ceramic material.\(^5\) In addition, the ceramic test groups In-A, In-Z, Tur-C, and Li-E used in our study showed a toxic effect over different time intervals using the MTT test. On the other hand, Messer et al\(^5\) reported, in the evaluation that they performed 2 weeks after a 96-hour aging process using 3% BSA, that proliferation of cells exposed to feldspathic ceramics (Vita Omega and Ducer-agold) did not change significantly, whereas cell proliferation measurements of leucide-content all-ceramic (IPS Empress 1) decreased significantly, and that of lithium-dilisilicate-based ceramics (Stylepress and IPS Empress 2) increased significantly. The initial cellular response to lithium disilicate was not clinically acceptable; however, cytotoxicity decreased significantly following the aging process.\(^5\) In the current study, it was observed that In-Z and Tur-C decreased cell proliferation on the 1st day, and Li-E and Zz decreased cell proliferation on the 2nd day and at the 1st week, respectively, indicating greater cytotoxic effects. However, contact with Zz and Li-E produced increased proliferation on the 1st day and at the 2nd week, respectively, and consequently indicated reduced cytotoxic effect. Moreover, aging process generally increases cell proliferation at other time intervals, but this increase is not statistically significant. The results of this study, similar to the findings of Messer et al,\(^5\) suggest that the aging process decreases the proliferation of cells in contact with some materials, whereas it increases or leaves unchanged the proliferation of cells in contact with other materials.

According to our results, various biological responses to all-ceramic materials used in dental restorations contradict the widespread belief that ceramic materials are always inert and biocompatible, and they substantiate reports indicating that different ceramics lead to different amounts of mass loss.\(^12,32\) Since the materials in this study were obtained from the manufacturers for use in the study without being characterized physically or chemically in a detailed manner, it was difficult to determine why the various materials exhibited different biologic effects. Therefore, future studies should focus on such characterization including microstructure, porosity, or component release to determine and predict the biologic response to ceramic materials.

In conclusion, In-A, In-Z, Tur-C, and Li-E displayed cytotoxic effects at different time intervals, while Fs and Zz did not show any evidence of cytotoxicity using the MTT test. After the aging process, In-Z and Tur-C decreased cellular proliferation on the 1st day, and Li-E and Zz decreased cellular proliferation on the 2nd day and at the 1st week, respectively, indicating greater cytotoxic effect. After the aging process, Zz and Li-E showed more cell proliferation on the 1st day and at the 2nd week, respectively, consequently indicating decreased cytotoxic effect.

Acknowledgments

The authors thank Dr Aslihan Usumez and Dr Ahmet Ozturk for their valuable contributions in the assessment of study results and preparation and writing of this manuscript. The authors gratefully acknowledge financial support from the Department of Scientific Research Projects of Erciyes University.

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