ORIGINAL ARTICLE

Cytotoxic effect of a nanohybrid restorative resin on lymphocytes

Upasana Jayaram Reddy, Devika Nair, Mitra N. Hegde

Department of Conservative Dentistry and Endodontics, A.B Shetty Institute of Dental Sciences, Mangalore, Karnataka, India

Abstract

Background: Over the last decade, a lot of anti-Bisphenol A (BPA) groups have emerged explaining the ill effects of its derivatives commonly used in dental products. Since these materials are used on a daily basis in our clinical practice, we must ensure their safety to all who are in contact with them. This is the primary motive of our study.

Aim: The purpose of the study was to analyze cell death and cytotoxicity caused by a dental composite on lymphocytes, comparing the response of cultured lymphocytes to substances leached from a nanohybrid restorative resin at different time intervals.

Methodology: The effect of composite on lymphocytes was studied in two groups, one at 24 h and the other at 72 h. Additional control groups were tested also for. On culturing the test materials with the cells, they were subjected to the ethidium bromide and acridine orange dyes to check vitality.

Results: Within the limitations of this *in-vitro* study, it was concluded that no significant apoptosis was detected in the control group. Early-stage apoptotic cells were detected in the first experimental group of 1 day/24 h. Increasing concentrations and treatment lengths showed a decrease in the number of early-stage apoptotic cells in the 3 day group.

Conclusions: The material tested at all intervals showed very less toxicity confirming low cell death.

Clinical significance: In light of recent awareness related to all materials with BPA and its derivatives, we conducted this study with a commonly used restorative material.

Introduction

Cytotoxicity by definition, "the cytotoxicity of a material or device refers to the toxicological risks caused by a material or its extract in a cell culture."[1]

Cytotoxicity tests are considered a rapid, sensitive, and standardized method to determine the toxicity of a material if it contains significant amounts of biologically harmful leachable compounds. The presence in cultures of isolated cells and the absence of important physiological effect present in *in-vivo* systems, which help to protect cells within the body, producing a test with high sensitivity, 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.[1]

Oral tissues are exposed to various kinds of dental restorative materials containing as many as 30 chemicals and monomers causing cell damage across dentin, and subsequently, causing adverse effects affecting both oral soft tissues adjacent to restorations and soft tissue reactions at distant sites to the restoration (Santerre *et al.*, 2001; Michelsen *et al.*, 2003; Bouillaguet *et al.*, 1996; Gerzina and Hume, 1996).[2]

Composites are ideal for anterior restorations where esthetics are essential and high strength composites as posterior restorations where occlusal forces are higher.[3] Since the polymerization reaction is never complete, methacrylic compounds in composite resin are released into the oral cavity tissues and body fluids where they could cause local adverse effects.

In the study, a direct filling restorative material Filtek Z-250, resin-based tooth colored material was evaluated for possible cytotoxicity at three different time intervals, and results were compared with each other. When evaluating the cytotoxicity effect, cell viability was evaluated using Mario Roderer's AO/EB (Acridine orange and ethidium bromide) test for viability.

Material and Methods

Materials

Reagents used in this study are acridine orange stock and EB stock. Commercially available human blood lymphocytes are used.

This study was conducted in 3 groups with dental composite material Filtek Z 250 XT; it is a Nano Hybrid Universal Restorative.

Composition of Filtek Z 250 XT

The filler system:[3]

Surface-modified zirconia/silica with a median particle size of approximately 3 microns or less

Non-agglomerated/non-aggregated 20 nanometer surface-modified silica particles

The filler loading is 82% by weight (68% by volume)

The resin system:[3]

BIS-GMA

UDMA

BIS-EMA

PEGDMA

TEGDMA

Methodology

Commercially available lymphocytes were chosen as the cell lines for all experiments in the study, which were obtained from the NITTE Research Laboratory where all experiments were done. They were stored and cultured in the carbondioxide incubator [Figure 2].

All experiments were performed in a vertical laminar air flow unit [Figure 1] observing all protocols to maintain a sterile environment. The resin material used for the experiment was also manipulated as per manufacturer's instructions.

10 ml PBS (1 ml PBS diluted in 9 ml of distilled water) and composite material were taken as two different groups and incubated for 24 h (1 day) and 72 h (3 days) at 37°C.

Group 1	24 h
Group 2	72 h
Group 3	Control

After incubation, human lymphocytes were added to the 3 groups (24 h, 3 days and control) and all these 3 groups were kept for 1 h incubation.

About 100 ml acridine orange and EB stains were added to all the groups, and they were mixed well. Precipitated cells from all the three groups were collected for to check their viability. They were observed under a compound microscope to check for cell death.

EB and acridine orange stain[4]

EB is used to identify apoptotic cells by staining condensed chromatin and appear as red.

Figure 1: Vertical laminar air flow unit

Figure 2: Carbon dioxide incubator

Acridine orange stains only living cells and appear as green. The cells stained under these dyes were examined under the microscope using low power (×100) and high power (×200 and ×400) magnification.

For each sample at least 100 cells were counted. Cells were counted under blue filter for apoptosis. Percentage of apoptotic cells were determined by:

$$\% \text{ cell viability} = \frac{\text{Average number of viable cells}}{\text{Average number of cells}} \times 100$$

Results

Lymphocytes were labeled by AO/EB, and dual staining was examined under a fluorescent microscope (Figure 3). No significant apoptosis was detected in the control group. (Diagram 1) Early-stage apoptotic cells, marked by crescent-shaped or granular yellow-green AO nuclear staining, were detected in the first experimental group of 1 day/24 h [Figure 1].

Staining was localized asymmetrically within the cells. With increasing concentrations and treatment lengths, the number of early-stage apoptotic cells decreased in the 3 day group.

Control

Viability	Trial 1	Trial 2	Trial 3	Trial 4
Living	100	117	157	187
Dead	3	5	4	4

1 day incubation

Viability	Trial 1	Trial 2	Trial 3	Trial 4
Living	98	83	103	96
Dead	1	3	2	3

3 day incubation

Viability	Trial 1	Trial 2	Trial 3	Trial 4
Living	72	84	46	59
Dead	2	1	1	2

$$\% \text{ cell viability} = \frac{\text{Average number of viable cells}}{\text{Average number of cells}} \times 100$$

Groups	Average viable cells counted	Average total cells counted	% viability
Control	139.5	143.5	97.2
1 day incubated	95	97.25	97.68
3 day incubated	65.25	65.75	97.75

Comparison of cellular viability at different time intervals

Intervals	n	Mean (SD)	Median (Q1-Q3)	Range	Friedman test Chi-square value	$P*$
Control	4	97.10 (0.87)	97.30 (96.20-97.81)	95.90-97.91	2.00	0.37 (NS)
1 day	4	97.64 (1.12)	97.53 (96.63-98.77)	96.51-98.99		
3 day	4	97.68 (0.90)	97.58 (96.87-98.59)	96.72-98.82		

*$P<0.05$ statistically significant, NS: Non-significant

At baseline the mean percentage viability was 97.10 (0.87) which improved to 97.64% (1.12) at the end of day 1.97.68 (0.90) at the end of day 3. There was no significance difference in the cellular viability during the period.

Discussion

Composites are defined as a three dimensional combination of two chemically different materials with a distinct interphase coupling agent, the inorganic material or fillers, and the organic portion is the resin component. It is an unusual combination. The coupling agent brings these two components into unison.

Figure 3: Compound microscope with attached camera with macro zoom
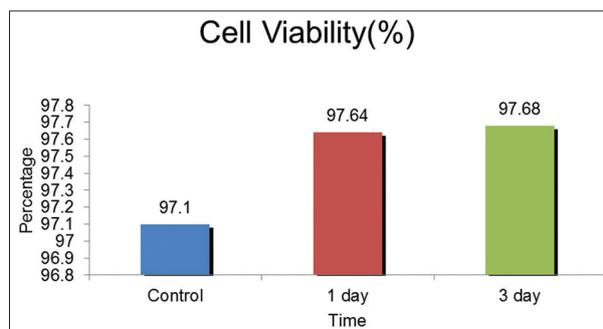

Diagram 1: Comparison of cellular viability at different time intervals

Composite restorative materials are a mixture of polymerized resin components reinforced by inorganic fillers.[5,6]

Resins bond to enamel which can be etched and create leeway to allow resin to flow into these leeway and form what is called as "resin tags."

Even today with all the deficiencies that BisGMA resins have it is still the most commonly used resin in restorative dentistry along with urethane dimethacrylates. The urgent need of the hour was to confirm whether these BisGMA resins are clinically safe. Most of the composites and sealants used in dentistry are based on BPA glycol dimethacrylate (Bis-GMA).[7] This had to be proved by tests for biocompatibility. Cytotoxicity tested *in-vitro*.

Research on several commercially available composite resins has shown the release of several monomers such as 2-hydroxyethyl methacrylate (HEMA) and triethylene glycol dimethacrylate (TEGDMA) even after polymerizing (Santerre *et al.*, 2001; Michelsen *et al.*, 2003).[2,8-10]

Concentrations of HEMA and TEGDMA that diffuse through the dentin layer could be estimated in the millimolar range in most resin-based materials.[5] These uncured resin

monomers leach out from the restorations and diffuses into the dentine targeting the odontoblastic layers of pulp as well as gingival or even into the saliva and circulatory blood.

Human blood lymphocytes were the cell line of choice for cytotoxicity testing in the study since they can be isolated as pure population from blood, are sensitive cells, and can be cultured in normal culture medium.

The chosen method for testing was of Mario Roderers, where a combination of AO and EB was used under a fluorescence scope, where green is live and red is dead.

MTT assay has most commonly been used in vitro for cytotoxicity testing, but this method has some disadvantages.[11] For example, this assay cannot detect the toxic effects of drugs on the body, therefore does not distinguish between apoptotic and necrotic cells and cannot differentiate between these mechanisms of cell death.[12,13] While tumor cells undergo apoptosis in the presence of anticancer drugs, normal cells become necrotic if the drug is toxic. Therefore, the effects of the drug may primarily be toxic, poisoning normal cells.

Dual AO/EB fluorescent staining can detect basic morphological changes in apoptotic cells. In addition, it allows for the distinction between normal cells, early and late apoptotic cells, and necrotic cells. Therefore, AO/EB staining is a qualitative and quantitative method to detect apoptosis.[14]

AO and EB are nucleic acid binding dyes that can be used to measure the cell viability.

Since AO is cell permeable, all stained nucleated cells generate a green fluorescence. EB (~668 Daltons) only enters cells with compromised membranes and therefore dying, dead, and necrotic nucleated cells stained with EB generate a red fluorescence.

This is due to Förster resonance energy transfer; where the EB signal absorbs the AO signal producing no spill-over or double positive results. In addition, other membrane-exclusion viability dyes such as propidium iodide can also be used instead of EB. (Spector *et al.*).[15]

Cell viability is calculated by examining the ratio of the number of live to the number of dead fluorescing cells. This assay is used to measure the viability of nucleated cells in cell culture and purified samples and also in complicated samples such as whole blood, bone marrow, and many more.

Conclusion

Based on the results of this study, it was concluded that good cell viability indicated less toxicity pattern for the material tested in all groups.

Hence, we hypothesize that the percentage of apoptotic cells caused by composite filling is insignificant at all tested durations.

This is in symmetry with the views of the American Dental Association which does not believe there is a basis for health concerns relevant to bisphenol-A or the exposure of its by-products from any dental material.

However, since it is a matter of possible harm to both the operator and patient, they fully support and encourage continued research into the safety of bisphenol-A related dental materials and its derivatives.

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