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Cytotoxicity of orthodontic bonding adhesive resins on human oral fibroblasts

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SUMMARY There is little information concerning the cytotoxic effects of no-mix and flowable adhesives used in orthodontics. The aim of the present study was to evaluate the cytotoxic effects of a no-mix (Unite), a light-cured (Tranbond XT), and a flowable (Denfil Flow) adhesives on human oral fibroblasts.

Twelve discs of each adhesive were prepared and aged for 1, 3, 5, and 7 days in Dulbecco's Modified Eagle's Medium (DMEM). Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the difference between the groups was tested by analysis of variance and Tukey tests ($\alpha = 0.05$).

After 1 day of storage, the no-mix adhesive showed moderate cytotoxic effects ($P < 0.05$), while the light-cured and flowable adhesives were essentially non-cytotoxic. Ageing considerably reduced the cytotoxicity of the no-mix adhesive. On days 5 and 7 of the experiment, the cell viability of three adhesives did not differ significantly ($P > 0.05$), but cell viability was slightly reduced on day 7.

Moderate cytotoxic effects of no-mix adhesive on the first day of the experiment suggest that care should be taken to protect dentists and patients when these adhesives are being handled. Despite higher resin components, the flowable adhesive showed excellent biocompatibility.

Introduction

Biocompatibility of dental materials is usually neglected in dental practice. Most practitioners purchase materials that are commercially available without any concerns about their biocompatibility. Today, while a number of orthodontic adhesives are used by clinicians, most relevant studies concentrate on their physical properties such as shear bond strength with less emphasis on biological compatibility.

Consistent exposure to dental monomers could cause allergic dermatitis (Estlander *et al.*, 1984; Munksgaard *et al.*, 1996; Geukens and Goossens, 2001), drowsiness, headache, anorexia (Anderson and Stasior, 1976), and behavioural alterations (Husain *et al.*, 1985). According to Jacobsen and Hensten-Pettersen (1989), most orthodontists' complaints about their job were related to skin reactions on the hands and fingers provoked by acrylic products, such as bonding materials and gloves.

Another concern regarding adhesive resins is their subtoxic effects, including oesterogenicity (Olea *et al.*, 1996), which is revealed in the long term. Bisphenol-A, one of the by-products resulting from degradation of adhesive resins, can act as a steroid hormone and cause biological effects, such as premature puberty in girls, ovarian cancer, or disruptive maturation of male reproductive organs (Eliades, 2007). However, some investigators believe that the quantity of bisphenol-A released from these materials is lower than that required to induce a biologic reaction (Gioka *et al.*, 2005).

There are only a limited number of studies concerning the cytotoxic effects of orthodontic adhesives. It has been shown that activator components of two no-mix adhesives had greater toxicity than other materials (Terhune *et al.*, 1983). Tang *et al.* (1999) evaluated the cytotoxicity of different orthodontic adhesives on human oral fibroblasts and showed that chemically cured liquid paste adhesives were more cytotoxic than light-cured and chemically cured two-paste materials. *In vivo* cytotoxic effects of six adhesives on hamster oral mucosa proved that the liquid component of one adhesive consistently caused an inflammatory response in all tested animals (Davidson *et al.*, 1982). Fredericks (1981) reported mutagenicity of the System I activator and Lee Unique primer, which were subsequently replaced with modified products. The study of Athas *et al.* (1979) also demonstrated the carcinogenic potential of orthodontic bonding materials. Most research on cytotoxicity in orthodontics has been carried out on monolayer cell cultures (Vande Vannet *et al.*, 2006). Recently, Vande Vannet and Hanssens (2007) used three-dimensional reconstructed human oral epithelium to determine the toxicity of orthodontic adhesives and found architectural and ultrastructural changes in epithelial cells due to penetration of uncured primers.

In most previous studies (Terhune *et al.*, 1983; Tell *et al.*, 1988; Tang *et al.*, 1999), the specimens were prepared using

the same dimensions as in operative dentistry, which are different from the adhesive dimensions in orthodontics. This may affect the amount of monomer release and biocompatibility of these adhesives and provide irrelevant data regarding the clinical situation in orthodontics (Gioka *et al.*, 2005). In addition, some researchers have studied different components of adhesive materials separately (Fredericks, 1981; Geurtsen *et al.*, 1998), but individual components of a compound may have different cytotoxic effects when compared with the total material (Gioka *et al.*, 2005).

Due to the shortcomings of two-paste adhesives, such as the limited working time and voiding during mixing, these are being replaced by no-mix and light-cured materials. Recently, there has been an increasing tendency to use flowable adhesives for bonding of orthodontic attachments because of their desirable characteristics, such as non-stickiness and fluid injectability (Tecco *et al.*, 2005). However, there is lack of information concerning the biocompatibility of no-mix and flowable adhesives used in clinical orthodontics. The mode of application of no-mix adhesives may result in unpolymerized monomer within the system that may have cytotoxic effects. In addition, flowable materials contain low-molecular weight resins that may cause excess monomer release and cell toxicity. The aim of the present study was therefore to evaluate the cytotoxicity of a no-mix, a light-cured, and a flowable orthodontic adhesives under *in vitro* conditions.

Materials and methods

Sample preparation

Three types of orthodontic adhesives, a no-mix (Unite bonding adhesive; 3M Unitek, Monrovia, California, USA), a light-cured (Transbond XT; 3M Unitek), and a flowable (Denfil Flow; Vericom Laboratories Ltd, Anyang, Korea), were tested in combination with their activators or primers as recommended by the manufacturers (Table 1).

Twelve samples were prepared from each adhesive using polyethylene discs with a diameter of 13 mm. To simulate the clinical situation, in the no-mix group, a thin layer of activator was brushed on the surface of two discs and no-mix adhesive was immediately placed on one of them while the other one was pressed against it for a few seconds followed by immediate excess adhesive removal with an explorer. The samples were then pressed consistently for 5 minutes. For the light-cured and flowable adhesives, a thin layer of primer or bonding agent was brushed on to one disc and light cured. The adhesive material was then placed and covered with another polyethylene disc and pressed with a glass slab. Excess material was removed with an explorer and the specimens were light cured for 20 seconds with a halogen light source of 420 mW/cm^2 intensity (Hilux 350; Express Dental Products, Toronto, Ontario, Canada), by applying the light tip close to the polyethylene disc. The sample thickness was measured in three areas using a digital calliper with an accuracy of 0.01 mm. Specimens with a thickness of more or less than 0.22 ± 0.03 mm were excluded from the study. All specimens were prepared by the same operator (FA). The samples were exposed to ultraviolet light for 45 minutes to prevent bacterial contamination and then aged for 1, 3, 5, and 7 days in Dulbecco's Modified Eagle's Medium (DMEM). For each experiment, one sample of adhesive material was immersed in 1 ml of sterile DMEM at 37°C. The surface area to volume ratio was 2.74 cm^2/ml, which is within the recommended range of 0.5–6.0 cm^2/ml suggested by the International Organization for Standardization (1996). After each ageing interval, the samples were transferred to fresh DMEM in an attempt to simulate the removal of soluble elements of the adhesives by saliva in the oral cavity. The extracts were filtered to eliminate solid particles and stored at −20°C until further use.

Table 1 Materials, manufacturers, and lot numbers.

Test groups	Components	Manufacturer	Lot number
Light-cured	Transbond XT Primer	3M Unitek	7EN
	Transbond XT Adhesive	3M Unitek	7AY
No-mix	Unite Activator	3M Unitek	060627
	Unite Adhesive	3M Unitek	070124
Flowable	BC Plus Bonding Agent	Vericom	BP781
	Denfil Flow Adhesive	Vericom	FR 7901122

Cytotoxicity assessment

Human gingival fibroblasts (HGF) obtained from the Pasteur Institute, Tehran, Iran, were cultivated in DMEM with 10 per cent foetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cytotoxic effects of the adhesive extracts were determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were seeded (7000 per well) onto 96-well plates and incubated for 24 hours at 37°C in a humidified atmosphere of 5 per cent CO_2 in air. The culture medium was then replaced with equal volumes (200 µl) of adhesive extracts, using the culture medium by itself as a control. After 24 hours incubation, 20 µl (5 mg/ml) MTT solution was added to each well and the plates were incubated for 4 hours. The MTT was then removed and 100 µl per well dimethyl sulphoxide was added to each well to dissolve the formazan crystals. Optical densities (OD) were measured at 570 nm in an ELISA reader and cell viability was calculated according to the following formula (Vande Vannet *et al.*, 2006):

$$\text{Cell Viability (\%)} = \frac{\text{OD of test group}}{\text{OD of control group}} \times 100$$

Cell viability was scored according to the method of Sjogren *et al.* (2000):

More than 90 per cent cell viability: non-cytotoxic,
60–90 per cent cell viability: slightly cytotoxic,
30–59 per cent cell viability: moderately cytotoxic, and
Less than 30 per cent cell viability: severely cytotoxic.

Six independent experiments were carried out in duplicate by one operator (AB) for each of the adhesive extracts.

Statistical analysis

Statistical significance was determined by one-way analysis of variance followed by Tukey multiple range tests using the Statistical Package for Social Sciences (SPSS, Version 13.0, Chicago, Illinois, USA). *P*-values less than 0.05 were considered significant.

Results

The MTT results of cell viability are presented in Figure 1. On day 1 of the experiment, the no-mix adhesive showed moderate cytotoxicity ($P < 0.05$), whereas the light-cured and flowable adhesives were essentially non-toxic to HGF (more than 90 per cent cell viability) despite their significant statistical differences ($P < 0.05$). On day 3, the light-cured adhesive showed mild cytotoxicity and a significant difference compared with the other groups ($P < 0.05$), while the no-mix and flowable adhesives were not cytotoxic ($P > 0.05$). The cell viability of the different adhesives were similar on days 5 and 7 of incubation ($P > 0.05$), but a reduction in cell viability was observed from days 5 to 7. On day 5, all specimens demonstrated almost 100 per cent cell viability, but on day 7, cell viability was 75, 78, and 80 per cent for the light-cured, flowable, and no-mix adhesives, respectively. Ageing considerably reduced the cytotoxicity of the no-mix adhesive so that after 1 day of storage, cell viability trends were almost similar in the three groups (Figure 2).

Discussion

Cytotoxicity of dental composites and their component materials has been shown in several studies (Caughman *et al.*, 1991; Schedle *et al.*, 1998; Bouillaguet *et al.*, 2002; Franz *et al.*, 2003; Issa *et al.*, 2004; Annunziata *et al.*, 2006; Vande Vannet and Hanssens, 2007). The major cause of cytotoxic effects is elution of residual unpolymerized monomers (Caughman *et al.*, 1991; Schedle *et al.*, 1998). Ferracane (1994) estimated that approximately 5–10 per cent of the residual monomer may elute in the solvent. Thompson *et al.* (1982) studied the amount of leachable materials from cured orthodontic adhesives and found that up to 14 per cent of the total material could leach after 48 hours. It has been found that Bis-GMA which is the main monomer eluted from dental composites (Rathbun *et al.*, 1991) is the most potent toxic component among dimethacrylate derivatives (Ratanasathien *et al.*, 1995; Thonemann *et al.*, 2002; Issa *et al.*, 2004). In addition, a recent study has shown that degradation derivatives of dental composites could cause comparable toxic effects as the raw monomers (Emmler *et al.*, 2008).

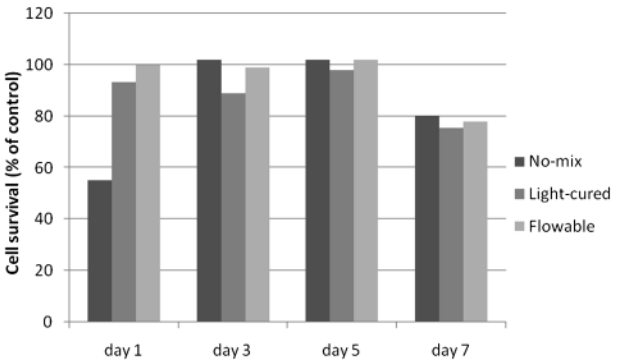

Figure 1 Cytotoxicity of the no-mix, light-cured, and flowable adhesives after 1, 3, 5, and 7 days of pre-incubation.

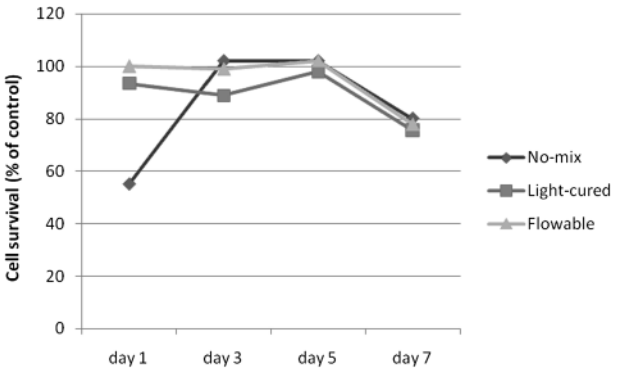

Figure 2 Cytotoxicity trends of the no-mix, light-cured, and flowable adhesives during the total period of the experiment.

In the present study, the effects of three types of adhesives on HGFs were investigated for a period of 7 days because it has been shown that incubation at 37°C in cell culture medium for 7 days is sufficient to abrogate cytotoxicity (Schedle *et al.*, 1998). Although some investigators have observed that orthodontic adhesives can be cytotoxic even after an experimental period of 2 years (Tell *et al.*, 1988), elution appears to be completed within a few days because, according to Ferracane (1994), subsequent weight loss is almost immeasurable. It should be noted that monolayer cell culture was used in the present study to test the toxicity of bonding adhesives. However, it has been demonstrated that three-dimensional cell cultures of human oral epithelium offer an improved system to imitate the human oral mucosa and make it possible to better understand the reality of the processes (Vande Vannet and Hanssens, 2007).

Significant differences were observed between the groups of this study after 1 day of pre-incubation, with the no-mix adhesive being moderately cytotoxic. However, ageing reduced the cytotoxicity of the no-mix adhesive. Previous studies have also demonstrated decreased cytotoxicity of

dental composites with increasing pre-incubation periods (Mohsen *et al.*, 1998) that is almost immeasurable after 7 days (Schedle *et al.*, 1998; Nalcaci *et al.*, 2004). On day 5 of the experiment, cell viability was almost 100 per cent in all groups; however, a mild cytotoxic effect was observed on day 7 in all three groups. This may be due to water absorption that provokes monomer release (Lassila and Vallittu, 2001). Variable cytotoxic trends over the total period of such experiments have been reported (Bouillaguet *et al.*, 2002; Sigusch *et al.*, 2007).

The low cytotoxicity of the light-cured adhesive is consistent with other studies (Tang *et al.*, 1999) and is probably due to sufficient surface curing of this material. It is clear that there is a negative relationship between the level of light curing and cytotoxicity (Caughman *et al.*, 1991; Mohsen *et al.*, 1998; Quinlan *et al.*, 2002). Jonke *et al.* (2008) demonstrated that different light-cured bonding systems resulted in cell toxicity levels significantly lower than that of a chemically cured system (Concise). In their study, the cytotoxicity of all substances diminished after 7 days of pre-incubation, with Concise still being the material with the highest cytotoxicity level.

Moderate cytotoxicity of the no-mix adhesive on day 1 of the present experiment was probably related to insufficient polymerization or severe toxicity of its liquid activator (Fredericks, 1981; Davidson *et al.*, 1982; Terhune *et al.*, 1983). This is contrary to the findings of Gioka *et al.* (2005) who did not find cytotoxic effects of no-mix and light-cured adhesives after 1, 3, and 6 days on HGFs but corroborates the findings of Tang *et al.* (1999) who concluded that chemically cured liquid paste materials were more cytotoxic than light- and chemically cured two-paste materials. In clinical application of no-mix adhesives, a layer of activator is applied both to the tooth surface and to the bracket base with the adhesive sandwiched between them. If the adhesive is very thin, the activator/adhesive ratio increases which may cause more residual unpolymerized monomers within the system. On the other hand, an increase in adhesive thickness produces an inhomogeneous polymerization pattern due to insufficient activator penetration, which may result in cytotoxicity. The latter phenomenon may have little significance in the current study because the dimensions of the specimens were controlled.

Since flowable composites contain higher ratios of resin diluents, higher cytotoxicity of this type of adhesive was expected in the present study, but the results showed excellent biocompatibility. This finding is in agreement with the results of Nalcaci *et al.* (2004) who compared hybrid, condensable, and flowable composites and found no toxic effects of flowable composite during the 2 day interval, while the other groups were moderately cytotoxic.

Franz *et al.* (2009) concluded that since cell culture toxicity data are highly model dependent, the test protocols to screen the toxicity of dental materials should be standardized to obtain comparable results. Thus, an attempt was made in this study to prepare specimens similar to clinical application to ensure the relevance of the data. The thickness of bonding materials in orthodontics is between 100 and 250 μm (Eliades *et al.*, 1991), while in some of the previous studies, the samples were prepared with a diameter of 6 mm and a thickness of 2 mm (Terhune *et al.*, 1983; Tell *et al.*, 1988) which is beyond clinical dimensions and may cause different results due to different activator/adhesive ratios.

The present findings suggest that additional care should be taken when manipulating adhesives, especially no-mix adhesives. This is emphasized by the fact that disposable gloves are permeable to methyl methacrylate and its derivatives (Pegum and Medhurst, 1971; Waegemaekers *et al.*, 1983; Afsahi *et al.*, 1988; Lonnroth *et al.*, 2003). It has been recommended that complete evacuation of remnant activators should be accomplished after adhesive setting with water spray and suction. Care should be taken to remove excess adhesive around the bracket base after polymerization, especially in areas where the adhesive may be in close contact with oral tissues, such as the subgingival and interproximal areas (Terhune *et al.*, 1983; Tell *et al.*, 1988).

It should be borne in mind that *in vitro* cytotoxicity tests do not completely represent the cytotoxic properties of materials in the oral environment. It is known that the oral mucosa is generally more resistant to toxic substances than cell cultures because of the musin and keratin layers (Sjogren *et al.*, 2000). However, in one study, the cytotoxicity of two uncured orthodontic primers was demonstrated on human keratonocytes in a three-dimensional cell culture model simulating epithelial tissue of oral mucosa (Vande Vannet and Hanssens, 2007). In addition, the sublethal effects of adhesive materials during prolonged exposure, which may cause oestrogenic effects, should not be neglected. Further research is required to focus on the long-term effects of these materials.

Conclusions

Within the limitation of the present study, the no-mix adhesive showed moderate cytotoxicity on day 1, which subsided considerably during longer incubation, while the light-cured and flowable adhesives showed excellent biocompatibility on day 1. The data suggest that care should be undertaken during the manipulation of no-mix orthodontic adhesives.

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