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Biocompatibility of a Fifth-Generation Adhesive System on Pulpal and Gingival Fibroblasts

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Abstract

The purpose of this study was to evaluate the cytotoxic and apoptotic potential of a fifth-generation, total-etch (etch & rinse) adhesive system, Prime&Bond XP. For this purpose, cultured human pulp and gingival fibroblast cells were used. Cells were kept in 1% antibiotic and 10% fetal calf serum-containing DMEM/F12 at 37°C and 95% air-5% CO_2 incubation atmosphere. The culture medium was replaced 2-3 times a week. The test materials were exposed to cells at 1:1000 and 1:4000 concentrations. Cell counting was performed on days 1, 3, 5 and 7; and untreated cells served as controls. Propidium iodide/acridine orange (PI/AO) staining was used to assess apoptosis of treated cells and of the control group at 7th day. The cells were visualized by fluorescence microscopy for the morphological determination of apoptosis. Compared with gingival fibroblasts, cultured pulp cells demonstrated more favorable response to the test solutions. However, at 1:1000 dilution, both cells showed significantly greater cell death and apoptosis.

INTRODUCTION

The use of dental bonding agents has enabled placement of minimally-invasive dental restorations that withstand fracture forces and microleakage. With the use of adhesive dental materials, less removal of sound tooth structure has also been possible. Despite such advantages, adhesive resins have also been shown to release unreacted monomers into the adjacent aqueous phase and diffuse through dentin to the pulp [1]. These residual unpolymerized monomers might be responsible for pulp inflammation and necrosis, especially when adhesive resins are placed directly over a thin remaining layer of dentin (i.e., < 500 μm) or when placed inadvertently on iatrogenic pulp microexposures [2]. In the latter scenario, tissue response to bonding resins might be even more severe [3,4] because complete polymerization of adhesive is hampered as a result of the high oxygen tension [5] and humidity (i.e., presence of blood, clot, and exudates) of the exposure site [6,7]. In addition to a plethora of structure-cytotoxicity evaluations published to date, studies have also shown that dental resin monomers might also promote pulpal hemorrhage [8-11]. Finally, adhesive resins have been shown to induce apoptosis [7] in cultured primary gingival fibroblasts.

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Etch and rinse adhesives, namely the fifth-generation dentin bonding systems adhesives require a separate etch and rinsing step. XP Bond is an etch and-rinse adhesive system that contains a new tertiary butanol solvent, which reduces inadequate penetration of the adhesive resin into exposed collagen network. With newer adhesives continuously being introduced for clinical use, their possible adverse effects on dental tissues need to be evaluated. In this study, we examined the effects of XP Bond cultured human pulp and gingival fibroblasts. We also evaluated the possible concentration-dependent apoptotic effect of this adhesive morphologically.

MATERIALS AND METHODS

Chemicals and Reagents

Dulbecco's Modified Eagle's Medium/Ham's F12 (DMEM/F12; 1:1), fetal bovine serum (FBS) (tested for mycoplasma and endotoxin) and trypsin/EDTA solution (0.05% : 0.02%) were obtained Biochrom AG, Germany; penicillin-streptomycin (powder, 10.000 units/mL penicillin and 10 mg/mL streptomycin), Dulbecco's phosphate buffered saline (PBS) and acridine orange (AO) were obtained from Sigma Chemical Co., St. Louis, MO, USA; propidium iodide (PI) was obtained from Applichem, Germany and mounting medium was obtained from Santa Cruz Biotechnology; cell culture dishes and other plastics were from Greiner bio-one (Germany).

Cytotoxicity Assay

Both the pulp-derived cells and HGF were obtained from stock frozen cell lines. The cells were incubated in 24-well plates at initial density of 20,000 cells/ml. Following 24 h incubation, the cell culture medium was removed and fresh medium containing two different dilutions (dilution 1: 1/1000 v/v; dilution 2: 1/4000 v/v; both passed through a 0.22-μm filter) of an etch and rinse adhesive (XP Bond, Dentsply, Konstanz, Germany) were added separately. Untreated cells served as controls. The cells were incubated for 7 days. Cell proliferation and cell morphology were investigated at the 1st, 3rd, 5th and 7th days. To measure cell growth, the adherent cells were detached from the surface of the culture plates using 0.25% trypsin-10 mM EDTA solution and counted with haemocytometer by using trypan blue dye exclusion method. The experiments (n=6 each) were conducted in triplicate.

Morphological Assessment of Apoptosis

Propidium iodide/acridine orange (PI/AO) staining was used to assess apoptosis of treated cells and of the control group at 7th days . At each evaluation period, the cell culture medium was removed and cells were washed briefly in sterile phosphate buffered saline (PBS). Approximately 25 μg/mL acridine orange and 25 μg/mL propidium iodide were mixed at a v:v ratio of 1:1 and added to the cells for 20 s. Thereafter, cells were washed in PBS for 10 s and mounted in PBS: glycerol (v:v; 1:1). The cells were visualized by fluorescence microscopy (Olympus IX70, Japan). Acridine orange-stained cells were observed under a narrow band fluorescein (FITC) filter (520–560 nm) in green color, and propidium iodide-stained cells were observed under rhodamine filter (510–560 nm) as stained red. Cells were visualized and photographed under the fluorescence microscope at 40X.

Statistical Analysis

Statistical analysis of the data was performed using One-Way ANOVA and Dunnet's T-tests. One Way ANOVA was used to compare the total cell number at respective time points for each cell line. For each dilution factor, differences in cell viability (total cell numbers) between pulp-derived cells and HGF were evaluated statistically using Dunnett's T-test.

RESULTS

Both cell typed elicited differential responses to XP Bond (Figures 1 and 2). Accordingly, the pulp fibroblasts responded more favorably to XP Bond in terms of time-dependent cell viability compared with the gingival fibroblasts ($p<0.05$). As expected, tissue response toward lower dilution of the test material was more severe in both cell types (Figures 3 and 4). On the other hand, this did not necessarily affect cell proliferation, which showed a significant increase over time ($p<0.05$).

In contrast to untreated cells (control group), pulpal and gingival fibroblasts exposed to XP bond demonstrated varying degrees of apoptosis, as verified morphologically (Figures 5 and 6) by the nuclei of apoptotic cells that exhibited yellow staining. Under AO/PI staining, unaffected cells displayed similar morphological characteristics as with untreated (control) cells. The qualitative assessment failed to show any difference between two cell types with respect to apoptosis, although there was a greater tendency for gingival fibroblasts to demonstrate morphologic evidence of apoptosis. The cellular response to XP was more pronounced at 1:1000 dilution in both cell types, and was

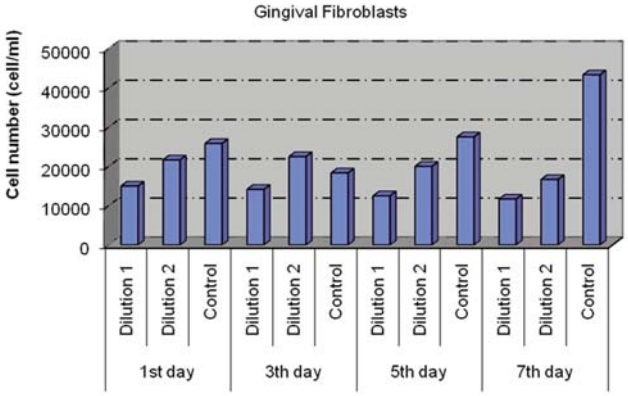

Figure 1. Time-dependent changes in the viability of gingival fibroblasts exposed to XP Bond (dilution 1= 1:1000; dilution 2= 1:4000). X20

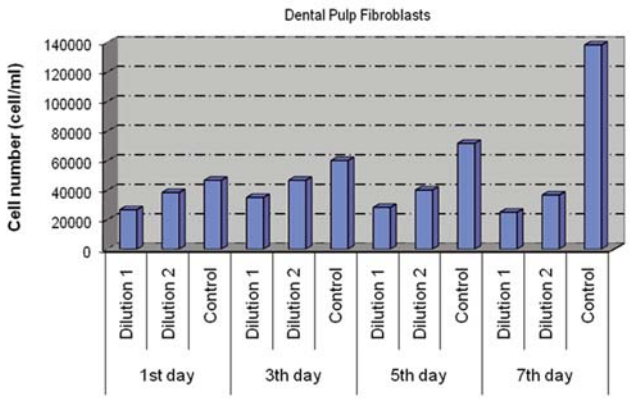

Figure 2. Time-dependent changes in the viability of pulp cells exposed to XP Bond (dilution 1= 1:1000; dilution 2= 1:4000). X20

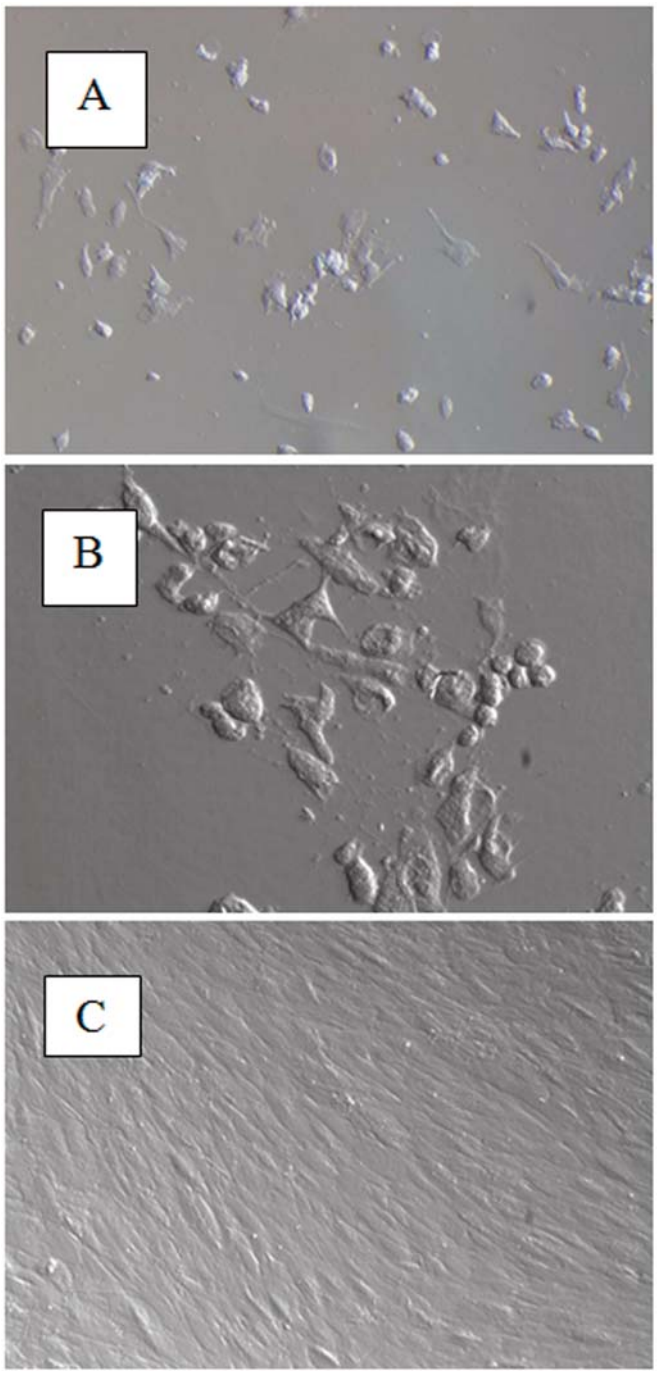

Figure 3. Morphological changes of gingival fibroblasts exposed to different dilutions of XP Bond at Day 1 (dilution 1= 1:1000; dilution 2= 1:4000, C= untreated cells [control]). X20

identified by fragmentation of the nuclei and apoptotic vesicle formation in the cell membrane.

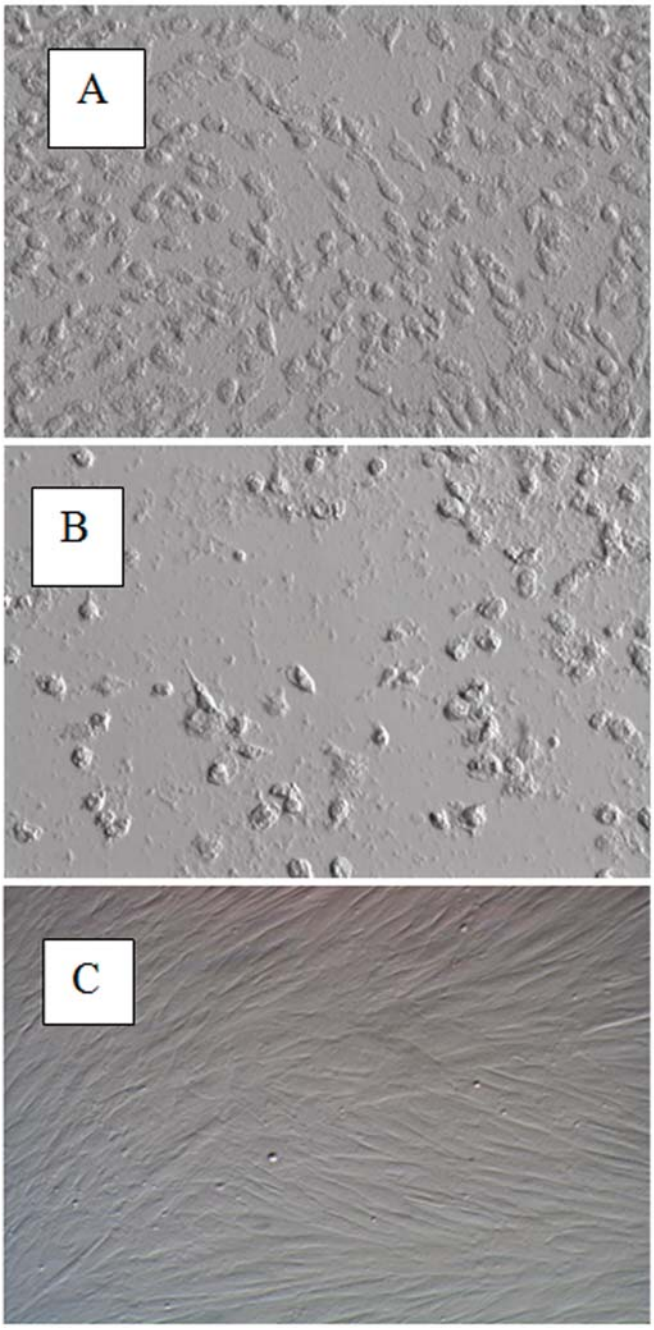

Figure 4. Morphological changes of pulp cells exposed to different dilutions of XP Bond at Day 7 (dilution 1= 1:1000; dilution 2= 1:4000, C= untreated cells [control]). X20

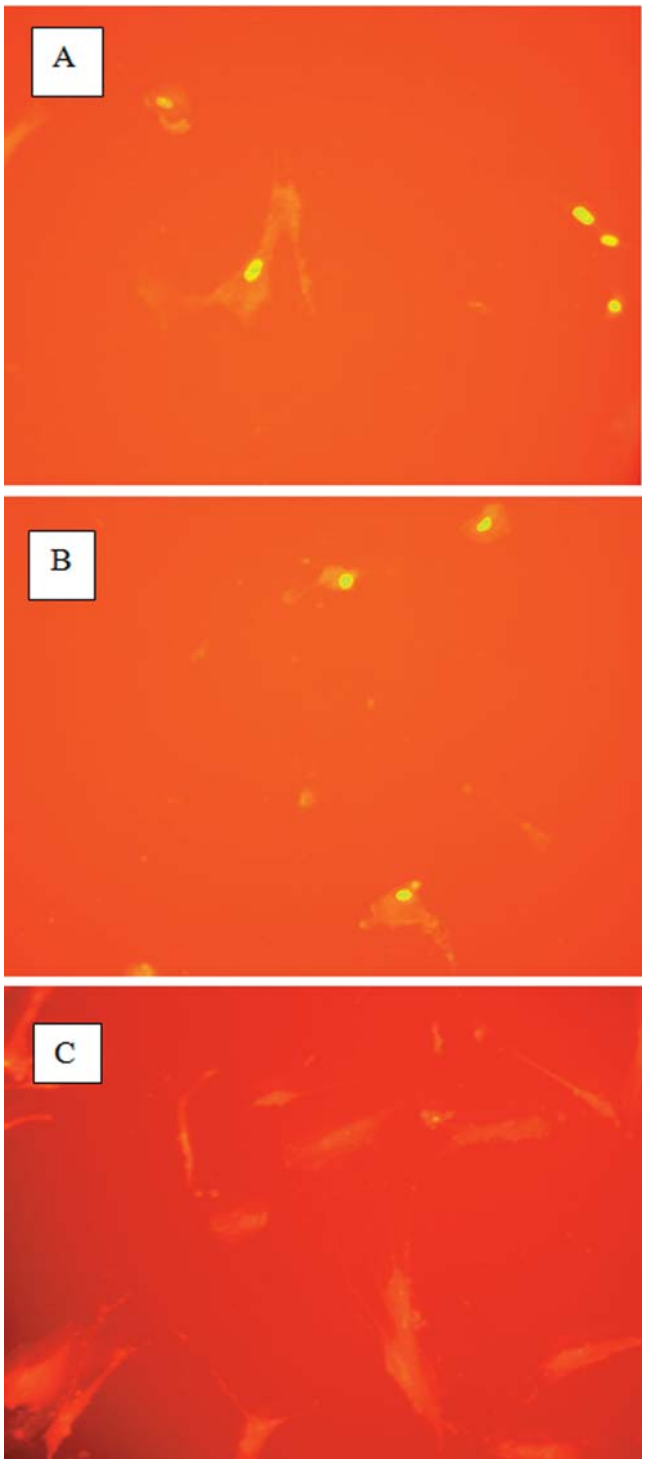

Figure 5. Morphological changes of gingival fibroblasts exposed to different dilutions of XP Bond at Day 7 (dilution 1= 1:1000; dilution 2= 1:4000, C= untreated cells [control]). AO/PI Staining, X20.

DISCUSSION

XP Bond is a self-priming, light-cured bonding agent that contains nanofillers of amorphous silicon dioxide, and di- and tri-methacrylate resins in a new tertiary butanol solvent. Although etch-and-rinse adhesives rely solely on micromechanical retention via hybridization with acid-etch-exposed Type-I collagen, XP Bond incorporates PENTA (dipentaerythritol penta acrylate monophosphate), which aids in chemical binding to dentin and enamel structures. Those monomers as well as other additives, even including photoinitiators [12] could all be responsible for both the cytotoxic response

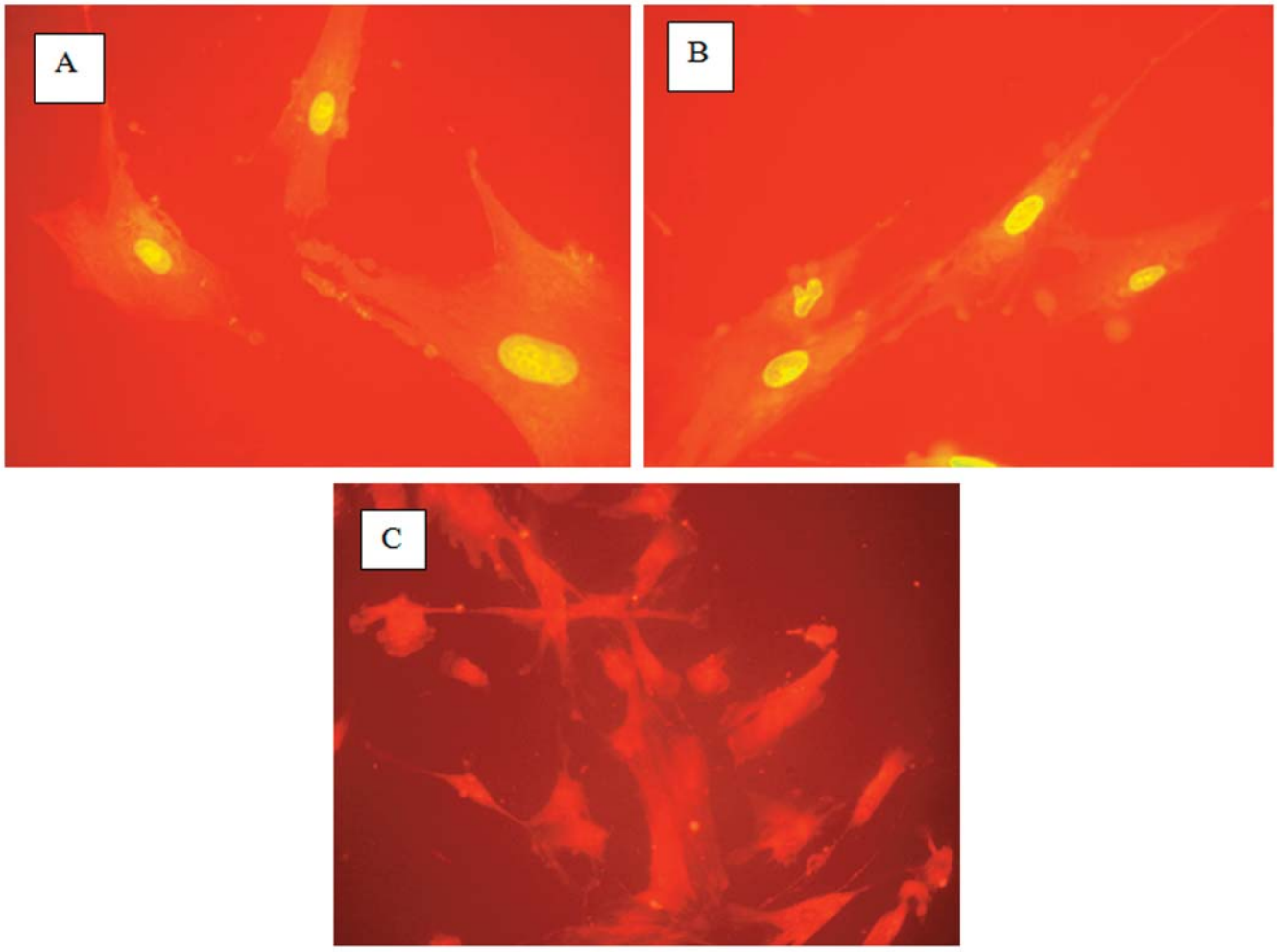

Figure 6. Morphological changes of gingival fibroblasts exposed to different dilutions of XP Bond at Day 7 (dilution 1= 1:1000; dilution 2= 1:4000, C= untreated cells [control]). AO/PI Staining, X20.

and apoptosis; either separately or in combinations. As such, identification of the constituent responsible for these cellular effects was kept beyond the scope of this study, since the clinical relevance of the present findings can only be appreciated on an overall basis. As known, dentin adhesives have been shown to release photoinitiator camphoroquinone [13], which has been found to contribute free radicals including reactive oxygen generation [13,14] and even exert mutagenic effect [15]. Mixture of resin monomers in proprietary adhesive systems have also been shown to induce apoptosis in established cell lines [6]. XP Bond contains three of the monomers commonly-used in contemporary dentin adhesives: 2-Hydroxyethyl methacrylate (HEMA), triethylene-glycol dimethacrylate (TEGDMA) and urethane dimethacrylate (UDMA). Of these, HEMA and TEGDMA can cause apoptosis following a rapid and intense depletion of intracellular glutathione and subsequent production of reactive oxygen species [16-18]. However, effects of monomer combinations on apoptosis still remain to be investigated. Ratanasathien et al [19] demonstrated synergistic effects of monomer combinations on the overall cellular response; while Szep et al. [20] showed that cytotoxicity was differentially affected by various proprietary monomer combinations.

AO/PI labeling is a dye exclusion method that facilitates discrimination of viable and dead cells and is preferred because of its reliability and simplicity [7]. Since the aim of this study was solely limited to investigate the existence of induced apoptosis, AO/PI staining which assays apoptosis by detecting DNA fragmentation provided a simple and effective approach. Our results indicate that there is no distinct difference between the two cell types (pulp

fibroblasts and gingival fibroblasts) with respect to apoptosis, although there was a greater tendency for gingival fibroblasts to demonstrate morphologic evidence of apoptosis. The cellular response to XP was more pronounced at 1:1000 dilutions in both cell types, and was identified by fragmentation of the nuclei and apoptotic vesicle formation in the cell membrane. Undoubtedly, further studies are required to provide insight into the mechanisms by which such apoptosis was induced ,because it is well appreciated that apoptosis is not always programmed under normal physiological conditions, and that injury or disease may also provoke the process [21,22]. Under these non-physiological conditions, both necrosis and apoptosis can be encountered following the same initial insult [7,23].

Provided that the results of this in vitro study can be directly extrapolated to the clinical situation, one may expect XP Bond to be a safe dentin adhesive in terms of pulpal toxicity. Nevertheless, the pulp should be protected with liners where the remaining dentin thickness is below 0.5 mm [24]. It should be cautioned that the curing light, whose affects was neglected in this study, may also contribute to the overall cellular effect [25]. Should the adhesive resin be used for the treatment of dentin hypersensitivity, the present results indicate that the marginal gingival tissue may be adversely affected by its direct application. Regardless of the type of cell examined, the present results also indicate that XP bond is capable of inducing apoptosis, whose effects on oral tissues need to be elucidated with more sophisticated techniques.

CONCLUSIONS

Within the experimental condition of the present study, the following conclusions were drawn:

1. The cytotoxic effect of XP Bond on cultured pulp cells was less pronounced compared to gingival fibroblasts. Except for functional exposure sites, this adhesive can be applied to deep human dentin without the need for pulpal protection. On the other hand, the use of this adhesive in the treatment of cervical tooth sensitivity may result in unfavorable gingival tissue response.

2. XP Bond elicited apoptotic response on both cell types, whose impact on cell survival needs to be further investigated.

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