Determination of cytotoxicity of dentine bonding agents, hydroxyethyl methacrylate and bisphenol alpha

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Abstract

Aim: The purpose of the study was to analyze cytotoxicity caused by dental adhesives on human gingival fibroblast (HGF) cell lines, compare the response of cultured HGFs to substances leached from a conventional total etch and self-etching adhesive system along with their individual components.

Methodology: Healthy gingival tissue biopsies (explants) were used to obtain isolates of HGFs, which were cultivated in flasks till subconfluent monolayers obtained. Serial dilutions for each compound tested were made and elutes at all concentrations tested for were obtained at 48 h for each material which were then added at different concentrations to the HGF cultures, and cytotoxicity was analyzed by 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Results: Within the limitations of the in-vitro study, we conclude: All of the materials tested showed moderately or slightly cytotoxicity at the lower serial dilutions except bisphenol alpha and One Coat-7.0, which proved to be strongly cytotoxic among almost all the dilutions. The lowest cytotoxicity was obtained from Clearfil SE Bond at 0.3125 mg/mL and the highest from One Coat-7.0 at 5.9 mg/mL, among the three dentin bonding systems tested for cytotoxicity.

Conclusions: Indicated that SE B had the lowest mean % of cell viability value and OC had the highest mean % of cell viability.

Introduction

Several dentin bonding systems have been developed over the last few decades with the aim to improve the bond between resin and tooth structures for more retentive restorations.

Bowen had proposed bisphenol alpha glycidyl methacrylate (BisGMA) resins to be bonded to enamel after etching, but he soon realized that resins would bond only to a clean substrate and not bond to dentin equally well. Clinically, bonding resins do not bond to dentin unless it is etched by a certain concentration of phosphoric acid and an intermediary dentin bonding system was required to bring together organic and inorganic components together.

Even today with all the deficiencies that Bis-GMA resins have it is still the most commonly used resin in restorative dentistry along with urethane dimethacrylate (UDMA) as primary components of bonding systems. Although the concept of ethical treatment of the patients extended back to Hippocrates (460-377 BC) the idea that new dental products have to be tested for safety before use is recent. The urgent need of the hour was to confirm whether these Bis-GMA based resins are clinically safe.

Cytotoxicity tests are considered a sensitive and standardized method to determine the toxicity of a material containing significant amounts of biologically harmful leachable compounds. Culture medium of mammalian cells is the preferred method for the extraction of substance that can be released from a material because it is a physiological solution capable of extracting a wide range of chemical structures, not only those soluble in water.

3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The MTT assay is a sensitive, most widely used method to determine the mitochondrial enzyme activity and the cell function. It is highly predictable in measuring the cell viability, growth, and proliferation by using tetrazolium dye that is reduced by metabolically active cells having intact mitochondrial dehydrogenase enzymes.

The resinous monomers used in dentistry are formed by different organic molecules, such as bisphenol alpha GMA (Bis-
GMA), triethylene glycol dimethacrylate (TEGDMA), UDMA, and hydroxyethyl methacrylate (HEMA), which work together as copolymeric chains. These monomers comprise the main organic basis of the majority of composite resins and dental adhesives.[3]

However, since various studies have been performed showing toxicity of most of the above-mentioned monomers, we have tested toxicity levels of HEMA and the chief precursor of Bis-GMA which is bisphenol alpha (BPA).

BPA was synthesized close to 100 years ago and recently data begun to emerge indicating BPA exposures, even those in the range generally experienced by any population, having adverse effects on human health. They are components of resin-based dental sealants and composites that are increasingly used in both preventive and restorative oral health care.[2]

However these components alone, as well as in combination with composite resins are capable to reach concentrations to damage pulp after diffusing through dentin.[14,15] As polymerization is incomplete free monomers can be detected by different analytic methods.[16,20]

In dentistry, more than 98% of the restorations are of polymers and monomers. This study was to focus on the toxicity levels of different unpolymerized monomers as single components or in their commercially available form which is used by practitioners on a day to day basis.

**Cytotoxicity study using MTT assay**

The list of armamentarium and materials used in the study [Table 1].

**Methodology**

Three commercially available dentin bonding systems were investigated and two individual components used in bonding agents were also tested namely BPA and HEMA. Of them two are self-etching adhesives and the third is a total etch adhesive. The primer and bond of each were taken as separate groups for the self-etch systems. The experiment was conducted in the following steps.

**Table 1:** Composition of different dentine bonding agents used in the study

<table>
<thead>
<tr>
<th>Product/manufacturer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clearfil Se Bond - Bond (Kuraray Noritake Dental Inc., Okayama, Japan)</td>
<td>MDP, Bis-GMA, HEMA, Hydrophobic aliphatic methacrylate Colloidal silica dl-camphorquinone</td>
</tr>
<tr>
<td>Clearfil Se Bond - Primer (Kuraray Noritake Dental Inc., Okayama, Japan)</td>
<td>MDP, HEMA, Hydrophobic aliphatic methacrylate dl-camphorquinone Water</td>
</tr>
<tr>
<td>Clearfil Protect Bond Bond (Kuraray Noritake Dental Inc., Okayama, Japan)</td>
<td>MDP, Bis-GMA, HEMA, Hydrophobic aliphatic dimethacrylate dl-Camphorquinino N, N-diethanol p-toluidine Silanated colloidal silica Sodium fluoride</td>
</tr>
<tr>
<td>Clearfil Protect Bond-Primer (Kuraray Noritake Dental Inc., Okayama, Japan)</td>
<td>MDP, 2-HEMA, 12-Methacryloyloxydecylpyridinium bromide Hydrophilic aliphatic dimethacrylate Water</td>
</tr>
<tr>
<td>One Coat 7.0 (Coltene/Whaledent AG, Altstatten, Switzerland)</td>
<td>HEMA, Ethanol</td>
</tr>
<tr>
<td>2-HEMA-HEMA (Sigma-Aldrich Chemicals Pvt. Limited, Bengaluru, India)</td>
<td>2-HEMA, Synonyms: 1,2-Ethanediol mono (2-methylpropenoate) Glycol methacrylate HEMA</td>
</tr>
<tr>
<td>BPA (Sigma-Aldrich Chemicals Pvt. Limited, Bengaluru, India)</td>
<td>BPA, Synonyms: 2,2-Bis (4-hydroxyphenyl) propane 4,4’-Isopropylidenediphenol</td>
</tr>
</tbody>
</table>

MDP: 10-Methacryloyloxydecyl dihydrogen phosphate, HEMA: Hyrdoxyethyl methacrylate, BPA: Bisphenol alpha, Bis-GMA: Bisphenol alpha glycidyl methacrylate
**Growth and maintenance of cell cultures**

Tissue pieces were minced to approximately 0.3 cm of the gingival tissue biopsy which was taken from the site of extraction of patients who required orthodontic extraction of teeth, and thus human gingival fibroblasts (HGFs) were isolated from them. These cells are frequently used in biological assessment of resin based materials because they are in close proximity with restorative dental materials in the oral cavity, more clinically relevant and are sensitive cells easy to isolate. These free monomers alter cell functions in the oral cavity.

Dulbecco’s Modified Eagle’s Medium (DMEM) containing 25 cm³ tissue flasks were used for explant cultivation, supplemented with 5% fetal bovine serum, penicillin, at 37°C, and 5% CO₂. Cells were counted on a Neubauer’s Chamber and seeded, followed with an incubation for 24 h in 5% CO₂ at 37°C.

**Sample preparation and elution**

Serial dilutions for each compound tested were made in Eppendorf and the testing components were placed in cell culture medium DMEM and subjected to the cytotoxicity assay.

**Addition of elute to cultured fibroblasts**

Cells were randomly divided and seeded into 96 well plates. There were seven experimental groups and two control groups.

After incubation for 48 h, the medium was removed and refilled with 100 µl in every well with test solution. The plates were incubated for another 24 h followed by evaluation of cytotoxicity.

**MTT assay and spectrophotometric analysis**

As first stated by Mosmann in 1983, the MTT assay outlines the capability of mitochondrial dehydrogenase enzymes in cells to convert the yellow water-soluble tetrazolium salt into dark purple formazan crystals. The number of surviving cells is thus, directly proportional to the level of the formazan product formed.

About 100 µl MTT dye was added to each well-containing cells treated with various extracts of bonding agents and the control wells. Optical density was determined by dissolving the MTT formazan intercellular reaction product with dimethyl sulfoxide and the spectrophotometric absorbance was measured at 630 nm using an enzyme-linked immunosorbent assay microplate reader (Lisa Plus, Aspen Diagnostics, India). Mean was calculated with three readings from every well.

The following formula was used to estimate the percentage of cell viability:

\[
\text{% of cell viability} = \frac{\text{Absorbance of sample} \times 100}{\text{Absorbance of control}}
\]

**Results**

The results of the percentage of cell survival for the single components HEMA and BPA revealed different patterns. With HEMA, the cell death increased as the concentration of the material was increased.

Whereas BPA demonstrated a different pattern, where there was almost no cell death at the highest concentration (20 mg/mL) tested, followed by a sudden increase in cell death at subsequent concentrations (i.e., 10, 5, 2.5, 1.25, 0.625 mg/mL) until 0.0156 mg/mL where the percentage of cell viability was again high indicating low cell death.

Scoring criteria are given by Dahl et al. was used to rate cytotoxicity. The percentage of cell viability for each component of bonding agent was recorded, and the results were tabulated and subjected to statistical analysis.

The mean value Graph 1 showing the cell viability of HEMA and BPA test groups at different concentrations on HGF cell lines.

The mean value Graph 2 showing the cell viability of all the concentrations of the five compounds tested for cytotoxicity.

**Discussion**

Several dentine bonding adhesives have been introduced into restorative dentistry and are formed by different organic molecules such as GMA, Bis-GMA, TEGDMA, UDMA, dipentaerythritol pentaacrylate monophosphate, and HEMA which function as copolymeric chains.
Most of the composites and sealants used in dentistry are based on Bis-GMA. Reports revealed that in-situ polymerization is not complete and that free monomers can be detected by different analytical methods.

The cytotoxicity of dentine bonding agents depends generally on dentine permeability, adhesive composition and time elapsed after their placement.

The cytotoxicity of dentin primers and bonding agents was determined on HGFs cell line by MTT assay in the current study (Figures 1 and 2).

The results of the percentage of cell survival for the single components HEMA and BPA, which were tested for cytotoxicity by MTT assay revealed different patterns. With HEMA, the cell death increased as the concentration of the material was increased, whereas BPA there was almost no cell death at the highest concentration (20 mg/mL) tested, followed by a sudden increase in cell death at subsequent concentrations until 0.0156 mg/mL where the percentage of cell viability was again high indicating low cell death.

A search of literature revealed that as of today very few have tested the cytotoxicity of BPA, found in composites and sealants for two reasons:
• As a by-product of other ingredients in dental composites and sealants that have degraded
• As a trace material left over from the manufacture of other ingredients used in dental composites and sealants.

"Dental sealant exposure to BPA occurs primarily with the use of sealants containing BPA dimethacrylate. This exposure is
considered an infrequent event with little relevance to estimating general population exposures."

All though earlier studies by Soderholm[6] suggest that the resins leached from materials are of no significance, however in 2012, the FDA reiterates that "recent studies provide reason for concern about the potential effects of BPA on the brain, behavior, and prostate gland of fetuses, infants, and children." Which resulted in this study on BPA and HEMA as individual components in the individual composite resins and their effect at cell levels in the laboratory. Almost all manufacturers add HEMA for additional bond strength to dentin.[9] The molecular weight of HEMA is 130.14 and is highly water soluble. High concentrations and early release of HEMA may release free unpolymerized HEMA and come in contact with cell cultures. In this study, cell viability was determined by the mitochondrial activity analysis carried out using MTT based cytotoxicity assay.

The studies by Bruno Neves et al. (2010)[10] demonstrated that the self-etching system was partially biocompatible since the primer was cytotoxic and observed that the adhesive resin was positioned in an intermediate level of biocompatibility.[10]

Based on the mean values, the results of the percentage of cell viability for the three dentin bonding systems used in the study showed that the cell viability was the highest for SE Bond followed by Protect Bond, SE Primer, Protect Primer and One Coat. Indicating that One Coat is the most cytotoxic and Clearfil SE Bond is the least cytotoxic among all the tested dentin bonding systems. Indicating that all the dentin bonding systems were cytotoxic especially the primers were most cytotoxic. However, One Coat 7 which contains both the primer and bonding agent in one bottle were found to be most cytotoxic in this study.

Most clinical researchers have observed the highest toxicity within the first 24 h after the placement. Hanks et al. (1991) and Krifka et al. (2012) have reported HEMA induced apoptosis in vitro at 24 h. In this study, the dentin bonding agents were kept in contact up to 48 h and recorded maximum toxicity at the end of 48 h. Demirci et al. (2008)[31] reported that dentin primers and dentin bonding agents of Clearfil SE Bond resin and Clearfil Protect Bond resin decreased cell survival in a dose-related manner. This applies to all materials used to test and of the resin based material used for testing.[22-26] A total-etching bonding system is more cytotoxic than a self-etching bonding system.[22,23,26]

**Conclusion**

It was observed that all the bonding systems and the two components HEMA and BPA showed a drastic variation in cytotoxic response, clearly indicating them to have cytotoxic effects at concentrations corresponding to concentrations released into the oral cavity. All materials were initially toxic at the highest concentration and the toxicity decreased over time except for BPA where the highest concentration showed no toxicity at all and as the concentration decreased there were strongly cytotoxic and again at the lowest concentration they proved to be moderately cytotoxic. One Coat-7.0 proved to be strongly cytotoxic at all the concentrations tested for.

The clinical significance of our study is that most of the byproducts of BPA based resin materials is a matter of harm to patient, dentist as well as the assistant handling the material. There is no one ideal material available to the clinician,[21] thus continued research is mandatory in this direction to avoid any complications.

**References**

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16. Theilig C, Tegtmeier Y, Leyhausen G, Geurtsen W. Effects of Bis-GMA and TEGDMA on proliferation, migration, and tenasin