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Comparative cytotoxicity of five current dentin bonding agents: Role of cell cycle deregulation

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Abstract

To compare the cytotoxicity of three nano-dentin bonding agents (nano-DBAs) and two non-nano-DBAs using Chinese hamster ovary (CHO-K1) cells. We found that nano fillers were not the major contributing factor in DBA cytotoxicity, as analyzed by colony forming assay and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Exposure of CHO-K1 cells to all three tested total-etching DBAs led to G_0/G_1 cell cycle arrest, whereas exposure to higher concentrations of two tested nano-DBAs induced G_2/M arrest. All five DBAs further induced apoptosis at the highest concentration, as analyzed by propidium iodide staining flow cytometry. The toxicity of all DBAs (1:4000 v/v or higher) is related to increased reactive oxygen species (ROS) production, as analyzed by single cell DCF fluorescence flow cytometry. These results indicate that clinical application of DBAs may be potentially toxic to dental pulp tissues. Cytotoxicity of DBAs is associated with ROS production, cell cycle deregulation and apoptosis. Presence of methacrylate monomers such as PENTA and UDMA is possibly the major cytotoxic factor for DBAs. Further studies on other toxicological endpoints of nano-DBAs are necessary to highlight their safe use.

Keywords: Apoptosis; Cell cycle; CHO-K1 cells; Cytotoxicity; Dentin bonding agents

1. Introduction

Deep penetration of dentin bonding agents (DBAs) into dentin is very important for good bonding and promotes clinical success of the restorative material. An ideal DBA must provide high initial and permanent bonding strengths with dentin/enamel and, moreover, have good biocompatibility [1,2]. If the polymerization of composite resin and resin-based dentin bonding agents is not complete, various resin monomers may be released from the unpolymerized resin matrix into the aqueous environment of the oral cavity or may directly affect the biological activity of dental pulp [3]. Furthermore, released resin monomers such as tri(ethylene glycol) dimethacrylate (TEGDMA) and 2-hydroxyethyl methacrylate (HEMA) may have differential cytotoxicity to fibroblasts cultured from dental pulp, gingiva and other tissues [4–6].

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Recently a marked advance in nanotechnology has changed the science of dental materials. Nanoparticles have been developed to improve the mechanical properties of dental resin-based composites, with some success [7]. However, the toxicological and adverse health effects of nano-products to humans and the environment have caused concern [8]. Various nanomaterials have been shown to induce tissue inflammation, lung fibrosis and cardiovascular disease [9,10]. Nanoparticles of cobalt–chromium alloy have been shown to induce more DNA damage, aneuploidy and cytotoxicity in human fibroblasts when compared with micron-sized particles [11]. Layered double hydroxide particles at 50 nm size showed higher cytotoxicity than 100–200 nm particles [12]. We hypothesize that conventional DBAs and nano-DBAs possibly showed differential toxicity due to variations in their content of monomers or nano fillers. The purpose of this investigation was, therefore, to compare the cytotoxicity induced by different DBAs, including three recently developed nano-DBAs, Futurabond NR (FB-NR), Single Bond 2 (SB-2) and Prime & Bond NT (PB-NT), as well as two conventional DBAs, Solobond M (SB-M) and Xeno III (Xeno III). Whether the toxicity of DBAs is related to cell cycle alterations, apoptosis and/or reactive oxygen species (ROS) production is also addressed in this study.

2. Materials and methods

2.1. Materials and chemicals

The sources, composition and major properties of the five dentin bonding agents, SB-M, FB-NR, SB-2, PB-NT and Xeno III, are shown in Table 1. Cell culture materials, such as DMEM/F12, penicillin/streptomycin and fetal bovine serum (FBS), were obtained from GIBCO (Life Technologies, Grand Island, NY). Propidium iodide, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were bought from Sigma (Sigma Chemical Co., St. Louis, MO). Reagents for flow cytometry analysis were purchased from Becton Dickinson (San-Jose, CA). Chinese hamster ovary cells (CHO-K1) from the American Type Culture Collection (ATCC) was kindly given by Professor Yang (National Hsin-Hua University, Hsin-Chu, Taiwan) and cultured in DMEM/F12 medium with 10% FBS and penicillin/streptomycin.

2.2. Preparation of five dentin bonding agents

Five DBAs from different companies were diluted with dimethylsulfoxide (DMSO) to obtain serial dilutions of stock DBAs solutions. They were then added to the culture medium to produce different final concentrations (1:400,000–1:2000 dilutions v/v) dependent on the cytotoxic potency of the DBAs. A final concentration of 0.5% (v/v) DMSO was used as a solvent control (C).

2.3. Colony forming efficiency

Two hundred CHO-K1 cells were seeded into 6 cm culture dishes. After 24 h attachment they were exposed to the five different DBAs at different dilutions and incubated for a further 24 h. The culture medium was changed for fresh medium and the cells were further cultured for 7–10 days. The culture medium was decanted and the cells were washed with phosphate-buffered saline (PBS). Thereafter the cells were fixed in 80% methanol for 5 min and stained with 10% Giemsa stain solution for 15 min. Colony numbers were counted to calculate colony forming efficiency as described previously [13,14].

2.4. Cytotoxicity of DBAs to CHO-K1 cells

Briefly, 1×10^5 CHO-K1 cells were inoculated into 6-well culture plates in DMEM/F12 medium with 10% FBS. After 24 h they were exposed to the five different DBAs at different dilutions and incubated for a further 24 h. Morphological alterations were photographed under a phase contrast microscope. Then MTT (final concentration 0.5 mg ml^{-1}) was added to all wells and the plates incubated for a further 3 h. The insoluble formazan produced from MTT by mitochondrial dehydrogenases in viable cells was dissolved in DMSO and read against a reagent blank (DMSO) using a microplate reader (Dynatech Labs Inc., Chantilly, VA) at a wavelength of 540 nm [4,13].

2.5. Cell cycle progression and apoptosis after exposure to DBAs

Briefly, CHO-K1 cells were inoculated as described above and treated with the five different DBAs in DMEM/F12 medium with 10% FBS for 24 h. Thereafter both floating and attached cells were collected together, resuspended and fixed for 30 min in 70% ice-cold ethanol containing RNase (2 mg ml^{-1}) as described previously [4,13]. The cells were rinsed with PBS and then stained with propidium iodide (PI) (40 μg ml^{-1}) for 10 min. The PI-elicited fluorescence of individual cells was measured using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). The excitation wavelength was 488 nm and emission fluorescence was measured at >590 nm. For each sample a total of 10,000 cells were analyzed. The percentage of cells residing in G_0/G_1 phase, S phase, G_2/M and sub-G_0/G_1 phase was counted using standard ModiFit software and Cell Quest programs.

2.6. Reactive oxygen species (ROS) production after exposure to DBAs

Briefly, 1×10^5 CHO-K1 cells were inoculated into 6-well culture plates in DMEM/F12 medium with 10% FBS. After 24 h they were exposed to the five different DBAs at different dilutions and further incubated for 24 h. The cells were then stained with 10 μM DCFH-

ID	Title	Pages
1868	Comparative cytotoxicity of five current dentin bonding agents: Role of cell cycle deregulation	7