

In vitro biocompatibility of denture relining materials

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Objective: The aim of the present study was to evaluate the *in vitro* biocompatibility of denture relining materials using cell culture tests and a test for irritation mechanisms.

Background: Denture relining materials contain non-reacted constituents that may leach out during use inducing local toxic or irritative effects.

Materials and methods: One chemically cured, four visible light cured and five dual-cured products were included. Cured test specimens were used for the filter diffusion test, and extracts of cured specimens were applied in the MTT and the irritation test using the hen's egg test-chorioallantoic membrane (HET-CAM) method.

Results: Five of the tested materials were slightly or moderately cytotoxic in the filter diffusion test, and one product coated with a liner induced severe toxicity. Cell cultures incubated for 24 hour with the test samples were more damaged than those incubated for 2 hour. In the MTT test, extracts of nine of the 11 products induced cytotoxicity. No extracts showed irritation, whereas the coating and two bonding agents tested were strong irritants.

Conclusion: Most of the tested materials contained water soluble, toxic substances that leach out of the products and that some time was needed to obtain cytotoxic amounts of the leachables. Many dental materials elicit cytotoxic response, but this does not necessarily reflect the long-term risk for adverse effects as the oral mucosa is generally more resistant to toxic substances than a cell culture.

Keywords: cell culture, dental materials, irritation, resin, prosthesis.

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Introduction

Denture relining is a very common procedure practised in removable prosthodontics. Two techniques are applied, the direct (intra oral) and the indirect (extra oral). By the direct technique, uncured relining material is placed on the denture that is subsequently placed in the patient's mouth and auto-polymerised. This technique is less time consuming than the indirect technique but the auto-polymerised denture relining materials used initially had some disadvantages relating to the chemical composition of the materials^{1–6}. The most commonly described disadvantages were irritation and burning sensation of the mucosa, porosities, odour, colour instability and poor bonding of the relining material to the denture base material. The introduction of visible light cured denture relining materials (VLC-materials)^{7,8} offered several advantages in relining compared with auto polymerised

acrylic resins⁹. These materials showed less mucosal irritation and improved accuracy of fit. The handling properties of working time, ease of correction and addition during relining were superior to those of the auto-polymerised materials⁹. The physical and mechanical properties of the VLC denture relining materials like wettability, colour stability, and roughness were also improved^{10–12}. However, the degree of monomer conversion of the VLC denture relining materials had a potential for improvement^{13–15}, and the so-called dual-cured relining materials (both visible light and chemically induced curing mechanisms) were introduced in order to improve the degree of conversion⁷. Also in these materials, the conversion of monomers to polymers is incomplete, and residual monomers and polymerisation reaction by-products may leach out irritating the oral mucosa¹⁶. This might be a particular problem for patients with mucosa that is infected, inflamed or lacerated as a result of concurrent

medications or nutritional problems. Thus, large areas of the oral mucosa may be exposed to these irritating or toxic compounds over an extended period that merits a biological evaluation of such products. At present, there is a lack of information regarding the biocompatibility of VLC and dual-cured denture relining materials as only a few studies have been undertaken^{17–20}.

Cell culture studies are usually the starting point of an evaluation of biocompatibility. They provide an investigation of toxicity in a simplified system that minimises the effect of confounding variables^{19,21}. For the evaluation of the irritative potential of such materials, the hen's egg test-chorioallantoic membrane (HET-CAM) method is applicable. The HET-CAM method was designed to replace the *in vivo* test for ocular irritation²², but has also been useful for investigating the potential for mucosal irritation like those of tissue adhesives for facial prosthesis²³, dental bonding agents²⁴, orthodontic materials²⁵ and denture adhesives²⁶. The main target in this test is the blood vessels of the chorioallantoic membrane (CAM) of the fertilised hen's egg, and the test detects substances that compromise the blood vessels and the circulating blood²⁷. The purpose of the present study was to evaluate the *in vitro* biocompatibility of denture relining materials on the European market using cell culture tests and the HET-CAM test for irritation.

Materials and methods

Sample preparation

The materials included in the study are listed in Table 1. The test samples were prepared according

to the manufacturers' instructions. A coating material was delivered with Lightdon U and this product was tested with and without the coating. Lightdon U and Tokuso Rebase were distributed with a bonding that should be applied to the denture before placing the relining materials. The coating and the bonding materials were tested separately in the HET-CAM method.

Extracts for the MTT test and the HET-CAM method

Extracts were prepared from cured specimens of all products according to ISO 10993–12²⁸ using minimal essential medium (MEM) supplemented with 2 mM L-glutamine, 100 units/ml of penicillin, 100 mg/ml of streptomycin and 5% foetal bovine serum (all Gibco BRL, Paisley, UK) for the MTT test and 0.9% NaCl for the HET-CAM test. The surface to liquid ratio was 3 cm²/ml²⁸. Extracts were shaken in a water-bath at 37°C for 24 h, and then filtered through a Millex-GS sterile filter (Millipore, Molsheim, France). The medium extracts were equilibrated for 30 min at 37°C in 5% CO₂ to stabilise the pH.

Filter diffusion test

The filter diffusion test²⁹ was carried out according to the international standards ISO 10993–5³⁰ and ISO 7405:1997, clause 6.2³¹. Millipore filters (Millipore, Bedford, MA, USA), 47 mm in diameter, with a pore size of 0.45 µm, were placed on the bottom of culture dishes (Falcon, NJ, USA) and covered with 6 ml of cell (L 929 mouse fibroblasts) suspension (1.7×10^5 cell/ml medium). The cultures were incubated at 37°C in air containing 5%

Table 1 Tested materials.

Product	Manufacturer	Curing method	Batch no.
Astron LC Soft	Astron Dental Corp. Lake Zurich, USA	Dual cure	Powder 588 Liquid 122
Astron LC Hard	Astron Dental Corp. Lake Zurich, USA	Dual cure	Powder 285 Liquid 537
Bosworth Lightliner Hard	Bosworth Company, Skokie, USA	Dual cure	0204–163
Bosworth Lightliner Soft	Bosworth Company, Skokie, USA	Dual cure	0202–057
Lightdon U	Dreve-Dentamid GmbH, Unna, Germany	VLC	100926
Lightdon U + Plaquit			
Plaquit (surface sealer)		VLC	106501
Bonding			0034181
Tokuso Rebase	Tokuyama Dental Corp, Tokyo, Japan	Chemical	x571232
Bonding			2021
Triad DuaLine	Dentsply International Inc, York PA, USA	Dual cure	020601A
Triad Hi-Flow Reline Material	Dentsply International Inc, York PA, USA	VLC	020129B
Triad Resiline Reline Material	Dentsply International Inc, York PA, USA	VLC	011026
Triad VLC Reline Material	Dentsply International Inc, York PA, USA	VLC	020306B

VLC, visible light cured.

CO₂ and 95% relative humidity for 24 hour to establish a cell monolayer on the filters. After incubation, filters were placed on an agar medium (Eagles MEM × 2 and 1.5% agar) cell side down. The relining materials (circular, diameter 5 mm) were placed on the filters together with a positive control (4% phenol) and a negative control (Teflon) then incubated at 37°C in an air containing 5% CO₂ and 95% relative humidity for 24 hour. Products that showed cytotoxicity at 24 hour of incubation were tested in a new experiment using 2 hour of incubation. After incubation, test samples were removed and the filter gently loosened from the agar layer. The cells, still adherent to the filters, were incubated at 37°C for 3 hour for demonstrating activity of succinate dehydrogenase. After incubation, the filters were rinsed in distilled water and left to dry. Filters were examined macroscopically and the stain intensity of the contact area of each test specimen was compared with the background stain. Each test was carried out twice using at least three replicates for each experiment. The cytotoxicity of the relining materials was rated, based on a scoring system that takes into account the staining intensity of the zone and the diameter or extension of the affected area as; 0–0.4 = not cytotoxic, 0.5–1.4 = slightly cytotoxic, 1.5–2.4 = moderately cytotoxic, and 2.5–3.5 = severely cytotoxic³¹.

MTT test

L 929 mouse fibroblasts (American Type Culture Collection CCL 1) were seeded at 15 000 cells/well in 100 µl. The 96 well dishes were then placed in a humid incubator with an atmosphere of 5% CO₂, 95% air for 24 hour. Thereafter, the medium from the 96-well plates was removed and replaced with the various extracts or control medium and incubated for 24 hour. After incubation, cytotoxicity was assessed using the MTT assay³². Twenty microlitre of a solution of 5 mg/ml MTT (Sigma, MO, USA) in warm phosphate-buffered saline (PBS) was added to each well, and incubated at 37°C, in an air containing 5% CO₂ and 95% relative humidity for 4 hour in the dark. After incubation MTT was aspirated and 0.1 ml 0.04 mol/l HCL in isopropanolol was added. Plates were agitated until thorough dissolution of the formazan had occurred. Absorbance was read at 570 nm, using a Multiskan EX spectrophotometer (Labsystem, Helsinki, Finland). Each extract was tested twice using at least eight replicas for each experiment. The mean absorbencies of the wells containing the same extract and their standard deviation were calculated.

Cytotoxicity was rated based on cell viability relative to controls as; non-cytotoxic >90% cell viability, slightly cytotoxic = 60–90% cell viability, moderately cytotoxic = 30–59% cell viability, and severely cytotoxic = <30% cell viability. The cytotoxicity of the different products was compared using a two-tailed *t*-test. The significance level was $\alpha = 0.05$.

HET-CAM procedure

The HET-CAM procedure was slightly modified from a previously published method²². Fertilised eggs were purchased (Samvirkekylling, N-2436 Valer, Norway) and placed in an automatically rotating incubator in a humidified atmosphere at 37°C until testing on day 9. Before testing, any defective eggs were discharged. The removal of the shell above the air cell and inner egg membrane using a dental drill saw blade and forceps gave access to the CAM. The test solution (300 µl) was applied directly on the CAM that was examined for 5 min using a photomicroscope (Wild M400, Wild, Heerbrugg, Switzerland). Irritation of the CAM was scored by recording haemorrhage, coagulation, and vascular lysis for a period of up to 5 min according to Kalweit et al.²². For details, the reader is referred to Dahl & Polyzois²³. Each product was tested in triplicate and the test repeated at least one time. Test solutions scored 0–0.9 were classified as non-irritant, score 1–4.9 as slight irritant, score 5–8.9 as moderate irritant, and score 9–21 as strong irritant²².

Results

The test results and the rating of cytotoxicity and irritation are given in Tables 2 and 3, respectively. Five of the tested materials were slightly or moderately cytotoxic in the direct contact test. Lightdon U coated with Plaquit induced a strong toxicity. Cell cultures incubated for 24 hour with the test samples were more damaged than those incubated for 2 hour. In the MTT test using extracts of the test samples, nine of the 11 products gave a cytotoxic response. None of the extracts of the relining materials showed irritation in the HET-CAM test, whereas the coating and the two bonding agents tested were all strong irritants.

Discussion

Both the filter diffusion test and the MTT test have the same endpoint; an evaluation of the mitochondrial function after exposure to potential toxic substances^{29,32}. In the filter diffusion test where the

Table 2 Cytotoxicity and HET-CAM tests.

Product	Filter diffusion test index (zone size in mm)		MTT test % viable cells ^d	HET-CAM method	
	2 hour	24 hour		IS	mtc100 ^d
Astron LC Hard	– ^b	0	99 ± 2	0	–
Astron LC Soft	0	1.9 (5 ± 1)	23 ± 1*	0	–
Bosworth Hard	0	0.5 (0/rsi) ^c	7 ± 1*	0	–
Bosworth Soft	0.9 (0/rsi)	1.5 (5 ± 1)	3 ± 2*	0	–
Lightdon U	0	0.8 (0/rsi)	84 ± 3*	0	–
Lightdon U coated with Plaquit	0	3.0 (19 ± 5)	4 ± 1*	0	–
Plaquit	–	–	–	12 ± 2	45 ± 16
Bonding	–	–	–	16 ± 2	35 ± 12
Tokuso Rebase	0	1.0 (rsi)	58 ± 4*	0	–
Bonding	–	–	–	18 ± 1	30 ± 0
Triad DuaLine	0	0	81 ± 3*	0	–
Triad Hi-Flow	0	0.1 (0/rsi)	92 ± 3*	0	–
Triad Resiline	–	0	64 ± 1*	0	–
Triad VLC	0	0.1 (0/rsi)	90 ± 2*	0	–

*Statistically significant from control ($p < 0.05$).

^aIn relation to control ($100 \pm 1\%$).

^bNot tested.

^crsi: reduced in staining intensity, but not totally discoloured.

^dmean time to coagulation.

Table 3 Cytotoxicity and irritation rating based on the results in Table 2.

Test substance	Filter diffusion test		MTT test Extract	HET-CAM method Extract
	Material	Material		
Product	2 hour cytotoxicity	24 hour cytotoxicity	Cytotoxicity	Irritation
Astron LC Hard		Non-toxic	Non-toxic	Non-irritant
Astron LC Soft	Non-toxic	Moderate	Severe	Non-irritant
Bosworth Hard	Non-toxic	Slight	Severe	Non-irritant
Bosworth Soft	Slight	Moderate	Severe	Non-irritant
Lightdon U	Non-toxic	Slight	Slight	Non-irritant
Lightdon U coated with Plaquit	Non-toxic	Severe	Severe	Non-irritant
Plaquit				Strong
Bonding				Strong
Tokuso Rebase	Non-toxic	Slight	Moderate	Non-irritant
Bonding				Strong
Triad DuaLine	Non-toxic	Non-toxic	Slight	Non-irritant
Triad Hi-Flow	Non-toxic	Non-toxic	Non-toxic	Non-irritant
Triad Resiline		Non-toxic	Slight	Non-irritant
Triad VLC	Non-toxic	Non-toxic	Slight	Non-irritant

test materials and the cells were in close contact, the toxicity was generally more prominent after long-term incubation (24 hour) than after short-term incubation (2 hour). For the MTT test, an extraction procedure for 24 hour was followed by cell exposure to the extract for another 24 hour. More products were found cytotoxic by this

method, and the cytotoxicity was generally given a more severe rating by this method compared with the filter diffusion test. This showed that most of the tested relining materials, but for two, contained water soluble toxic substances that leached out of the products and that some time was needed to obtain toxic amounts of the leachables.

Table 4 Summary of results for cytotoxicity testing of soft relining materials compiled from present and previous studies. Data from two or more studies was required to be included.

Endpoint	Results ^a						
	Product	Astron LC Hard	Extoral	Triad	Triad Hi-Flow	Control	Reference
Mitochondrial function ^b		Non-toxic			Non-toxic	Unexposed	Present study
Mitochondrial function ^c		Non-toxic			Non-toxic	Unexposed	Present study
Mitochondrial function ^c		Slight	Moderate	Slight	Slight	Unexposed	20
Protein synthesis		Slight	Slight	Moderate		Unexposed	17
Protein synthesis		Slight	Slight	Moderate		Lucitron 199 ^d	19
DNA synthesis		Moderate	Moderate	Moderate		Lucitron 199	19
DNA synthesis		Moderate	Slight	Moderate		Lucitron 199	18
RNA synthesis		Moderate	Slight	Moderate		Lucitron 199	19
RNA synthesis		Moderate	None	Slight		Lucitron 199	18

^aThe effects are compared with control and classified according to the cytotoxicity rating given in material and methods in present study.

^bFilter diffusion test.

^cMTT test.

^dHeat polymerised methyl methacrylate denture base resin.

The *in vitro* toxic effects of three relining materials [Triad (Dentsply, York, PA, USA), Astron LCH (Astron Dental, Wheeling, IL, USA) and Extoral (Pro-Den Systems, Portland, OR, USA)] on oral epithelial cells have been examined in two different studies. In these studies, cellular protein synthesis¹⁷ and the RNA and DNA synthesis¹⁸ were determined after radiolabelling and by scintillation counting. In both investigations, toxic effects on oral epithelial cells were reported and related to the specific formulation of the materials rather than to the type of polymerisation required, although the dual-cured resins generally appeared to be less cytotoxic than those polymerised by light only^{17,19}. No relationship was found between the type of curing and cytotoxicity in the present study.

Some of the materials in our study have also been examined earlier (Table 4). The MTT result for Triad Hi-Flow concurred with the results of an earlier study²⁰ including the same relining materials and evaluating cytotoxicity of eluates. The Astron LC Hard was rated as non-cytotoxic whereas cytotoxic reaction was reported in earlier studies^{17–20}. Since there has been more than 10 years between the various studies, it is reasonable to believe that the composition of the products has been modified reducing the amount of leachables.

Most products used for denture base lining have a cytotoxic potential (Tables 3 and 4). The clinical implication of a cell culture finding is often difficult to interpret. The oral mucosa is generally more resistant to toxic substances than a cell culture, because of the mucin and the keratin layers. Many

denture materials elicit cytotoxic response³³, but this does not necessarily reflect the long-term risk for adverse effects.

Another aspect is the risk of irritation of the mucus membrane^{23,26}. None of the denture relining materials gave any response with the HET-CAM method. This test was made with the same extracts as the cell culture study implying that the cytotoxic substances did not affect the blood vessels of the CAM. The bonding agent included for two of the product was to be placed on the prosthesis in the contact area for the denture relining material. Their strong responses in the HET-CAM test suggest that the patient should not be exposed to these bonding agents. The uncured surface sealing resin was a strong irritant. The product is intended to be applied on the top of the relining material and cured. However, curing in air will result in an uncured surface layer because of oxygen inhibition that could result in mucosal irritation for the patient.

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