DENTAL MATERIALS XXX (2015) XXX–XXX

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.intl.elsevierhealth.com/journals/dema

Evaluation of cell responses toward adhesives with different photoinitiating systems

Kirsten L. Van Landuyt[a,b,*], Stephanie Krifka[a], Karl-Anton Hiller[a], Carola Bolay[a], Claudia Waha[a], Bart Van Meerbeek[b], Gottfried Schmalz[a,c], Helmut Schweikl[a]

[a] Department of Operative Dentistry and Periodontology, University Hospital Regensburg, D-93042, 93042 Regensburg, Germany

[b] KU Leuven BIOMAT, Department of Oral Health Sciences, University of Leuven & Dentistry University Hospitals Leuven, Kapucijnenvoer 7, 3000 Leuven, Belgium

[c] School of Dental Medicine -ZMK Bern, University of Bern, Freiburgstrasse 7, 3010 Bern, Switzerland

ARTICLE INFO

Article history:
Received 14 August 2014
Received in revised form
13 March 2015
Accepted 28 April 2015
Available online xxx

Keywords:
Dental adhesive
Photoinitiator
TPO
CQ
Cytotoxicity
Reactive oxygen species
Apoptosis
Biocompatibility

ABSTRACT

Objectives. The photoinitiator diphenyl-(2,4,6-trimethylbenzoyl)phosphine oxide (TPO) is more reactive than a camphorquinone/amine (CQ) system, and TPO-based adhesives obtained a higher degree of conversion (DC) with fewer leached monomers. The hypothesis tested here is that a TPO-based adhesive is less toxic than a CQ-based adhesive.

Methods. A CQ-based adhesive (SBU-CQ) (Scotchbond Universal, 3 M ESPE) and its experimental counterpart with TPO (SBU-TPO) were tested for cytotoxicity in human pulp-derived cells (tHPC). Oxidative stress was analyzed by the generation of reactive oxygen species (ROS) and by the expression of antioxidant enzymes. A dentin barrier test (DBT) was used to evaluate cell viability in simulated clinical circumstances.

Results. Unpolymerized SBU-TPO was significantly more toxic than SBU-CQ after a 24 h exposure, and TPO alone (EC_{50} = 0.06 mM) was more cytotoxic than CQ (EC_{50} = 0.88 mM), EDMAB (EC_{50} = 0.68 mM) or CQ/EDMAB (EC_{50} = 0.50 mM). Cultures preincubated with BSO (L-buthionine sulfoximine), an inhibitor of glutathione synthesis, indicated a minor role of glutathione in cytotoxic responses toward the adhesives. Although the generation of ROS was not detected, a differential expression of enzymatic antioxidants revealed that cells exposed to unpolymerized SBU-TPO or SBU-CQ are subject to oxidative stress. Polymerized SBU-TPO was more cytotoxic than SBU-CQ under specific experimental conditions only, but no cytotoxicity was detected in a DBT with a 200 μm dentin barrier.

Significance. Not only DC and monomer-release determine the biocompatibility of adhesives, but also the cytotoxicity of the (photo-)initiator should be taken into account. Addition of TPO rendered a universal adhesive more toxic compared to CQ; however, this effect could be annulled by a thin dentin barrier.

* Corresponding author at: University of Leuven, Oral Health Sciences, Sint-Rafaël, Kapucijnenvoer 7, 3000 Leuven, Belgium. Tel.: +32 16 33 27 45; fax: +32 16 33 27 52.
E-mail address: kirsten.vanlanduyt@med.kuleuven.be (K.L. Van Landuyt).

http://dx.doi.org/10.1016/j.dental.2015.04.016
0109-5641/

1. Introduction

During the past decade, most developments in the field of dental adhesive technology have been based on the simplification of multi-step systems. In line with this, so-called 'universal adhesives', which recently have been introduced onto the market, represent one further step in simplification. Typically, universal adhesive systems can be used for bonding not only to enamel and dentin, but also to ceramics, metal and composites. Universal adhesives are actually not new, but new is that the latest generation of universal adhesives come as one-component, one-bottle systems [1].

Even though application of adhesives on exposed pulp tissue is nowadays advised against [2,3], the biocompatibility of adhesives remains very important. There is ample evidence that adhesive ingredients such as monomers and additives may be toxic for pulp cells as they were shown to seriously disrupt vital cell functions [4].

The dentin substrate should be regarded as a permeable substrate, through which ingredients may permeate to the pulp. Self-evidently, the thickness of the remaining dentin after cavity preparation plays an important role [5], and a remaining dentin thickness of 300 μm is considered critical to maintain pulp health [6]. Permeation of monomers can occur during the application of the unpolymerized adhesive, but also after polymerization ingredients may be released [7]. In this regard, the degree of polymerization, often also called 'degree of conversion (DC)' is important. The higher the DC, the lower is the release of unpolymerized monomers [8].

The monomers in methacrylate-based adhesives polymerize thanks to a radical polymerization reaction, for which purpose photoinitiators are added in small amounts to the composition of adhesives [9]. Conventionally, the co-initiator camphorquinone/teriary amine is added to adhesives, but a major drawback of this photoinitiator system is its intense yellow color [10]. Diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide (TPO) is an alternative photoinitiator belonging to the group of acylphosphine oxides, whose initiating system is based on photofragmentation [9]. In contrast to the camphorquinone/co-initiating system, which is characterized by a broad absorption spectrum with peak absorption around 468 nm, the absorption spectrum of TPO is situated more toward the UV spectrum (380–425 nm). Several studies showed that methacrylate composites obtained similar [11,12] or higher degree of conversion [13,14] when TPO was used as photoinitiator. It was also shown that TPO is more reactive than camphorquinone [15]. Significantly fewer monomers eluted from a TPO-based methacrylate resin compared to a CQ-based material in ethanol-based extraction solutions [16,17].

This specific finding is also of particular relevance considering biological effects of these two dentin adhesives. It has been clearly established that resin monomers disrupt the redox homeostasis in cells of the oral cavity through the generation of elevated levels of reactive oxygen species (ROS). As an adaptive response, cells modify the expression of enzymatic antioxidants like superoxide dismutase (SOD1), which eliminates superoxide anions, and glutathione peroxidase (GPx1/2) or catalase, which reduce increasing levels of hydrogen peroxide (H_2O_2) to water. In addition, an increased expression of the stress-responsive haem oxygenase (HO-1) supports antioxidant defense by the generation of the antioxidant bilirubin. Remarkably, the expression of these cytoprotective enzymes depends on the availability of glutathione (GSH), a non-enzymatic antioxidant [18]. Moreover, monomer-induced oxidative burden exceeding the cells antioxidant capacities to regain balanced intracellular redox homeostasis finally leads to cell death via apoptosis through the intrinsic mitochondrial pathway [4,19].

The objective of this study was to use these parameters for a detailed analysis of oxidative stress related cellular responses toward a CQ/amine or TPO based universal adhesive. To this end, cytotoxicity, generation of ROS and expression of enzymatic antioxidants were analyzed, and the raw photoinitiators were evaluated as well. It could be hypothesized that the TPO-based adhesive is biologically less active as it releases fewer monomers, but the initiator itself is a leachable compound whose biological activity should also be taken into account. The null hypothesis tested in the current investigation was that the TPO adhesive would be less cytotoxic than the CQ/amine-based adhesive.

2. Materials and methods

All chemicals and reagents have been listed in Table 1.

2.1. Adhesives tested

One commercial camphorquinone-based adhesive (Scotchbond Universal, 3 M ESPE, Seefeld, Germany) and its experimental counterpart were included in this study, which were also used in the study by Pongprueksa et al. [16]. Their compositions can be found in Table 2. Both adhesives were identical in composition, except that they contained a different photoinitiator. Whereas the commercial adhesive contained camphorquinone and ethyl 4-(dimethylamino)benzoate (EDMAB) as co-initiator, the non-commercialized experimental version contained diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide (TPO). In the remainder of the text, they will be referred to as SBU-CQ and SBU-TPO, respectively.

Dissolved unpolymerized adhesive (i), 24h-extracts of polymerized adhesive (ii) and the raw photoinitators (iii) were used for further testing.

(i) *Unpolymerized adhesives*: the uncured adhesives were dissolved in pure ethanol (0.5 g/ml; w/v) at room temperature and stock solutions were prepared in culture medium at a concentration of 10 mg/ml following ISO standards [20,21]. Serial dilutions in cell culture medium were prepared. In a pilot study, it was found that the ethanol in the tested concentrations was not toxic for the cells used in following experiments.

(ii) *Extracts of polymerized adhesives*: Polymerized adhesive disks were prepared in a standardized teflon mold (diameter 5 mm and heigth 0.5 mm). After applying the uncured adhesive in the mold and gently air-blowing for 5 s (as per manufacturer's instructions), the adhesive was covered by a glass plate to prevent incomplete polymerization

Please cite this article in press as: Van Landuyt KL, et al. Evaluation of cell responses toward adhesives with different photoinitiating systems. Dent Mater (2015), http://dx.doi.org/10.1016/j.dental.2015.04.016

Table 1 – Chemicals and reagents used.

Productname	CAS no. (if available)	Company	City, country
MEMα		Gibco Life Technologies	Karlsruhe, Germany
Fetal bovine serum		Gibco Life Technologies	Karlsruhe, Germany
Penicillin/streptomycin		Gibco Life Technologies	Karlsruhe, Germany
Phosphate buffered saline (PBS)		Gibco Life Technologies	Karlsruhe, Germany
CMF-PBS (calcium- and magnesium-free)		Gibco Life Technologies	Karlsruhe, Germany
PBS-EDTA		Gibco Life Technologies	Karlsruhe, Germany
Genetecin		Gibco Life Technologies	Karlsruhe, Germany
RPMI 1640 containing L-glutamine		Pan Biotech	Aidenbach, Germany
$NaHCO_3$ (2.0 g/l)	144-55-8	Pan Biotech	Aidenbach, Germany
2-hydroxyethyl methacrylate (HEMA)	868-779	Merck KGaA	Darmstadt, Germany
DMSO (dehydrated, SeccoSolv)	67-68-5	Merck KGaA	Darmstadt, Germany
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	57360-69-7	Sigma Aldrich	Steinheim, Germany
L-Buthionine sulfoximine (BSO)	83730-53-4	Sigma Aldrich	Steinheim, Germany
Accutase		PAA	Cölbe, Germany
2′-7′ dichlorofluorescin (H_2DCF)	4091-99-0	MoBiTec	Goettingen, Germany
BCA protein assay		Sigma Aldrich	Steinheim, Germany
Polyclonal antibodies anti-catalase (H-300, sc-50508)		Santa Cruz Biotechnology	Santa Cruz, CA, USA
Polyclonal antibodies anti-catalase (H-300, sc-50508)		Santa Cruz Biotechnology	Santa Cruz, CA, USA
Polyclonal antibodies anti-haem oxygenase-1 (HO-1, M-19, sc-1797)		Santa Cruz Biotechnology	Santa Cruz, CA, USA
Monoclonal antibodies: anti-Cu-Zn superoxide dismutase (SOD-1, B-1, sc-271014)		Santa Cruz Biotechnology	Santa Cruz, CA, USA
Monoclonal antibodies: anti-glutathione peroxidase 1/2(GPx1/2, D-12, sc-133152)		Santa Cruz Biotechnology	Santa Cruz, CA, USA
Monoclonal antibodies: anti-glutathione peroxidase 1/2(GPx1/2, D-12, sc-133152)		Santa Cruz Biotechnology	Santa Cruz, CA, USA
Anti-rabbit IgG horseradish peroxidase linked antibodies (n° 7074)		Cell Signaling NEB	Frankfurt, Germany
Goat anti-mouse IgG with horseradish peroxidase conjugate		Bio-Rad Laboratories	Munich, Germany
Amersham hyperfilm enhanced chemiluminiscence		GE Healthcare	Munich, Germany
Protease inhibitor cocktail (complete mini)		Roche Diagnostics	Mannheim, Germany
Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (clone 6C5)		Millipore	Schwalbach, Germany
CHEMICON re-blot plus mild antibody stripping solution		Millipore	Schwalbach, Germany

Table 2 – Composition of the adhesives tested.

Adhesive	Composition	Photoinitiator
SBU-CQ	Bis-GMA 15–25 wt% (cas: 1565-94-2) HEMA 15–25 wt% (cas: 868-77-9) Water 10–15 wt% (cas: 7732-18-5) Ethanol 10–15 wt% (cas: 64-17-5) Silanized silica 5–15 wt% (cas: 122334-95-6) DMDMA 5–15 wt% (cas: 6701-13-9) 2-Proprionic acid, 2-methyl-,reaction products with 1,10-decanediol and phosphorus oxide (P_2O_5) 1–10 wt% (cas: 1207736-18-2) copolymer of acrylic and itaconic acid 1–5 wt% (cas: 25948-33-8) Ethyl 4-dimethylaminobenzoate <2 wt% (cas: 10287-53-3) Butanone <0.5% (cas: 78-93-3) (Dimethylamino)ethyl methacrylate <2 wt% (cas: 2867-47-2)	Camphorquinone ~2 wt% (cas: 10373-78-1) EDMAB ~2 wt% (cas: 10287-53-3)
SBU-TPO	idem	TPO ~2 wt% (cas: 75980-60-8)

Abbreviations: Bis-GMA: bisphenol A diglycidyl methacrylate; CQ: camphorquinone; EDMAB: ethyl 4-(dimethylamino)benzoate; HEMA: 2-hydroxyethyl methacrylate; DMDMA: 1,10 decamethylene dimethacrylate, TPO: diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide.

Please cite this article in press as: Van Landuyt KL, et al. Evaluation of cell responses toward adhesives with different photoinitiating systems. Dent Mater (2015), http://dx.doi.org/10.1016/j.dental.2015.04.016

due to oxygen inhibition, and light-cured for 20 s with a Bluephase C8 (Ivoclar-Vivadent, Schaan, Liechtenstein; output = 1220 mW/cm^2). One minute after light curing, the disks were weighed to monitor variations in weight and each disk was immersed in either 150, 200 or 300 μl MEMα medium per well in a 48-well plate following ISO standards [20,21]. After 24 h, the media were collected for further testing.

(iii) *Photoinitiator*: The photoinitiators CQ (cas: 10373-78-1) and TPO (cas: 75980-60-8), and the co-initiator EDMAB (cas: 10287-53-3) were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie, Steinheim, Germany). Stock solutions of 100 mM in ethanol were prepared for further testing.

2.2. Cell culture

Clonal SV 40 large T-antigen transfected human pulp-derived cells (tHPC) [22] were cultivated using a minimal essential medium (MEMα) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (100 U/ml penicillin, 100 μg/ml Streptomycin), and 0.2% geneticin (0.1 mg/ml) at 37 °C and 5% CO_2. The medium was refreshed twice per week.

RAW264.7 macrophages (ATCC TIB71) were cultivated in RPMI 1640 medium containing L-glutamine, sodium-pyruvate and 2.0 g/l $NaHCO_3$ supplemented with 10% fetal bovine serum (FBS), and penicillin-streptomycin at 37 °C and 5% CO_2. These cells were only used for Western blotting, which are considered 'golden standard cells' [18], to confirm the results obtained with the pulp-derived cells.

2.3. Cytotoxicity testing

To test the influence of the unpolymerized adhesives, the extracts of the polymerized adhesives and the raw photoinitiators on cell viability, the MTT assay, which is based on the reduction of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), was used [23]. To assess the role of oxidative stress on cytotoxicity, BSO, an inhibitor of glutathione synthesis, was added while testing the unpolymerized adhesives [18]. Briefly, to assess the cytotoxicity of the unpolymerized adhesives, tHPCs were seeded in 96-well plates at a density of 1×10^4 cells/well in 100 μl and cultivated for 4 h at 37 °C and 5% CO_2. Then 100 μl of 50 μM BSO or medium was added to beforehand determined wells, to pre-incubate half of the cell cultures with BSO for at least 20 h. The next day, the cells that were pre-incubated in medium were exposed to 200 μl of diluted unpolymerized adhesives and those pre-incubated to BSO were exposed to unpolymerized adhesives in the presence of 50 μM BSO. Either after 1 h or 24 h, the supernatant was removed, and the cells were incubated with 0.5 mg/ml MTT in PBS solution for 1 h before absorbance reading at 540 nm using a spectrophotometer (Infinite F200, TECAN, Männedorf, Zurich, Switzerland) after complete solubilization of the formazan crystals in DMSO.

To evaluate the cytotoxicity of the extracts of the polymerized adhesives and the raw photoinitators, tHPCs were seeded in 96-well plates in 200 μl culture medium and cultivated at 37 °C and 5% CO_2. After 24 h, the medium was removed and replaced by the undiluted or diluted extracts (in cell culture medium), or by different concentrations of the photoinitators originally prepared as a stock solution in ethanol and further diluted in cell culture medium. After 24 h, cell viability was determined using MTT as described above. The effect of ethanol concentrations was tested in separate experiments. While ethanol concentrations present in extracts and dilutions of unpolymerized materials had no influence on cell viability, a very small effect on viability was detected for the highest concentrations with photo-initiators. This effect was taken into account for each individual photo-initiator solution while calculating the final viability. HEMA (6 and 8 mM) was included here as a positive control substance [18].

Each experiment was performed in duplicate or triplicate as specified in the figure legends and four replicate cell cultures were analyzed in each experiment. The optical density readings obtained from treated cell cultures were expressed as the percentage of untreated cells. The half-maximum-effect concentrations (EC_{50}) were calculated by plotting the viability results onto a dose-effect sigmoidal curve (Table Curve 2D, Version 5.01, Systat Software, San Jose, CA, USA). Differences between the EC_{50} values were statistically analyzed using the Tukey interval method (SPSS 22.0, SPSS, Chicago, IL, USA).

2.4. Cellular reactive oxygen species (ROS) generation and expression of antioxidant enzymes

The generation of ROS in tHPCs was measured following a previously described protocol based on the intracellular oxidation of 2′–7′ dichlorofluorescin (H_2DCF) to 2′–7′dichlorofluorescein (DCF) [24,25]. tHPCs seeded in six-well plates at a density of 2×10^5were exposed to different concentrations (0.1, 0.25, 0.5 and 1 mg/ml) of unpolymerized adhesives prepared as described above. 2-hydroxyethyl methacrylate (HEMA, 6 and 8 mM) was applied on the cells as positive control, whereas cell culture medium only was applied as negative control. DCF fluorescence was determined by flow cytometry (Becton Dickinson FACSCanto, San Jose, CA, USA) at an excitation wave length of 495 nm and an emission wave length of 530 nm. Main fluorescence intensities were obtained by histogram statistics using the FACSDiva™ 5.0.2 software. Individual values of fluorescence intensities were normalized to fluorescence detected in untreated control cultures (=1.0). At least four independent experiments were performed, individual values were summarized as medians (with 25–75% quartiles), and differences between medians were statistically analyzed with the Mann–Whitney *U* test ($\alpha = 0.05$) (SPSS 22.0).

The expression of several antioxidant enzymes in tHPCs and RAW264.7 cells after exposure to the unpolymerized adhesives was determined by Western blot analysis following a previously described protocol [18]. Briefly, the cells were seeded at a density of 1.5×10^6 in petri-dishes and cultivated for 24 h. Subsequently, they were exposed to 0.25 mg/ml or 0.5 mg/ml SBU-CQ or 0.1 mg/ml or 0.25 mg/ml SBU-TPO for 24 h. These concentrations were selected based on the results of cell viability measurements. After exposure, cells were detached, and collected through centrifugation and lysed as

Please cite this article in press as: Van Landuyt KL, et al. Evaluation of cell responses toward adhesives with different photoinitiating systems. Dent Mater (2015), http://dx.doi.org/10.1016/j.dental.2015.04.016

described before [18]. The supernatant was collected by centrifugation, and the amount of proteins present in the cell lysates was determined by BCA protein assay (Sigma) using bovine serum albumin as a standard. Subsequently, Western blot analysis was performed as described before [18]. Proteins (10 μg/lane) were separated by electrophoresis on a sodium dodecyl sulfate polyacrylamide gel and transferred to a nitrocellulose membrane (blot), which was incubated with primary antibodies specific for the detection of SOD-1, GPx1/2, catalase or HO-1. These primary antibodies were detected by horseradish peroxidase-conjugated secondary antibodies visualized by enhanced chemiluminescence (ECL). Finally, the nitrocellulose membranes were stripped using an antibody stripping solution and the enzymes were reprobed with an anti-GAPDH antibody as described previously [26]. All details have been described before [18].

2.5. *Dentin barrier test*

Three dimensional cultures of tHPCs were cultivated according to a previously described protocol on polyamide meshes [27]. Bovine incisors were cut to obtain dentin disks (200 ± 20 μm), and the pulpal side of the disks was etched with 50% citric acid to remove the smear layer. They were then autoclaved and applied in a cell culture perfusion chamber (Minucells and Minutissue GmbH, Bad Abbach, Germany) to obtain two compartments. After an incubation time of 14 days ± 2 days, the three-dimensional tHPC cultures were introduced into the lower 'pulpal' compartment in direct contact with the pulpal side of the dentin disk. This pulpal compartment was perfused with perfusion medium (MEMα with 5.96 g/l HEPES and 20% FBS) at a rate of 0.3 ml/h and left for 24 h before starting the experiment. The next day, perfusion was briefly stopped and SBU-CQ and SBU-TPO were applied onto the dentin disks at the cavity side following the instructions of the manufacturer. After light-curing the adhesives, a flowable composite (Tetric EvoFlow, Ivoclar-Vivadent, Schaan, Liechtenstein) was applied and light-cured for 20 s. As positive control, an experimental light-curing glass ionomer was prepared (Table 3) [28] and as negative control, a polyvinysiloxane impression material (President Regular, Coltene-Whaledent AG, Altstätten, Switzerland) (100% cell viability) was used. The perfusion rate through the pulpal compartment was subsequently increased to 2 ml/h. After an exposure time of 24 h, cell viability in the three-dimensional tHPC cultures was assessed by the MTT assay as described above. Each experiment was performed with five replicates and carried out at least two times. Medians (with 25–75% percentiles) were calculated from individual values, and differences between medians were statistically analyzed with the Mann–Whitney *U* test ($\alpha = 0.05$) (SPSS 22.0).

Exposed three-dimensional cell cultures were also analyzed by confocal laser microscopy (LSM 510 Meta, Zeiss, Jena, Germany) and managed with a LSM Image VisArt software after dying with the a viability/cytotoxicity kit for mammalian cells (Invitrogen, Karlsruhe, Germany). Images were obtained using 10× magnification and further adjusted with an electronic zoom, and image analysis was performed using Zeiss LSM Image Browser software.

3. Results

3.1. *Cytotoxicity of the adhesives*

Unpolymerized, both adhesives exhibited cytotoxicity after 1 h and 24 h under the current experimental conditions (Fig. 1A and B). Both adhesives were similarly toxic to the cells after 1 h exposure because 5 mg/ml of each material reduced cell survival to about 20% compared to untreated cell cultures both in the presence or absence of BSO. The EC_{50} values of extracts of uncured SBU-CQ and SBU-TPO calculated from dose-response curves after a 1 h exposure period were not significantly different independent of the presence of BSO (Fig. 1C).

In contrast, cytotoxicity of extracts of both materials increased after a 24 h exposure period. EC_{50} values of extracts of uncured SBU-CQ were reduced about 13-fold from 2.77 mg/ml after a 1 h exposure to 0.204 mg/ml after a 24 h exposure period. Likewise, the EC_{50} values of SBU-TPO extracts decreased about 20-fold from 3.05 mg/ml after a 1 h exposure to 0.153 mg/ml after a 24 h exposure. Thus, EC_{50} values calculated after a 24 h exposure period indicated that unpolymerized SBU-TPO is 25% more toxic than SBU-CQ (Fig. 1D).

Addition of BSO led to slightly increased cytotoxicity of the unpolymerized adhesives at almost all concentrations. There was, however, only on few occasions a statistically significant difference between cell viability in cultures treated without BSO compared to the cell viability observed in the presence of BSO. EC_{50} of extracts of uncured SBU-CQ (0.204 mg/ml) decreased to 0.164 mg/ml in the presence of BSO, and a reduction of the EC_{50} value from 0.153 mg/ml to 0.122 mg/ml for SBU-TPO in the presence of BSO was statistically significant (Fig. 1D). Remarkably, different to the observations with unpolymerized SBU-CQ or SBU-TPO, cytotoxic effects of the resin monomer HEMA (6 and 8 mM) drastically increased in the presence of BSO.

Polymerized adhesives reduced cell viability in a dose-related manner (Fig. 2A and B). As expected, the cytotoxicity of the extracts of both polymerized adhesives differed consistently depending on the amount of culture medium in which the adhesive disks had been immersed. Whereas the undiluted extracts of both adhesives prepared in 150 μl led to a complete reduction of cell viability, extracts of both materials prepared in a volume of 300 μl reduced cell viability almost equally to about sixty percent (Fig. 2A and B). A clear difference, however, was detected between materials when 200 μl extracts were prepared and tested. In this case, original extracts and its 50% dilution of SBU-TPO were significantly more cytotoxic than SBU-CQ. This finding indicated that the cytotoxic dose range was much narrower for SBU-TPO than for SBU-CQ. Consequently, the EC_{50} value for 200 μl extracts of SBU-TPO was significantly lower than for SBU-CQ (Fig. 2C).

The photoinitiators CQ, EDMAB, and TPO reduced cell viability in a dose-dependent manner (Fig. 2D). Concentrations leading to a reduction of cell viability to 50% (EC_{50}) indicated that TPO (EC_{50} = 0.06 mM,) was about 10 times more toxic than CQ (EC_{50} = 0.88 mM) or EDMAB (EC_{50} = 0.68 mM) (Fig. 2D). A mixture of CQ/EDMAB (1:1) was slightly but significantly more cytotoxic than CQ or EDMAB (EC_{50} = 0.50 mM) but still less effective than TPO.

Please cite this article in press as: Van Landuyt KL, et al. Evaluation of cell responses toward adhesives with different photoinitiating systems. Dent Mater (2015), http://dx.doi.org/10.1016/j.dental.2015.04.016

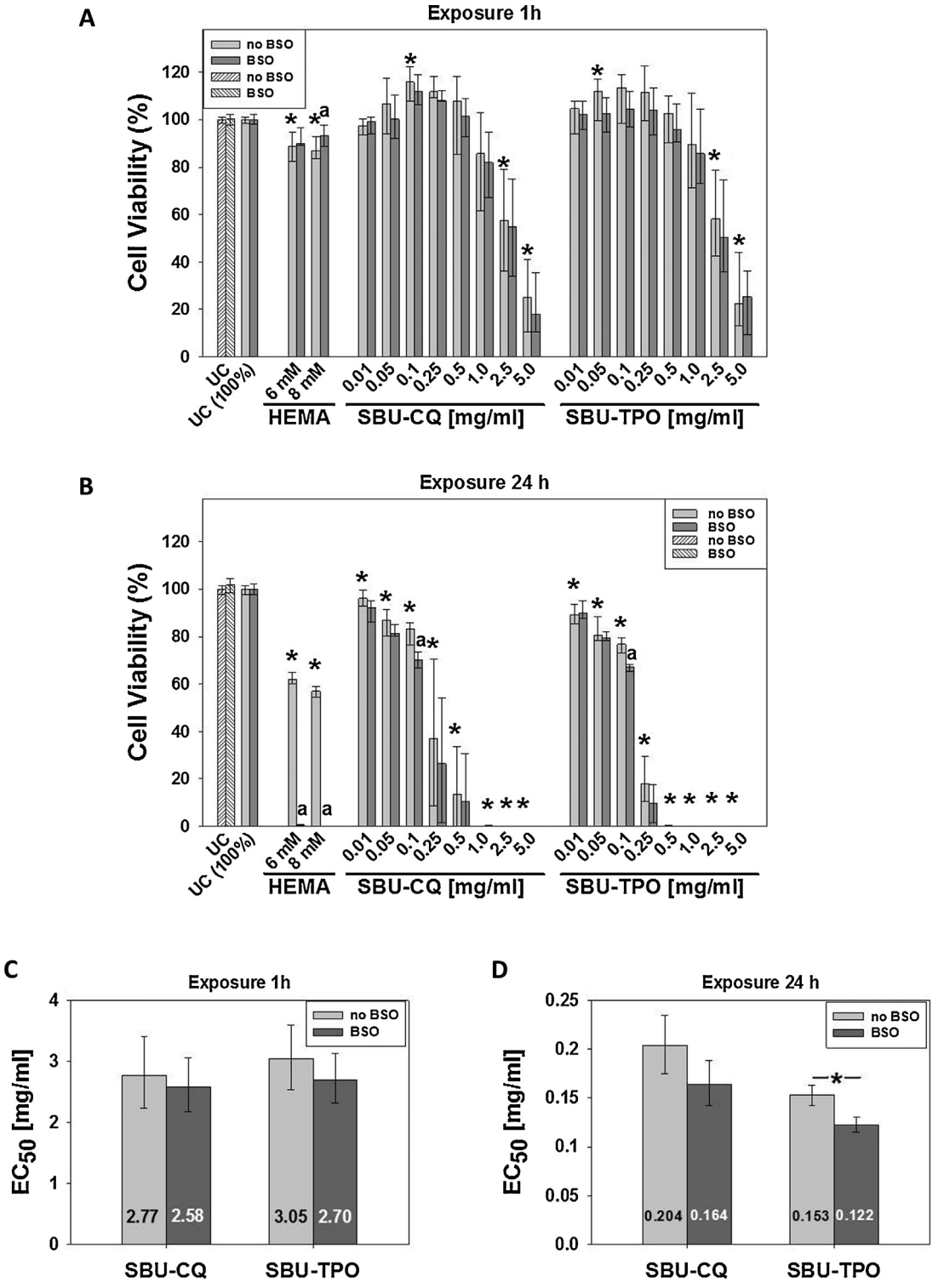

Fig. 1 – Cell viability of human pulp-derived cells (tHPCs) after exposure to unpolymerized adhesives SBU-CQ or SBU-TPO. Cells were exposed for 1 h (A) or 24 h (B) in the presence or absence of BSO (50 μM), and original optical density readings (absorbance at 540 nm) were normalized. Bars represent median values (with 25% and 75% percentiles) calculated from two independent experiments with quadruplicate cultures for each concentration ($n = 8$). Half-maximum-effect concentrations (EC_{50}) after a 1 h (C) or after a 24 h (D) exposure period were calculated from fitted dose-response curves as described in Section 2. UC = untreated control (medium); * significant difference between cell viability found in untreated controls (UC) and cultures treated with HEMA, SBU-CQ or SBU-TPO in the presence or absence of BSO. a = significant difference between cell viability found in cultures treated in the presence or absence of BSO.

Please cite this article in press as: Van Landuyt KL, et al. Evaluation of cell responses toward adhesives with different photoinitiating systems. Dent Mater (2015), http://dx.doi.org/10.1016/j.dental.2015.04.016

Table 3 – Composition of the experimental light-curing glass ionomer.

	Ingredient	Manufacturer	Final wt% after mixing (%)
Powder 1	Glass particles	Schott (GM 35429)	66.3
	Diphenyliodoniumchlorid	Sigma Aldrich (cas 10287-53-3)	2.0
Powder 2	Polyacrylic acid	Sigma Aldrich (cas 9003-01-4)	11.7
Liquid	CQ	Sigma Aldrich (cas 10373-78-1)	0.05
	EDMAB	Merck (cas 10287-53-3)	0.05
	HEMA	Merck (cas 868-77-9)	15.0
	Water		4.9

Abbreviations: CQ: camphorquinone; EDMAB: ethyl 4-(dimethylamino)benzoate; HEMA: 2-hydroxyethyl methacrylate.

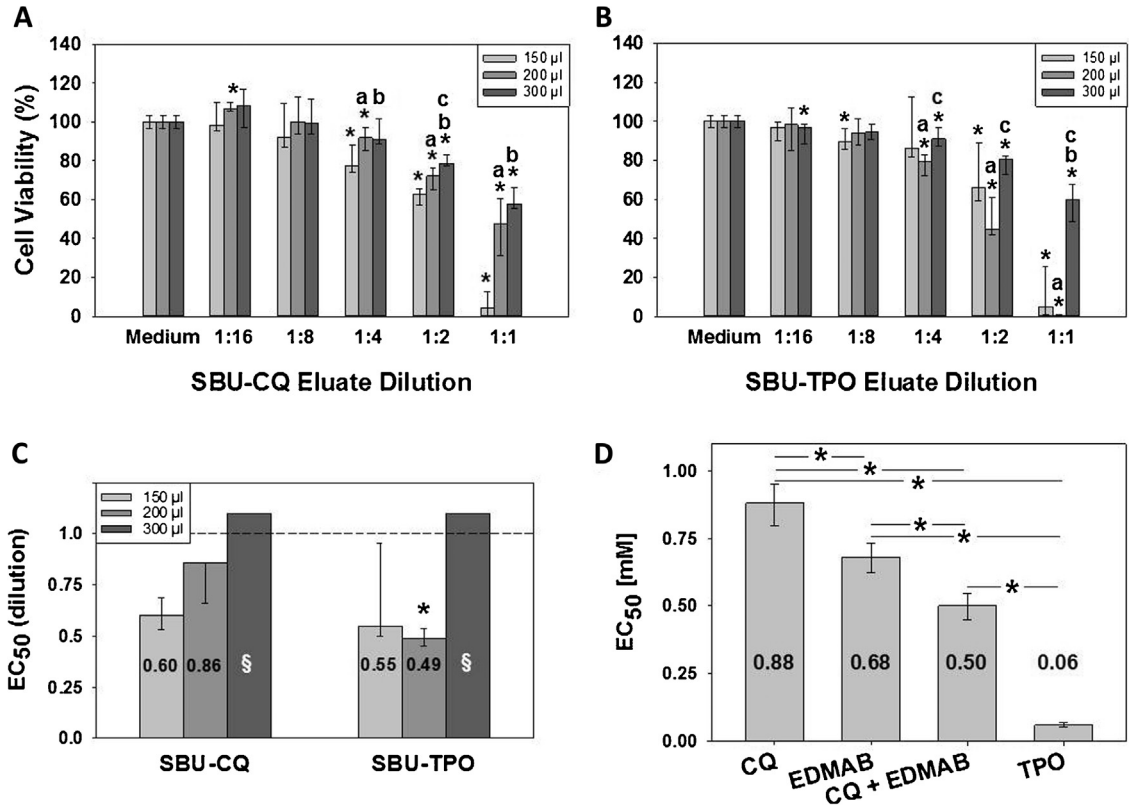

Fig. 2 – Cell viability of human pulp-derived cells (tHPCs) after exposure to the extracts of polymerized adhesives and to the raw photoinitiators. Extracts in 150 µl, 200 µl or 300 µl culture medium were prepared as described in Section 2, and cells were exposed to serially diluted extracts of SBU-CQ (A) or SBU-TPO (B) for 24 h. Original optical density readings (absorbance at 540 nm) were normalized and related to untreated control cell cultures (100%). Bars represent median values (with 25% and 75% percentiles) calculated from three independent experiments with quadruplicate cultures for each concentration ($n = 12$). * = significant difference between cell viability found in untreated controls (medium) and cultures treated with diluted extracts; a = significant difference between cell viability found in untreated controls (medium) and cultures treated with extracts in 150 µl culture medium. b = significant difference between cell viability found in untreated controls (medium) and cultures treated with extracts in 200 µl culture medium. c = significant difference between cell viability found in untreated controls (medium) and cultures treated with extracts in 300 µm culture medium. (C) Half-maximum-effect concentrations (EC_{50}) of SBU-CQ or SBU-TPO extracts shown as median values (25% and 75% percentiles) were calculated from fitted dose-response curves with individual values presented in (A) and (B). Correlation coefficients of fitted cures in (A) were $r^2 = 0.916$ (150 µl), $r^2 = 0.761$ (200 µl), and $r^2 = 0.731$ (300 µl). Correlation coefficients of fitted curves in (B) were $r^2 = 0.861$ (150 µl), $r^2 = 0.955$ (200 µl), and $r^2 = 0.776$ (300 µl). The dashed line indicates the non-diluted original extracts ($EC_{50} = 1.0$) of SBU-CQ or SBU-TPO. Differences between the median EC_{50} values shown by an asterisk were statistically analyzed using the Tukey interval method as described in Section 2. § = no EC_{50} values were calculated for 300 µl extracts of SBU-CQ or SBU-TPO, because cell viability was higher than 50% with original extracts (1:1 dilution). (D) Half-maximum-effect concentrations (EC_{50}) for the raw photoinitiators were calculated from fitted dose-response curves. Correlations coefficients of fitted cures were $r^2 = 0{,}895$ (A), $r^2 = 0{,}919$ (B), $r^2 = 0{,}885$ (C), $r^2 = 0{,}979$ (D). * Significant differences between EC_{50} values (in mM) found in cell cultures treated with a photoinitiator.

Please cite this article in press as: Van Landuyt KL, et al. Evaluation of cell responses toward adhesives with different photoinitiating systems. Dent Mater (2015), http://dx.doi.org/10.1016/j.dental.2015.04.016

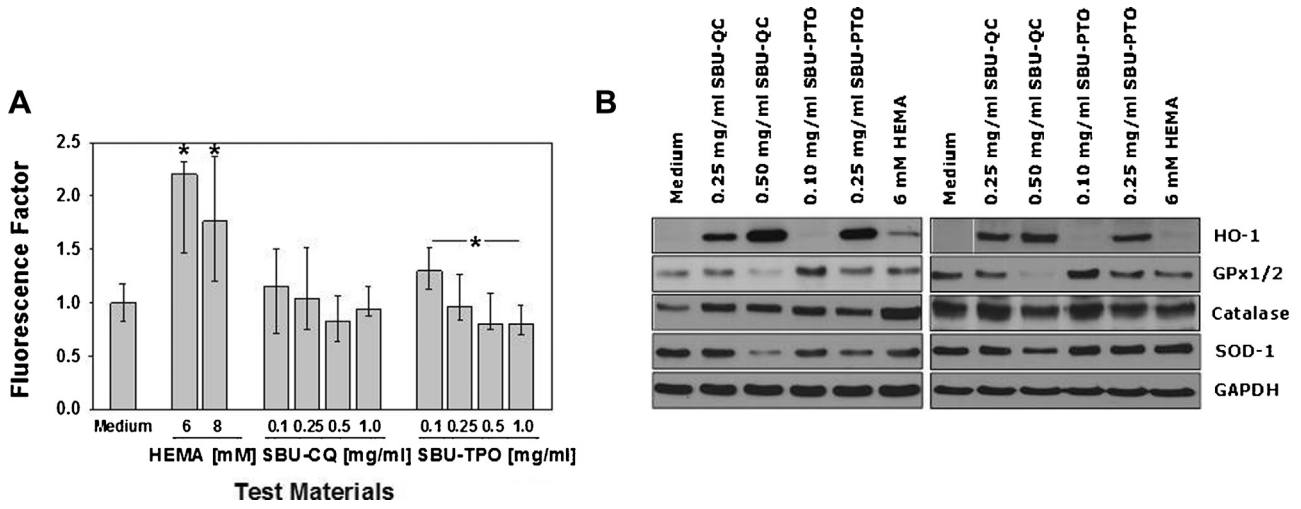

Fig. 3 – Analyses of oxidative stress caused by dentin adhesives. (A) Generation of reactive oxygen species (ROS) indicated by fluorescence factors in tHPC after exposure to dental adhesives was measured using the oxidation-sensitive fluorescent probe 2′7′-dichlorodihydrofluorescin diacetate (H_2DCF-DA) and flow cytometry. The cell cultures were exposed to increasing concentrations of SBU-CQ or SBU-TPO in cell culture medium for 1 h, and HEMA (6 and 8 mM) was used as a control. Mean fluorescence intensities were obtained by histogram statistics, and mean fluorescence intensities were normalized to untreated control cultures (=1.0). Bars represent median fluorescence factors (with 25% and 75% percentiles) calculated from individual histograms ($n = 4$), and asterisks (*) indicate significant differences. (B) Expression of antioxidant enzymes in tHPCs and RAW 264.7 mouse macrophages is shown by Western blotting in a representative experiment. Adaptive changes in the expression of enzymes involved in the cell redox homeostasis could be observed in a dose-dependent manner.

3.2. *ROS generation and expression of antioxidant enzymes*

Whereas the positive control HEMA induced a more than two-fold increase in DCF fluorescence in cell cultures exposed for 1 h compared to untreated cultures, no such significant increase was detected in cultures exposed to extracts of unpolymerized SBU-CQ or SBU-TPO at concentrations tested here (Fig. 3A). No effects of lower concentrations of extracts of both adhesives than shown here were detected as well (not shown). Only the lowest concentration of SBU-TPO tested (0.1 mg/ml), which slightly increased fluorescence, was significantly different from 1 mg/ml SBU-TPO.

Nevertheless, Western blot analyses of extract concentrations of SBU-TPO or SBU-CQ that were effective in cell viability analyses as shown before revealed some changes in the expression of enzymes involved in maintaining a stable intracellular redox state (Fig. 3B). These changes were observed both in tHPC cells as well as in RAW 264.7 mouse macrophages, which are considered the 'golden standard cells' because of previous Western blot analyses to evaluate antioxidant enzymes [18]. First, the expression of glutathione peroxidase (GPx1/2), one of the most important intracellular antioxidant enzymes to decompose H_2O_2, was downregulated in a dose-dependent way. In contrast, the expression of catalase, a parallel enzyme with a similar function was upregulated only in RAW 264.7 mouse macrophages treated with extracts of both adhesives and HEMA as well. A relatively strong expression of catalase in untreated tHPC was not further enhanced in cultures exposed to materials or HEMA (Fig. 3B). Haem oxygenase (HO-1), an enzyme that is involved in the degradation process of haem and that can be induced by oxidative stress, could not be observed in untreated cells, but was detected in cells exposed to the unpolymerized adhesives in a dose-dependent way. Last, the expression of the enzyme superoxide dismutase (SOD-1), an enzyme responsible for destroying superoxide radicals, was downregulated in tHPC and RAW 264.7 mouse macrophages at the highest concentrations of both tested adhesives (Fig. 3B).

3.3. *Dentin barrier test*

Cytotoxicity of both adhesives was also assessed using a model in which a 3D-cell culture is separated from the adhesives by dentin disks, so as to imitate the dentinal pulp and remaining dentin acting as a barrier (Fig. 4). Cell survival determined by MTT was 76% for SBU-CQ and 100% for SBU-TPO, but this was statistically not different from each other or from the negative control material. As expected, the positive control material, a light-cured glass ionomer cement, was most toxic and reduced cell viability to 30% (Fig. 4).

4. Discussion

Dental adhesives are typically complex mixtures of functional and cross-linking monomers dissolved in organic solvent(s) [9]. They also contain low concentrations of additives, such as (photo-)initiators and inhibitors to regulate the polymerization reaction. Previous research revealed that monomers

Please cite this article in press as: Van Landuyt KL, et al. Evaluation of cell responses toward adhesives with different photoinitiating systems. Dent Mater (2015), http://dx.doi.org/10.1016/j.dental.2015.04.016

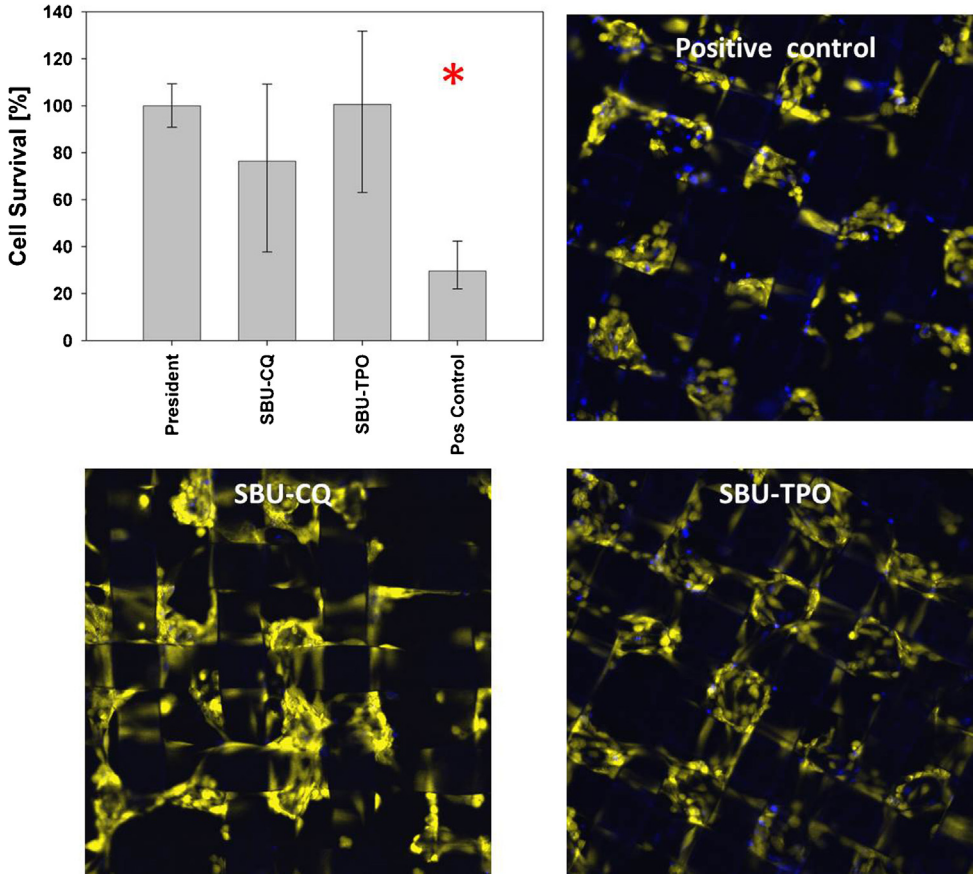

Fig. 4 – Cell viability of the three-dimensional cultures in a dentin barrier test device after exposure to both dental adhesives. Data are expressed as percentage of the negative control, in which the silicone impression material President was applied onto the dentin. The bars represent medians (with 25% and 75% percentiles) ($n = 10$). Only the experimental light-curing glass ionomer cement, which served as positive control, reduced cell viability significantly (*). Representative images of the three-dimensional cell cultures are shown after live/dead staining and confocal analysis. Green cells represent living cells, whereas blue cells indicate dead cells.

are able to interfere with cellular adaptive responses, and that they could severely disturb vital cell functions through the generation of oxidative stress and exhaustion of the cells' innate antioxidant defense mechanisms [18].

It could thus be hypothesized that a good strategy to decrease toxicity of adhesives is to increase their degree of polymerization, which should inevitably lead to a decreased release of uncured monomers. Much research has already been dedicated to the use of alternative photoinitiators in composites and adhesives. One of the most investigated alternatives for the CQ/amine system is the photoinitiator TPO, whose polymerization capacity has mostly been described as very promising and superior to that of CQ/amine [13–17]. However, this study, in which two identical adhesives were used that only differed for their photoinitiating system, showed that the TPO-based adhesive behaved in a more toxic way than did the CQ/amine-based adhesive. The null hypothesis must thus be declined. Not only the unpolymerized TPO-based adhesive, but also extracts of the cured adhesive in medium were found to be more cytotoxic under specific experimental conditions than its CQ/amine-based counterpart after a 24 h exposure period. This last finding is rather surprising at first sight, since the TPO-based adhesive was observed to release lower concentrations of HEMA and BisGMA, [16]. A plausible explanation for the higher cytotoxicity of uncured SBU-TPO in particular compared to SBU-CQ may be the fact that the photoiniator TPO itself apparently interacts more efficiently with the cells than the CQ system. Indeed, the EC_{50} of TPO was more than 10 times lower than that of CQ, which indicates that TPO is 10 times more cytotoxic than CQ. Also the amine EDMAB was much less cytotoxic than TPO, and TPO was still almost 10 times more effective than a mixture of CQ/EDMAB. Thus, it appears unlikely that the effect of a CQ/EDMAB mixture in a complete material might account for the moderate differences in cytotoxicity observed with uncured complete adhesives. In spite of their low proportion in the composition of adhesives, additives such as photoinitiators are usually not bound to the resin matrix and may easily leach from the polymer [29–31]. Previous research already revealed that photoinitiators such as camphorquinone are cytotoxic at high concentrations, which was attributed to their reactive and radical-inducing nature [29,31,32]. Compared to the roughly tenfold differences observed with TPO and CQ alone or in combination with EDMAB (CQ/EDMAB), extracts of the unpolymerized SBU-TPO

Please cite this article in press as: Van Landuyt KL, et al. Evaluation of cell responses toward adhesives with different photoinitiating systems. Dent Mater (2015), http://dx.doi.org/10.1016/j.dental.2015.04.016

were only about 1.3-fold more effective than SBU-CQ based on EC_{50} values calculated from dose-response curves after a 24 h exposure period. These observations suggest that TPO could contribute to the higher cytotoxic potential of unpolymerized SBU-TPO compared to SBU-CQ by overcompensating a considerable lower amount of monomers released from TPO-based resins [17].

Beside resin monomers, camphorquinone has been shown to rapidly activate the oxidative stress pathway, even without irradiation [29,33]. We therefore also assessed the extent of oxidative stress in the cytotoxic response of the tHPC cells. Oxidative stress indeed seemed to play a role in the toxic response of the cells to the adhesives, but probably to a minor extent. First, addition of BSO, an inhibitor of the glutathione synthesis, consistently decreased viability of the cells exposed to the adhesives as detected by the MTT assay. BSO leads to depletion of glutathione, which is the most important enzymatic antioxidant that protects the cells from oxidative damage [18]. Addition of BSO may thus help to reveal the extent of oxidative stress. The concentration of BSO used here was efficient because cytotoxicity of HEMA, which was used as a positive control, drastically increased in the presence of BSO after a 24 h exposure as observed before [18]. In contrast, the decrease in viability caused by extracts of the adhesives in cells preincubated with BSO was only moderate. Second, the evaluation of the expression of enzymatic antioxidants indicated that the cells exposed to effective concentrations of extracts of unpolymerized SBU-TPO or SBU-CQ are subject to oxidative stress. Consistent with a previous study on cell responses after exposure to the monomer HEMA [18], the expression of GPx1/2 and SOD-1 was downregulated while catalase expression increased in RAW 264.7 mouse macrophages and catalase and HO-1 expression was upregulated in a dose-dependent manner in both cell lines used here. This pattern of enzyme expression would suggest that both adhesives tested here induce adaptive cell responses discussed in a hypothetic model of mechanisms caused by HEMA [18]. Cells exposed to adhesives would be depleted of glutathione which leads to the downregulation of GPx1/2 expression. Consequently, increased H_2O_2 formation would enhance the expression of catalase and inhibit SOD-1 expression by a feedback mechanism. Finally, oxidative stress also increases the expression of the stress-inducible haem oxygenase (HO-1) and results in the generation of the antioxidant bilirubin [18]. Since our tested adhesives were mixtures of many different ingredients, it is difficult to pinpoint which component(s) triggered these adaptive changes in the cells, and it is very well possible they were induced by monomers such as HEMA [18]. Notably, oxidative stress was not detected in cultures exposed to extracts of both adhesives using DCF fluorescence. Despite different exposure periods, this seemingly discrepancy might point to the fact that expression of enzymatic antioxidants is a very sensitive adaptive response to the perturbation of the intracellular redox balance which is crucial for vital cell functions. Such small changes are obviously below the detection limit of the assay using DCF fluorescence. In this respect the findings presented are in accordance with an earlier investigation which showed the induction of oxidative stress by dental adhesives as well [24].

While evaluating cytotoxicity of the extracts of the polymerized disks of adhesives, it became clear that the amount of solvent in which the adhesives had been immersed played an important role in cytotoxicity testing. When the samples were immersed in 150 μl of medium, which was the minimal volume of medium necessary to fully wet the samples, both adhesives were equally cytotoxic, with a similar EC_{50} of 60 and 55% dilution for SBU-CQ and SBU-TPO, respectively. When the samples were immersed in 200 μl of medium, SBU-TPO was significantly more toxic than SBU-CQ, with an EC_{50} of 49 versus 86% dilution, respectively. When the samples were placed in 300 μl, the resulting original extracts (1:1) were so diluted that they only reduced viability by 40%. The low EC_{50} values of the 150 μl-extracts must be explained by the fact that these solutions were so concentrated that they induced a maximum cytotoxic response for both adhesives. The 200 μl extracts on the other hand showed a differentiated toxic response between the two adhesives. Whereas SBU-TPO still induced maximal cytotoxicity, the extracts of SBU-CQ were less cytotoxic. This finding shows that the cytotoxic dose-range for extracts SBU-TPO is narrower than that for SBU-CQ which gradually decreased cell viability. In other words, the amount of solvent to prepare extracts for cytotoxicity testing of dental biomaterials plays a very important role, and using too concentrated solutions might lead to different conclusions when a dose-dependent effect is not shown. These findings might be better understood while considering that a cytotoxic response toward a substance is not linear with the dose, but can best be described by a sigmoidal curve (typical 'dose-response curve'). The guidelines issued by ISO 10993 on the 'biological evaluation of medical devices' (part 12) indeed also recommend to use a surface/volume extraction ratio of 3 cm^2/ml for samples with a thickness of 0.5–1.0 mm [34]. This would correspond to 300 μl for the samples in this study, which only elicited a moderate cytotoxic reaction.

A plausible explanation for the observed higher toxicity of the extracts of SBU-TPO is the release of redundant TPO molecules in excess. As suggested as a cause of the higher cytotoxic effects of unpolymerized materials, a lower amount of monomers released from TPO-based resins could be overcompensated by the release of unbound TPO. In this regard, it is important to mention that the concentration of TPO in the experimental adhesive was probably not optimized with regard to obtaining the maximum degree of conversion, and that a relatively high concentration of 2% TPO was used in SBU-TPO. The rationale for this was that the concentration of TPO was the same as that of CQ in the commercial adhesive, Scotchbond Universal (SBU-CQ), which allowed comparing between the two adhesives. Previous research, however, showed that in TEGDMA/BisGMA systems, much lower concentrations (<1 wt%) already resulted in a maximal polymerization degree [15,17,35–37], and that the degree of conversion does not increase with higher concentrations [38]. Leaching of redundant TPO molecules may thus be responsible for the higher observed toxicity seen in the extracts of SBU-TPO. It will thus be important for future research to also test the toxicity of adhesives in which the amount of TPO has been optimized.

We also tested cytotoxicity of both adhesives in a so-called 'dentin barrier test' set-up to simulate major parameters

Please cite this article in press as: Van Landuyt KL, et al. Evaluation of cell responses toward adhesives with different photoinitiating systems. Dent Mater (2015), http://dx.doi.org/10.1016/j.dental.2015.04.016

relevant for a clinical situation. Not only the three-dimensional tHPC cultures, but in particular the dentin disk that acts as a physical barrier are important for the clinical performance of adhesives. With a thickness of 200 μm, a clinical situation with a relatively deep cavity near to the pulp was simulated [5]. It was previously shown that the thickness plays an important role in mitigating the toxic effect of dental adhesives [6] and it was hypothesized that the barrier function of dentin may be ascribed to the buffering of acidic compounds by hydroxyapatite in dentin, and by the binding of noxious compounds to dentin [5]. In this study, in spite of the thin dentin disks, both adhesives proved to be very little to not toxic in the DBT, which indicated that the dentin may protect pulp tissues from immediate damage. The light-curing glass ionomer on the other hand reduced viability to 40%. These findings correspond very well to previous observations with a variety of dentin adhesives [24].

To conclude, not only the degree of conversion and the release of monomers determine the biocompatibility of adhesives, but also the toxicity of the (photo-)initiator should be taken into account, as these reactive compounds are not bound to the matrix and may leach from adhesives. Even though TPO was more reactive and increased the polymerization degree, this photoinitiator may also render a universal adhesive more cytotoxic compared to an adhesive with the conventional CQ/amine photoinitiator. Future research should focus on the toxicity of TPO-adhesives with lower concentrations of TPO, optimized for a maximum degree of conversion. Although generation of ROS was not directly detected with unpolymerized adhesives, cytotoxicity was to some extent associated with the depletion of glutathione and oxidative stress as indicated by the expression of enzymatic antioxidants. However, the toxic effects of both the CQ/amine and TPO adhesive could be annulled by a thin dentin barrier and the use of a three-dimensional cell culture.

Acknowledgments

This research was supported by the Research Foundation Flanders (FWO) travel stipend LK2.014.13N. We would like to thank 3 M ESPE for providing the commercial and experimental adhesive.

REFERENCES

[1] Ilie N, Obermaier J, Durner J. Effect of modulated irradiation time on the degree of conversion and the amount of elutable substances from nano-hybrid resin-based composites. Clin Oral Invest 2014;18:97–106.
[2] Accorinte Mde L, Loguercio AD, Reis A, Muench A, de Araujo VC. Adverse effects of human pulps after direct pulp capping with the different components from a total-etch, three-step adhesive system. Dent Mater 2005;21:599–607.
[3] Accorinte ML, Loguercio AD, Reis A, Costa CA. Response of human pulps capped with different self-etch adhesive systems. Clin Oral Invest 2008;12:119–27.
[4] Krifka S, Spagnuolo G, Schmalz G, Schweikl H. A review of adaptive mechanisms in cell responses towards oxidative stress caused by dental resin monomers. Biomaterials 2013;34:4555–63.
[5] Galler K, Hiller KA, Ettl T, Schmalz G. Selective influence of dentin thickness upon cytotoxicity of dentin contacting materials. J Endod 2005;31:396–9.
[6] Hebling J, Giro EM, Costa CA. Human pulp response after an adhesive system application in deep cavities. J Dent 1999;27:557–64.
[7] Bouillaguet S, Wataha JC, Hanks CT, Ciucchi B, Holz J. In vitro cytotoxicity and dentin permeability of HEMA. J Endod 1996;22:244–8.
[8] Durner J, Obermaier J, Draenert M, Ilie N. Correlation of the degree of conversion with the amount of elutable substances in nano-hybrid dental composites. Dent Mater 2012;28:1146–53.
[9] Van Landuyt KL, Snauwaert J, De Munck J, Peumans M, Yoshida Y, Poitevin A, et al. Systematic review of the chemical composition of contemporary dental adhesives. Biomaterials 2007;28:3757–85.
[10] Miletic V, Pongprueksa P, De Munck J, Brooks NR, Van Meerbeek B. Monomer-to-polymer conversion and micro-tensile bond strength to dentine of experimental and commercial adhesives containing diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide or a camphorquinone/amine photo-initiator system. J Dent 2013;41:918–26.
[11] Cadenaro M, Antoniolli F, Codan B, Agee K, Tay FR, Dorigo Ede S, et al. Influence of different initiators on the degree of conversion of experimental adhesive blends in relation to their hydrophilicity and solvent content. Dent Mater 2010;26:288–94.
[12] Ilie N, Hickel R. Can CQ be completely replaced by alternative initiators in dental adhesives? Dent Mater J 2008;27:221–8.
[13] Miletic V, Santini A. Micro-Raman spectroscopic analysis of the degree of conversion of composite resins containing different initiators cured by polywave or monowave LED units. J Dent 2012;40:106–13.
[14] Arikawa H, Takahashi H, Kanie T, Ban S. Effect of various visible light photoinitiators on the polymerization and color of light-activated resins. Dent Mater J 2009;28:454–60.
[15] Schneider LF, Cavalcante LM, Prahl SA, Pfeifer CS, Ferracane JL. Curing efficiency of dental resin composites formulated with camphorquinone or trimethylbenzoyl-diphenyl-phosphine oxide. Dent Mater 2012;28:392–7.
[16] Pongprueksa P, Miletic V, Janssens H, Van Landuyt KL, De Munck J, Godderis L, et al. Degree of conversion and monomer elution of CQ/amine and TPO adhesives. Dent Mater 2014;30:695–701.
[17] Randolph LD, Palin WM, Bebelman S, Devaux J, Gallez B, Leloup G, et al. Ultra-fast light-curing resin composite with increased conversion and reduced monomer elution. Dent Mater 2014;30:594–604.
[18] Krifka S, Hiller KA, Spagnuolo G, Jewett A, Schmalz G, Schweikl H. The influence of glutathione on redox regulation by antioxidant proteins and apoptosis in macrophages exposed to 2-hydroxyethyl methacrylate (HEMA). Biomaterials 2012;33:5177–86.
[19] Schweikl H, Petzel C, Bolay C, Hiller KA, Buchalla W, Krifka S. 2-Hydroxyethyl methacrylate-induced apoptosis through the ATM- and p53-dependent intrinsic mitochondrial pathway. Biomaterials 2014;35:2890–904.
[20] International Organization for Standardization (ISO). 10993-5, biological evaluation of medical devices—Part 5: Tests for in vitro cytotoxicity. International Organization for Standardization (ISO); 2009.
[21] International Organization for Standardization (ISO). 10993-12, biological evaluation of medical devices—Part 12: Sample preparation and reference materials. International Organization for Standardization (ISO); 2012.

Please cite this article in press as: Van Landuyt KL, et al. Evaluation of cell responses toward adhesives with different photoinitiating systems. Dent Mater (2015), http://dx.doi.org/10.1016/j.dental.2015.04.016

[22] Galler KM, Schweikl H, Thonemann B, D'Souza RN, Schmalz G. Human pulp-derived cells immortalized with Simian Virus 40 T-antigen. Eur J Oral Sci 2006;114:138–46.
[23] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
[24] Demirci M, Hiller KA, Bosl C, Galler K, Schmalz G, Schweikl H. The induction of oxidative stress, cytotoxicity, and genotoxicity by dental adhesives. Dent Mater 2008;24: 362–71.
[25] Schweikl H, Hiller KA, Eckhardt A, Bolay C, Spagnuolo G, Stempfl T, et al. Differential gene expression involved in oxidative stress response caused by triethylene glycol dimethacrylate. Biomaterials 2008;29:1377–87.
[26] Krifka S, Seidenader C, Hiller KA, Schmalz G, Schweikl H. Oxidative stress and cytotoxicity generated by dental composites in human pulp cells. Clin Oral Invest 2012;16:215–24.
[27] Schuster U, Schmalz G, Thonemann B, Mendel N, Metzl C. Cytotoxicity testing with three-dimensional cultures of transfected pulp-derived cells. J Endod 2001;27:259–65.
[28] International Organization for Standardization (ISO). 7405: 2008/Amendment 1. International Organization for Standardization (ISO); 2013.
[29] Volk J, Ziemann C, Leyhausen G, Geurtsen W. Non-irradiated campherquinone induces DNA damage in human gingival fibroblasts. Dent Mater 2009;25:1556–63.
[30] Van Landuyt KL, Nawrot T, Geebelen B, De Munck J, Snauwaert J, Yoshihara K, et al. How much do resin-based dental materials release? A meta-analytical approach. Dent Mater 2011;27:723–47.
[31] Atsumi T, Iwakura I, Fujisawa S, Ueha T. The production of reactive oxygen species by irradiated camphorquinone-related photosensitizers and their effect on cytotoxicity. Arch Oral Biol 2001;46:391–401.
[32] Atsumi T, Ishihara M, Kadoma Y, Tonosaki K, Fujisawa S. Comparative radical production and cytotoxicity induced by camphorquinone and 9-fluorenone against human pulp fibroblasts. J Oral Rehabil 2004;31:1155–64.
[33] Volk J, Leyhausen G, Wessels M, Geurtsen W. Reduced glutathione prevents camphorquinone-induced apoptosis in human oral keratinocytes. Dent Mater 2014;30:215–26.
[34] International Organization for Standardization (ISO) 10993, Biological evaluation of medical devices. Part 12: Sample preparation and reference materials. 2012 ISO 10993–12.
[35] Randolph LD, Palin WM, Watts DC, Genet M, Devaux J, Leloup G, et al. The effect of ultra-fast photopolymerisation of experimental composites on shrinkage stress, network formation and pulpal temperature rise. Dent Mater 2014;30:1280–9.
[36] Albuquerque PP, Moreira AD, Moraes RR, Cavalcante LM, Schneider LF. Color stability, conversion, water sorption and solubility of dental composites formulated with different photoinitiator systems. J Dent 2013;41(Suppl. 3):e67–72.
[37] Leprince JG, Hadis M, Shortall AC, Ferracane JL, Devaux J, Leloup G, et al. Photoinitiator type and applicability of exposure reciprocity law in filled and unfilled photoactive resins. Dent Mater 2011;27:157–64.
[38] Miletic V, Santini A. Optimizing the concentration of 2,4,6-trimethylbenzoyldiphenylphosphine oxide initiator in composite resins in relation to monomer conversion. Dent Mater J 2012;31:717–23.

Please cite this article in press as: Van Landuyt KL, et al. Evaluation of cell responses toward adhesives with different photoinitiating systems. Dent Mater (2015), http://dx.doi.org/10.1016/j.dental.2015.04.016