Original Article

Cytotoxicity of Two Bonding Adhesives Assessed by Three-Dimensional Cell Culture

Bart Maurice Rene Antoine Vande Vannet[a]; Jean Louis Hanssens[b]

ABSTRACT

Objective: To determine the toxicity of orthodontic adhesives assessed on in vitro three-dimensional reconstructed human oral epithelium (RHOE).

Materials and Methods: Two adhesive primers, Transbond XT (3M, Monrovia, Calif) and Excite (Vivadent, Schaan, Liechtenstein), were tested. After topical exposure, the cell cultures were fixed, cut, and stained for light microscopy (LM) and transmission electron microscopy (TEM). Detection of cytotoxicity by measuring lactate dehydrogenase (LDH) activity was performed. Toxicity was assessed by evaluating the morphological changes with LM and TEM. Copper wires and Triton X-100 served as positive controls, and native cell cultures as negative control.

Results: Morphological evaluation of the native cell cultures revealed no toxic reactions. The LDH assay revealed the following mean values for viability: native cell line (negative control), 1.51; Triton X-100 (positive control), 3.06; Transbond XT polymerized, 1.15; Excite polymerized, 1.11; Transbond XT primer, 2.67; and Excite primer, 0.04. Acute toxicity was observed for Triton X-100 and Transbond XT primer ($P < .001$). Histological evaluation of the RHOE showed toxicity for both primers and mild changes after topical application of polymerized adhesives. The biocompatibility ranking of the adhesive primers was the same after histological analysis and LDH assay except for Excite™ noncured.

Conclusions: RHOE proved to be a valuable model for topical exposure. The toxicity of both uncured primers was demonstrated.

KEY WORDS: 3-D cell culture; LDH assay; Orthodontics; Toxicity; TR 146; Bis-GMA

INTRODUCTION

Intraoral reactions such as redness, soreness, and swelling of the oral mucosa, gingiva, and/or lips have been associated with metal brackets, labial wires, bonding procedures, or acrylic appliances.[1] The clinical use of orthodontic appliances often goes together with an increase in gingivitis score, mostly as a consequence of insufficient oral hygiene.[2,3] Appliances or their corrosion products can cause local tissue damage, which cannot clinically be distinguished from gingivitis of a bacteriological etiology, and erases the question about the biocompatibility of the used materials.[4,5] Estimation of cytotoxicity is part of the initial evaluation of biocompatibility.[6]

The use of cell cultures, mostly on monolayers, to test the toxicity of dental materials was reviewed.[7–9] 3-D cell cultures of reconstituted human oral epithelium (RHOE; Skinethic Laboratories, Nice, France) offer an in vitro system imitating the human oral mucosa. The aim of this study was to analyze two adhesives used in orthodontic practice in their unpolymerized and polymerized fractions to evaluate their possible toxic effect on 3-D human cell cultures and eventually their clinical possible harm to patient or practitioner. Primers are monomers dissolved in water and acetone or ethanol that convert the hydrophilic tooth surface to a hydrophobic surface, permitting hydrophobic adhesive bondings to infiltrate.[10]

3-D cell cultures offer a unique system, without taking into account in vivo parameters (plaque, bacterial infections), to imitate a natural situation. In contrast to monolayer cell cultures on L929 cells or human gingival fibroblast,[11,12] this multilayer model could procure

[a] Lecturer, Department of Orthodontics, Faculty of Medicine and Pharmacy, Vrije Universiteit Brussel, Brussels, Belgium.

[b] Research Assistant, Department of Orthodontics, Faculty of Medicine and Pharmacy, Vrije Universiteit Brussel, Brussels, Belgium.

Corresponding author: Dr Bart Vande Vannet, Department of Orthodontics, Faculty of Medicine and Pharmacy, Vrije Universiteit Brussel, Laarbeeklaan 103, 1090 Jette, Belgium

Accepted: September 2006. Submitted: May 2006.

 DOI: 10.2319/052706-212.1

Table 1. Composition of the Tested Adhesive Primers with Weight Percentages as Delivered by the Manufacturers

Adhesive Primer	Composition	Weight Percentage
Transbond™ XT Primer (3M Unitek, Monrovia, Calif)	Bisphenol a diglycidyl ether dimethacrylate	45–55
	Triethylene glycol dimethacrylate (TEGDMA)	45–55
Excite™ Dental adhesive (Ivoclar Vivadent, Schaan, Liechtenstein)	Phosphonic acid acrylate	11
	Hydroxyethylmethacrylate (HEMA)	30
	Bisphenol-glycidine methacrylate (Bis-GMA)	32.6
	Dimethacrylate	10
	High dispersed silicon dioxide	0.5
	Ethanol	25
	Catalysts and stabilizers	0.9

additional information that makes it possible to understand the reality of the processes under study. The 3-D architecture of the cell cultures makes it possible to effect histological research by using light and transmission electron microscopy.

MATERIALS AND METHODS

Reconstituted Human Oral Epithelium

The RHOE in vitro model was supplied by Skinethic. These multilayer cultures consist of 0.5-cm^2 oral mucosa of RHOE on an inert microporous polycarbonate substrate. The transformed human keratinocytes derived from squamous cell carcinoma of the buccal mucosa (cell line TR146) form an epithelial tissue devoid of stratum corneum, resembling microscopic oral mucosa.[13–15] The in vitro model and culture media were prepared without antibiotics and antimycotics and was controlled for biological safety. The defined medium is based on the MCDB-153 medium (Clonetics, San Diego, Calif) and was supplemented with 5 $\mu g\ mL^{-1}$ insulin.

Adhesive Primers

The following materials were tested (Table 1):

1. Transbond XT Primer (3M Unitek, Monrovia, Calif).
2. Excite™ dental adhesive (Ivoclar Vivadent, Schaan, Liechtenstein).[16,17]

Cytotoxicity Assay Kit

Positive control. The following substances were used as positive controls:

1. Copper oxide: Copper oxide scales were used as a second positive control in order to test their validity in this model.
2. Triton™ X-100 solution (Roche, Mannheim, Germany): This alkylaryl-polyether-alcohol known to cause membrane disruption was used as a positive control.[18] The maximum amount of releasable lactate dehydrogenase (LDH) enzyme activity was determined by lysing the cells with Triton™ X-100.

Negative control. Native cells were used as a negative control.

Procedures

Determination of the optimal cell concentration before the LDH assay. Eight cell layers were assessed: 400,000 × 8 = 3,200,000 cells. This cell concentration was optimal for a good absorbance according to the LDH assay protocol.

LDH assay. The wells were refreshed to remove the LDH activity released from the cells during overnight incubation. Two wells contained no inserts and were provided with 600 μL of medium (they served as background). Two wells contained 600 μL, and each well was provided with native cells to obtain a standard volume. One hundred microliters of medium was supplied to each well (negative control 1 and 2). Positive control 1 was performed by covering the cell culture with copper scales. For positive control 2, 100 μL Triton™ X-100 diluted to 2% was added. For both polymerized adhesives, pieces were deposited covering the RHOE. For both primers, three drops of liquid were applied to the RHOE. All cell cultures of RHOE were incubated overnight (37°C, 5% CO_2, and 90% humidity). One insert was transferred in glutaraldehyde for transmission electron microscopy (TEM) and one in formalin for light microscopy (LM).

After homogenization, 100 μL of supernatant (containing LDH) was transferred into triplicate in an optically clear 96-well titer plate.

To determine LDH activity, 100 μL of reaction mixture (250 μL of catalyst mixed with 11.25 mL of dye solution) was added and light-protected (room temperature, 30 minutes). The absorbance of the samples was measured at 492 nm using an enzyme-linked immunosorbent assay reader (reference filter 620 nm).

Manipulation for LM. Cultures were fixed overnight (formalin 10% buffered saline solution). Tissue blocks (4 μm) were cut using a Microm™ HM 340 E (Histotronix Inc, Atlanta, Ga) and colored following normal hematoxylin (0.7%)/eosin (1%) staining procedure. Sections were evaluated under LM (Leica™, DMR, Wetzlar, Germany; 20×, 40×, and 63× magnification) and the sections were viewed by three persons.

Manipulation for TEM. Tissues were fixed overnight in 4% electron-microscopic grade glutaraldehyde in 0.1 mol/L of cacodylate buffer (Sigma, St. Louis, Mo) at pH 7.4.[19] After fixation with 2% osmium tetroxide (EM Sciences, Fort Washington, Pa) buffered in 0.1 mol/L of cacodylate buffer, sections were dehydrated

Table 2. Content of the Microtiter Plate[a]

BG1	BG 1	BG 1	BG 2	BG 2	BG 2			
neg ctrl 1	neg ctrl 1	neg ctrl 1	neg ctrl 2	neg ctrl 2	neg ctrl 2			
pos ctrl 1	pos ctrl 1	pos ctrl 1	pos ctrl 2	pos ctrl 2	pos ctrl 2			
Tr cur 1	Tr cur 1	Tr cur 1	Tr cur 2	Tr cur 2	Tr cur 2	Tr cur 3	Tr cur 3	Tr cur 3
Ex cur 1	Ex cur 1	Ex cur 1	Ex cur 2	Ex cur 2	Ex cur 2	Ex cur 3	Ex cur 3	Ex cur 3
Tr n-c 1	Tr n-c 1	Tr n-c 1	Tr n-c 2	Tr n-c 2	Tr n-c 2	Tr n-c 3	Tr n-c 3	Tr n-c 3
Ex n-c 1	Ex n-c 1	Ex n-c 1	Ex n-c 2	Ex n-c 2	Ex n-c 2	Ex n-c 3	Ex n-c 3	Ex n-c 3

[a] BG1 indicates background in triplicate descended from the same well; BG2, background in triplicate descended from another well; neg ctrl 1, negative control in triplicate descended from the same insert containing native cells; neg ctrl 2, negative control in triplicate descended from another insert; pos ctrl 1, positive control, copper scales, in triplicate descended from the same insert; pos ctrl 2, positive control, Triton™ X-100, in triplicate descended from the same insert; Tr cur 1, 2, and 3, medium in triplicate descended from, respectively, inserts 1, 2, and 3, containing the tested substance Transbond™ XT polymerized; Ex cur 1, 2, and 3, medium in triplicate descended from, respectively, inserts 1, 2, and 3 containing the tested substance Excite™ polymerized; Tr n-c 1, 2, and 3, medium in triplicate descended from, respectively, inserts 1, 2, and 3 containing the tested substance Transbond™ XT primer adhesive; and Ex n-c 1, 2, and 3, medium in triplicate descended from, respectively, inserts 1, 2, and 3 containing the tested substance Excite ™ primer adhesive.

with graded alcohol to 2× 100% alcohol and 2× propylene oxide (EM Sciences) and embedded in pure epon resin. Ultrathin sections were cut at a maximum of 60 nm with an ultramicrotome (Ultracut, Reichert, Wien, Austria). Areas of interest were stained with lead citrate and 3.5% uranyl acetate for 30 minutes (EM Sciences), and observed in TEM (Tecnai 10™, Philips, Eindhoven, The Netherlands).

RESULTS

Quantitative Evaluation by LDH Assay

Readout (Multiscan RC™, Labsystems, Farnborough, United Kingdom) is described in Table 2. Absorption values of the optic density (OD) are presented in Tables 3 and 4. For every product the mean of the results from the three cultures was calculated. The mean OD of the negative control measured 1.51, and the mean of positive control 2 was 3.06. Transbond™ XT noncured had an OD of 2.67, whereas Excite had an OD of 0.04. Polymerized adhesive primers had about the same results; the absorption values were even lower compared to the negative control (respectively 1.15 and 1.11).

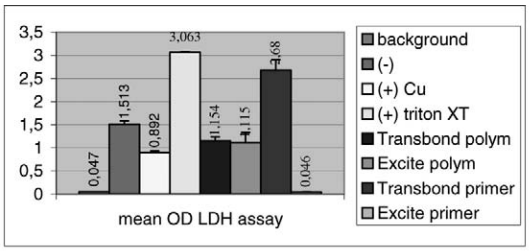
The results of positive control 1, copper scale, were not taken into consideration because the values were so low that copper was not accepted as positive control for this in vitro model.

Table 3. Quantitative Cell Viability: Absorption Values of LDH at 492 and 620 nm and the Calculated Netto Absorption Values[a]

Optical density	R_1	R_2	R_3	R_1	R_2	R_3	R_1	R_2	R_3
492 nm	0.092	0.086	0.086	0.085	0.086	0.088			
	1.775	1.573	1.604	1.69	1.597	1.58			
	1.01	0.994	0.933	3.456	3.377	3.377			
	1.365	1.266	1.265	1.285	1.252	1.034	1.268	1.294	1.291
	1.425	1.349	1.338	0.925	0.898	0.892	1.381	1.376	1.351
	3.09	3.266	3.868	3.391	3.391	3.391	3.391	3.169	3.266
	0.094	0.092	0.093	0.095	0.093	0.094	0.085	0.085	0.083
620 nm	0.041	0.04	0.039	0.039	0.04	0.042			
	0.134	0.122	0.121	0.128	0.12	0.119			
	0.09	0.087	0.083	0.38	0.325	0.317			
	0.107	0.102	0.103	0.105	0.102	0.091	0.103	0.106	0.111
	0.111	0.106	0.107	0.085	0.083	0.083	0.11	0.11	0.108
	0.775	0.718	0.723	0.751	0.739	0.707	0.563	0.567	0.564
	0.044	0.044	0.045	0.047	0.042	0.045	0.045	0.043	0.042
Calculated optical density	0.051	0.046	0.047	0.046	0.046	0.046			
	1.641	1.451	1.483	1.562	1.477	1.461			
	0.92	0.907	0.85	3.076	3.052	3.06			
	1.258	1.164	1.162	1.18	1.15	0.943	1.165	1.188	1.18
	1.314	1.243	1.231	0.84	0.815	0.809	1.271	1.266	1.243
	2.315	2.548	3.145	2.64	2.652	2.684	2.828	2.602	2.702
	0.05	0.048	0.048	0.048	0.051	0.049	0.04	0.042	0.041

[a] LDH indicates lactate dehydrogenase.
R[b] indicates reading value.

Table 4. Absorption Means (OD) of the Results of the LDH Assay*



OD, optical density; polym, polymerised; LDH, lactate dehydrogenase; Cu, copper.

[a] OD indicates optical density; polym, polymerized; LDH, lactate dehydrogenase; and Cu, copper.

Table 5. Peered Assessment of the Results of the Histological Interpretation Using a Histological Index

Tested material	A	B	C	D
Native cells (−)	✓			
Transbond primer				✓
Excite primer			✓	✓
Transbond polymerized		✓		
Excite polymerized		✓	✓	
Copper wire (+)				✓

[a] A indicates that the epithelial tissues have a constant thickness, devoid of terminally differentiated cells, with a regular and compact shape; cells are attached to others via multiple desomosomes; B, minimal changes occur with slight edema; C, the beginning of spongious tissue development in the upper layers, architectural atropy, and cellular irregularity; and D, most of the upper cell layers of the epithelial tissue have become disintegrated, and the remaining basal cells demonstrate loose adherence to the polycarbonate substratum; there is spongious tissue development, cellular necrosis, and loss of cellular junctions in the basal layer together with cellular edema and necrosis in all other cell layers.

Histological Evaluation—LM

The results of the LM histological evaluation are presented in Figure 1. Native cell cultures (Figure 1A) show healthy, homogenous layers with some vacuoles and some dark nuclei. The in vitro tissues have a constant thickness devoid of terminally differentiated cells, and of a regular and compact shape. Cells are attached to each other via multiple desmosomes. About seven cell layers were counted. For the positive control (Figure 1B), most of the upper cell layers of the tissue had disintegrated, and the remaining basal cells were loosely adhering to the polycarbonate substratum. There was spongious development, cellular necrosis, and loss of cellular junctions in the basis layer, together with cellular edema and necrosis in all cell layers. This necrosis consisted of dark cell nuclei without vacuoles.

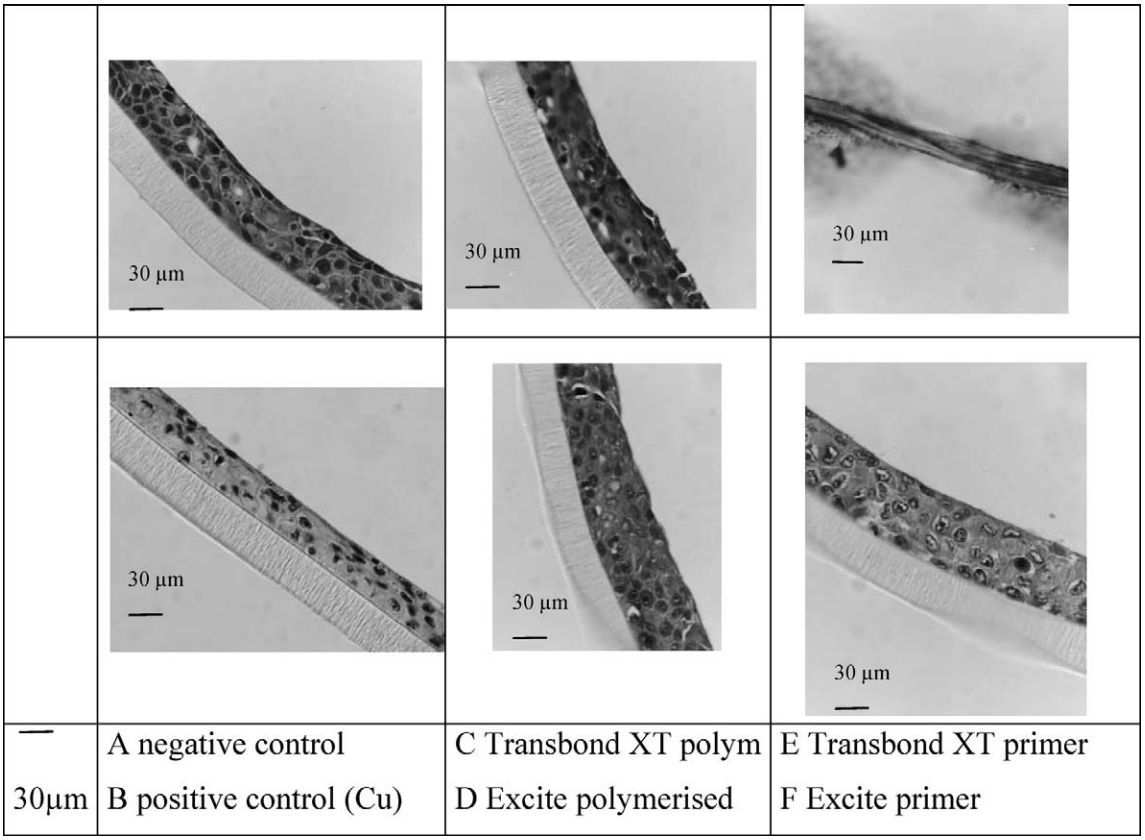


Figure 1. Presentation of LM histology.

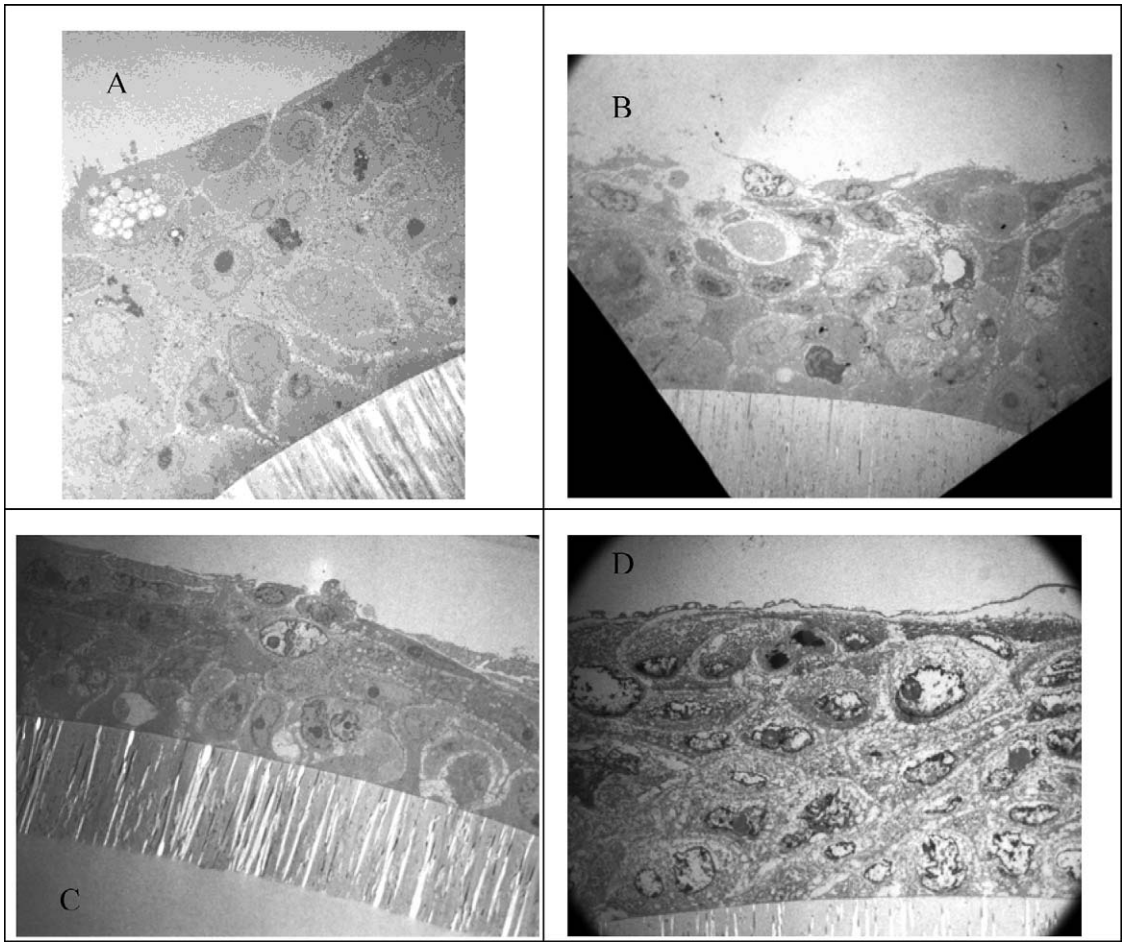


Figure 2. Ultrastructural appearance RHOE in vitro. TEM, magnification 1250×.

The cell cultures that were loaded with Excite™ (polymerized) (Figure 1C) showed clear cell nuclei; the cells were not stuck together and some vacuoles were located in between the cell junctions. The same remarks apply to the tissue covered with Transbond™ XT (Figure 1D) polymerized, but, compared to the negative control, this tissue was less healthy. Excite™ (primer) showed the beginning of spongious tissue development with architectural atrophy and cellular irregularity (Figure 1F). The nuclei were dark bordered. RHOE covered with Transbond™ XT (Figure 1E) (primer) didn't have cells anymore. Peer assessment of the results is presented in Table 5.

Transmission Electron Microscopy

TEM results are presented in Figure 2A through D. Figure 2A (negative control) shows a transverse section (seven cell layers), resting on a polycarbonate filter. The cells contain mitochondria, intermediate filaments, ribosomes, and numerous mature intercellular desmosomal junctions, as seen in normal vital cell behavior. Topical application of Excite™ is shown in Figure 2B. Normal viability was lost and disturbance of the cell junction was present for three-fourths of the depth. The culture surface was no longer homogenous. Figure 2C shows RHOE exposed to Transbond™ XT (polymerized). At the surface layers a light disturbance of the cell junctions was observed, but this structure could be accepted as normal. In Figure 2D (Excite™ primer), loss of architecture caused by penetration of the primer in the layers was demonstrated. Cells appeared necrotic and indication of apoptotic cells was observed. The morphological features of apoptosis were resumed as a loss of microvilli, smooth-surfaced protuberances, chromatin condensation, nuclear and cytoplasm condensation, shrinkage, and nuclear fragmentation. For topical application of Transbond™ XT primer, with the exception of the filter, no cells could be traced. It appears that the RHOE was dissolved because of this application. Infiltration of the RHOE in the pores is observed in Figure 2A,B.

Statistical Analysis

Statistical evaluation was performed using SPSS (SPSS®, Chicago, Ill, version 12.0 for Windows) using

Table 6. Results of the Paired *t*-Test Obtained for the Four Tested Chemicals

Chemicals[a]	*P* Value	Significance
Transbond XT polym-Excite polym	.7617	No significance
Transbond XT polym-Transbond XT	.0003	***
Transbond XT polymer-Excite	.0009	***
Excite polym-Excite	.0187	*
Excite polym-Transbond XT	.0076	**
Transbond XT-Excite	.0001	***

[a] polym indicates polymerized.
* $P = .05$; ** $P = .01$; *** $P = .001$.

Scheffe's method to test all possible contrasts at the same time. Normal distribution of the data was confirmed and the results were checked for homogeneity. The *P* value for the products was .0001 ($P < .001$), which means that there was a highly significant difference between the adhesives (Table 6). There was no statistical difference between Transbond™ XT and Excite™ in their polymerized fraction.

DISCUSSION

Detection of cytotoxicity by measuring LDH activity released from the cytosol-damaged cells into the supernatant is a colorimetric assay for the quantification of cell death and cell lysis. Dental materials can be screened for biocompatibility by the use of tests such as MTT [3-(4.5-dimethylthiazol-2-yl) 2.5-diphenyl trazolium bromide] and LDH on cultures of RHOE. MTT is mainly converted by the basal and suprabasal cells in RHOE. Necrosis of the upper superficial layers of the tissue model is not detected, and requires histological tissue analysis.[20,21] It was demonstrated in the LM and TEM results that superficial layers were affected. Adhesive primers were applied directly to the RHOE, and the irritation potential of the adhesives was assessed in a reproducible way by multiple endpoint analysis.[12,22] LDH served as a potential marker of cell injury and death.[23,24]

A review of ethanol (15%–30%) in relation with the oral mucosa showed increased mucosal permeability[25–27] and penetration of potential carcinogens across the mucosal permeability barrier. Howie[27] has provided further evidence that the association of alcohol with the development of oral cancer may be, in part, because of the topical action of ethanol on oral mucosa, and may also suggest a possible mechanism for the recently reported association between higher levels of alcohol consumption and increased incidence of oral cancer.[28–30] It was reported that topical application of ethanol on the oral mucosa affects epithelial cell homeostasis[31–33] and alters mucosal structure.[34,35] This was confirmed in this study. However, the results of the LDH assay for Excite™ are perhaps attributable to an ethanol concentration of up to 30%, provoking a fixating effect on the RHOE. Excite™ may have an irritating effect and also may cause a sensitizing reaction in patients with a hypersensitivity to any of its ingredients (material safety data sheet) (Ivoclar, Vivadent, Schaan, Liechtenstein). Chemicals remaining within the mucosa can become systemically available.[36]

The present study shows that penetration of both primers caused architectural changes and ultrastructural changes. Epithelial cells expressed features of accessory cells, such as expression of HLA-DR (human leukocyte antigen DR), costimulatory molecules, and molecules of the antigen-processing machinery, and displayed an ability to internalize the antigen.[37] Schendel et al[38] revealed that after 30 days in situ, all adhesives and primers used for their study were not toxic anymore. Nevertheless, both primers can be toxic at the phase of application to the tooth surface and accidental application to the gingiva of the patient or practitioner.

Identification of apoptotic cells was done at the TEM level. Blebbing and apoptotic bodies were observed. Surface blebbing is considered a pattern specific to apoptosis, caused by a deep cytoskeleton rearrangement, causing progressive changes in cell shape and organelle distribution. Blebs are occasionally also described on the surface of cells undergoing necrosis, but in this condition they are followed by the rapid appearance of membrane discontinuities, causing water influx and strong ion distribution.[39] If a toxic agent is present in an excessive concentration that is able to damage the cell, the cell will die by necrosis. Toxic chemicals generating oxidative stress or inducing a pathophysiological increase in cellular calcium levels can kill their target cells by necrosis or by apoptosis.[40] However, under more controlled conditions, as in the present study, the same toxic agents may be capable of inducing apoptosis. Practitioners need to be aware of the toxicity of primers and handle these very carefully.

CONCLUSIONS

- In vitro testing on RHOE is a valuable tool for understanding the biological behavior of adhesive primers.
- LDH release is a noninvasive means for quantification and identifying plasma membrane damage.
- The biocompatibility ranking of the adhesive primers was the same after histological analysis and LDH assay except for Excite™ noncured.
- Triton™ X-100 is a valuable positive control.
- Transbond™ XT showed acute toxicity.

REFERENCES

1. Jacobson N, Hensten-Pettersen A. Occupational health problems and adverse patient reactions in orthodontics. *Eur J Orthod.* 1989;11:254–264.
2. Zachrisson BU, Zachrisson S. Gingival condition associated with orthodontic treatment. *Acta Odontol Scand.* 1972;30: 127–136.
3. Dubey R, Jalili VP, Garg S. Oral hygiene and gingival status in orthodontic patients. *J Pierre Fauchard Acad.* 1993;7:43–54.
4. Owin K, Diedrich P. Changes in periodontal parameters during orthodontic treatment. *Dtsch Zahnarztl Z.* 1990;45:109–112.
5. Hensten-Pettersen A. Occupational health problems and adverse patient reactions in orthodontics. *Eur J Orthod.* 1981;11:254–264.
6. Babich H, Sinensky MC. Indirect toxicity of dental materials: study with Transwell inserts and the neutral red uptake assay. *Altern Lab Anim.* 2001;29:9–13.
7. Schmalz G. Use of cell cultures for toxicity testing of dental materials—advantages and limitations. *J Dent.* 1994; 22(suppl 2):S6–S11.
8. Locci P, Lilli C, Marinucci L, et al. In vitro cytotoxic effects of orthodontic appliances. *J Biomed Mater Res.* 2000;53: 560–567.
9. Tomakidi P, Koke U, Kern R, Erdinger L, Kruger H, Kohl A, Komposch G. Assessment of acute cyto- and genotoxicity of corrosion eluates obtained from orthodontic materials using monolayer cultures of immortalized human gingival keratinocytes. *J Orofac Orthop.* 2000;61:2–19.
10. Bottenberg P. Toward an ideal composite: is every modification a synonym for improvement? *Rev Belge Med Dent.* 2003;58:172–188.
11. Kostoryz EL, Eick JD, Glaros AG, Judy BM, Welshons WV, Burmaster S, Yourtee DM. Biocompatibility of hydroxylated metabolites of BISGMA and BFDGE. *J Dent Res.* 2003;82: 367–371.
12. Reichl FX, Simon S, Esters M, Seiss M, Kehe K, Kleinsasser N, Hickel R. Cytotoxicity of dental composite (co)monomers and the amalgam component Hg(2+) in human gingival fibroblasts. *Arch Toxicol.* 2006;11:1–8.
13. Vande Vannet B, Mohebbian N, Wehrbein H. Toxicity of used orthodontic archwires assessed by three-dimensional cell culture. *Eur J Orthod.* 2006; doi: 10.1093 ejo cj1002.
14. Jacobsen J, Van Deurs B, Pedersen M, Rassing MR. TR146 cells as a model for human buccal epithelium: I. Morphology, growth, barrier properties, and permeability. *Int J Pharm.* 1995;125:165–184.
15. Jacobsen J, Pedersen M, Rassing MR. TR146 cells as a model for human buccal epithelium: II. Optimization and use of a cellular sensitivity MTS/PMS assay. *Int J Pharm.* 1996; 141:217–225.
16. Asscherickx K, Elaut J, Vande Vannet B, Wehrbein H. Low viscosity composites as materials for orthodontic bonding. *Rev Belge Med Dent.* 2003;58:137–144.
17. Elaut J, Asscherickx K, Vande Vannet B, Wehrbein H. Flowable composites for bonding lingual retainers. *J Clin Orthod.* 2002;36:597–598.
18. Weyerman J, Lockmann D, Zimmer A. A practical note on the use of cytotoxicity assays. *Int J Pharm.* 2005;288:369–376.
19. Wisse E. An electron microscopy study of the fenestrated endothelial lining of rat liver sinusoids. *J Ultrastruct Res.* 1970;31:125–150.
20. Mosmann T. Rapid colorimetric assay for cellular growth and survival application to proliferation and cytotoxic assays. *J Immunol Methods.* 1983;65:55–63.
21. Lobner D. Comparison of the LDH and MTT assays for quantifying cell death: validity for neuronal apoptosis? *J Neurosci Methods.* 2000;96:147–152.
22. Van Goethem F, Adriaens E, Alépée N, et al. Pre-validation of a new in vitro reconstituted human cornea model to assess the eye irritation potential of chemicals. *Toxicol In Vitro.* 2006;20:1–17.
23. Legrand C, Bour JM, Jacob C, et al. Lactate dehydrogenase (LDH) activity of the cultured eukaryotic cells as marker of the number of dead cells in the medium. *J Biotechnol.* 1992; 25:231–243.
24. Allen M, Millett P, Dawes E, Rushton N. Lactate dehydrogenase activity as a rapid and sensitive test for the quantification of cell numbers in vitro. *Clin Mater.* 1994;16:189–194.
25. Squier CA, Cox P, Hall BK. Enhanced penetration of nitrosonornicotine across oral mucosa in the presence of ethanol. *J Oral Pathol.* 1986;15:276–279.
26. Du X, Squier CA, Kremer MJ, Wertz PW. Penetration of N-nitrosonornicotine (NNN) across oral mucosa in the presence of ethanol and nicotine. *J Oral Pathol Med.* 2000;29: 80–85.
27. Howie NM, Trigkas TK, Cruchley AT, Wertz PW, Squier CA, Williams DM. Short-term exposure to alcohol increases the permeability of human oral mucosa. *Oral Dis.* 2001;7:349–354.
28. Ogden GR, Wight AJ. Aetiology of oral cancer: alcohol. *Br J Oral Maxillofac Surg.* 1998;36:247–251.
29. Hindle I, Downer MC, Speight PM. The association between intra-oral cancer and surrogate markers of smoking and alcohol consumption. *Community Dent Health.* 2000;17:107–113.
30. Rosenquist K. Risk factors in oral and oropharyngeal squamous cell carcinoma: a population-based case-control study in southern Sweden. *Swed Dent J Suppl.* 2005;179:1–66.
31. Huston GJ. The effects of aspirin, ethanol, indomethacin and 9alpha-fludrocortisone on buccal mucosal potential difference. *Br J Clin Pharmacol.* 1978;5:155–160.
32. Slomiany BL, Piotrowski J, Slomiany A. Alterations in buccal mucosal endothelin-1 and nitric oxide synthase with chronic alcohol ingestion. *Biochem Mol Biol Int.* 1998;45:681–688.
33. Axford SE, Ogden GR, Stewart AM, Saleh HA, Ross PE, Hopwood D. Fluid phase endocytosis within buccal mucosal cells of alcohol misusers. *Oral Oncol.* 1999;35:86–92.
34. Valentine JA, Scott J, West CR, St Hill CA. A histological analysis of the early effects of alcohol and tobacco usage on human lingual epithelium. *J Oral Pathol.* 1985;14:654–655.
35. Maier H, Tisch M. Epidemiology of laryngeal cancer: results of the Heidelberg case-control study. *Acta Otolaryngol Suppl.* 1997;527:160–164.
36. Reddy MS, Geurs NC, Jeffcoat RL, Proskin H, Jeffcoat MK. Periodontal disease progression. *J Periodontol.* 2000;71: 1583–1590.
37. Roggen El, Lindstedt M, Borrebaeck C, Verheyen GR. Interactions between dendritic cells and epithelial cells in allergic disease. *Toxicol Lett.* 2006;162:71–82.
38. Schendel KU, Lenhardt M, Fusenig NE, Komposch G. The testing of the toxicity of the plastics used in orthodontics. *Fortschr Kieferorthop.* 1992;53:263–272.
39. Hoorens A. *Distinction between Necrosis and Apoptosis of Pancreatic Beta Cells* [doctoral thesis]. Brussels, Belgium: Free University of Brussels; 2000.
40. Raffray M, Cohen GM. Apoptosis and necrosis in toxicology: a continuum or distinct modes of cell death? *Pharmacol Ther.* 1997;75:153–177.