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Original Article

Depth of cure, flexural strength, microhardness, and cytotoxicity of light activated pit and fissure resin-based sealant experimental prototypes

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Abstract

Objective To evaluate the light-activated pit and fissure resin-based sealant prototypes (LAS-clear and LAS-opaque) for depth of cure, flexural strength, microhardness, and cytotoxicity compared with commercial light-activated pit and fissure resin-based sealants.

Material and methods LAS-clear, LAS-opaque, Delton-clear (DC), Helioseal clear (HC), Helioseal opaque (HO), and Clinpro (CL) were investigated for depth of cure per ISO 6874:2005. Their flexural strength and microhardness were also evaluated. Cytotoxicity was determined using an MTT based colorimetric assay. Statistical analysis was performed using the SPSS program.

Results All materials met ISO 6874:2005 requirements. The mean depth of cure of DC, HC, and LAS-clear were significantly higher than that of HO, CL, and LAS-opaque ($p<0.05$). The flexural strength and microhardness values were not significantly different between each group. The MTT assay showed that CL conditioned media significantly reduced cell viability at 24 and 48 hours compared with the control and other sealants ($p<0.05$). LAS-clear, LAS-opaque, DC, HC, and HO conditioned media slightly decreased cell viability.

Conclusions LAS-clear and LAS-opaque met the ISO 6874:2005 requirements for depth of cure. LAS-clear and LAS-opaque conditioned medium were biocompatible with gingival fibroblasts.

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Key words: *Cytotoxicity; depth of cure; flexural strength; microhardness; resin-based dental sealant*

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Introduction

In 2009, Thailand imported dental materials and related instruments valued at approximately 50 million US dollar (NIA, 2012). Due to an increased lifespan, high quality of life, increased access to dental care, and financial coverage by the Health Security System and Society Security System, this expense has been rapidly rising and will be a burden on the budgets of the government, families, and individuals. The development of low cost basic dental materials is an important strategy in solving this problem and enhancing the quality of people's oral health from children to the elderly.

Light activated pit and fissure resin-based sealants are commonly used to seal caries susceptible pits and fissures in teeth, forming a physical barrier to prevent caries development (Beauchamp et al., 2009). This type of sealant is composed of a liquid, which is a mixture of light-and chemical-sensitive dimethacrylate monomers with optional pigmentation. Light-activated polymerization gives these sealants more favorable clinical properties, such as a longer working time and a shorter setting time compared with autopolymerized

Table 1 *Components of the experimental pit and fissure dental sealants.*

Material	Composition	Recommend light activating duration (sec)	Manufacturer
Delton®-clear (Lot no. 140218)	Aromatic and aliphatic dimethacrylate monomers; Light activators	20	Dentsply Professional USA
Helioseal® clear (Lot no. S03682)	Bis-GMA and TEGDMA (99%); Photoinitiator	20	Ivoclar Vivadent Liechtenstein
Helioseal® opaque (Lot no. S39123)	Bis-GMA and TEGDMA (97%); Titanium dioxide Photoinitiator	20	Ivoclar Vivadent Liechtenstein
Clinpro™ Sealant (Lot no. N478759)	Bis-GMA and TEGDMA Titanium dioxide; Coloring agent Photoinitiator	20	3M ESPE, USA
LAS-clear	Bis-GMA and TEGDMA (99%); Photoinitiator	20	Research Unit of Herbal Medicine, Biomaterial, and Material for Dental Treatment, Faculty of Dentistry Chulalongkorn University
LAS-opaque	Bis-GMA and TEGDMA (97%); Titanium dioxide Photoinitiator	20	Research Unit of Herbal Medicine, Biomaterial, and Material for Dental Treatment, Faculty of Dentistry Chulalongkorn University

resin-based sealants.

Our research group has developed the light activated resin-based sealant prototype I (LAS-clear) and light activated resin-based sealant prototype II (LAS-opaque) as clear and opaque sealants, respectively. The cost of these materials is much lower than that of similar imported products. However, the basic properties of these new materials need to be investigated prior to their use in animals or patients. Thus, in this study, the depth of cure, microhardness, flexural strength, and cytotoxicity of LAS-clear and LAS-opaque were evaluated. The results for LAS-clear and LAS-opaque were compared with those of four commercial resin-based sealants.

Materials and methods

Four commercial light activated resin-based sealants, Delton®-clear (DC; Dentsply), Helioseal® clear (HC; Ivoclar Vivadent), Helioseal® opaque (HO; Vivadent), and Clinpro™ Sealant (CL; 3M™ ESPE™), were used as reference materials in this study. The expiration date of the commercial sealants was more than 6 months after the completion of the study. The components of DC, HC, HO, CL, LAS-clear, and LAS-opaque are shown in Table 1. A light activator (Halogen Curing Light, Elipar™ 2500, 3M ESPE, USA) was used to activate the materials at an intensity of 700 mW/cm^2.

Depth of cure

Each dental sealant (N=10) was loaded into a customized circular stainless steel mold (6 mm high and 4 mm in diameter) placed on a glass slide. The sealant was covered with a polyester film and a second glass slide was placed on top of the mold under gentle pressure to displace excess material. The sealant was light activated for 20 seconds per the manufacturer's instructions. The specimen was then removed from the mold and the uncured material was immediately and gently removed with a plastic spatula. The height of the cured sealant was determined with a digital caliper (Mitsutoyo Co., Kawasaki, Japan). According to ISO 6874:2005, the minimal depth of cure of a dental sealant is 1.5 mm.

Flexural strength

The flexural strength test was performed as previously described with some modifications (Thunyakitpisal et al., 2011). Each dental sealant (N = 10) was placed into a customized stainless steel mold (24 x 2 x 2 mm^3). A polyester film and a glass slide were placed on both sides of the mold. The dental sealant was cured using three overlapping activating-light exposures of 20 seconds each per the manufacturer's instructions. The specimen was light-cured on the opposite side of the mold in the same fashion. The slides were clamped to the mold and the specimen was placed in a water bath at 37°C for 15 minutes. After removing the specimen from the mold, any flash was removed using sandpaper, and the specimen was immersed in distilled water at 37°C for 24 hours.

Flexural strength was determined using a three-point bending testing device (Universal Testing Machine 8872, Instron, High Wycombe, UK) with a cross-head speed of 50 N/min, a span of 20 mm, and 1000 N load cell. The specimens were loaded until fracture occurred. The flexural strength was calculated using the following formula[4]: $\delta = 3FI/2bh^2$, where δ = flexural strength (MPa), F = maximum load (N), I = span length between the supports (mm), b = specimen width (mm), and h = specimen height (mm).

Microhardness test

Each material was prepared as described above in 12 x 3 x 2 mm^3 stainless steel blocks, and kept at room temperature for 24 h (N = 10). Subsequently, each specimen was mounted in a resin block. The specimen's surface was ground to provide a flat surface using water cooled abrasive discs with grit sizes 400, 800,

and 1,200, followed by 0.05 μm deagglomerated alumina powder polishing paste with a polishing cloth. After polishing, the samples were sonicated for 30 minutes, and washed for 5 minutes with deionized water. Using a microhardness tester (FM-700e, Future-Tech, Japan), each specimen was impressed with a 300-g load for 15 seconds using a Vickers indenter. The distance between indentations was approximately 2 mm and each indentation was at least 1 mm from the edge of the specimen. Each specimen was subjected to four indentations on the top surface, and the average microhardness value was calculated.

Cell culture

The study protocols were approved by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University. Human gingival fibroblasts were explanted from gingiva obtained during the surgical removal of impacted third molars as previously described (Jettanacheawchankit, et al., 2009). Briefly, the gingiva was minced and the pieces were placed on 35-mm culture plates. The outgrown cells were cultured in complete media (Dulbecco's Modified Eagle Medium supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin, 2 mM L-glutamine, and 10% fetal bovine serum). The medium was changed every two days. The cells were cultured at 37°C in a humidified 5% CO_2 atmosphere. When the cells reached confluence, the cells were subcultured using 0.25% trypsin-EDTA. Cells from three donors were used in this study. All cell culture media were purchased from GibcoBRLTM (Invitrogen™, Grand Island, NY, USA).

MTT cytotoxicity assay

Four specimens of each material were prepared according to the manufacturer's instructions in an autoclaved 2 x 2 x 2 mm^3 mold. The sealant was light-activated as described in the flexural strength test. After sterilization by UV exposure for 30 minutes on each side, each specimen was immersed in 1 mL of growth media at 37°C with gentle agitation for 24 h. For the control group, the same volume of growth media was incubated under the same conditions. The conditioned media were passed through 0.2 μm sterile filters (Acrodisc® Syringe Filters with Supor® Membrane, Pall Corporation, USA).

The MTT test was performed as previously described with some modifications (Zhang et al., 2013). Briefly, 12,000 cells/well were seeded into a 48 well culture plate (Nunc™ cell culture plate, Thermo Scientific, USA). After 24 h, the cells were washed twice with phosphate buffered saline (PBS) and then incubated with the conditioned medium of each sealant type for 24 or 48 h. Cells incubated with growth medium were used as the control group. Subsequently, the cells were washed twice with PBS and incubated with 0.5 mg/mL of 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) solution for 10 min. The precipitated formazan crystals were dissolved in dimethyl sulfoxide and the optical density was determined by measuring the light absorbance at 570 nm. The assay was carried out in three independent experiments.

Statistical analysis

The data were collected and presented as mean ± SD for depth of cure, flexural strength assays, microhardness test, and the number of viable cells. The data was analyzed by one-way analysis of variance using the SPSS program for Windows, version 17 (SPSS, Inc., Chicago, IL, USA). The Scheffé multiple comparison test was used for *post-hoc* analysis. Significance was assumed at $p < 0.05$.

Results

Depth of cure, flexural strength, and microhardness of the sealants

The results of the depth of cure, flexural strength, and microhardness assays of the dental sealants are

presented in Table 2. The depth of cure assay showed that the DC, HC, LAS-clear, HO, CL, and LAS-opaque groups had a mean depth of cure of 5.88 ± 0.11, 5.88 ± 0.08, 5.75 ± 0.08, 3.35 ± 0.15, 3.58 ± 0.11, and 3.68 ± 0.15 mm, respectively. Our data showed that the mean depth of cure of DC, HC, and LAS-clear were significantly higher than that of HO, CL, and LAS-opaque ($p < 0.05$). The DC and HC groups had the highest mean curing depth of the dental sealants. However, there was no significant difference between DC, HC, and LAS-clear, and between HO, CL, and LAS-opaque ($p > 0.05$). According to ISO 6874:2005, a light activated dental sealant should have a depth of cure greater than or equal to 1.5 mm. Therefore, LAS-clear and LAS-opaque surpassed the minimum depth of cure requirement.

In addition, there was no significant difference in the mean flexural strength and the mean microhardness of all light activated dental sealant materials ($p > 0.05$) (Table 2).

Cytotoxicity of the sealants to gingival fibroblasts

After 24 h culture in conditioned media of each material, the mean number of viable cells in the control, DC, HC, HO, CL, LAS-clear, and LAS-opaque groups were 15,320 ± 1,231, 15,305 ± 1,531, 14,824 ± 1,219, 14,729 ± 516, 9,557 ± 1,009, 14,679 ± 745, and 14,532 ± 1003, respectively (Fig. 1). The CL group demonstrated significantly reduced cell viability compared with the growth media-treated control, DC, HC, HO, LAS-clear, and LAS-opaque groups ($p < 0.05$). There was no significant difference in cell viability between the control, DC, HC, HO, LAS-clear, and LAS-opaque groups ($p > 0.05$).

After 48 h incubation, the mean number of viable cells in the growth media-treated control, DC, HC, HO, CL, LAS-clear, and LAS-opaque groups were 28,383 ± 2,740, 28,070 ± 2,542, 27,684 ± 1,848, 26,367 ± 1,709, 20,029 ± 974, 27,397 ± 1,504, and 26,867 ± 2,122, respectively (Fig. 1). The CL group showed significantly decreased cell viability compared with that

Table 2 Depth of cure, flexural strength, and microhardness of Delton®-Clear (DC), Helioseal®-Clear (HC), Helioseal®-opaque (HO), Clinpro™Sealant (CL), LAS-clear, and LAS-opaque. The data is expressed as Mean ± SD.

Sealants	Depth of cure (mm)	Flexural strength* (MPa)	Microhardness* (Vicker hardness number)
DC	5.88 ± 0.11[a]	81.51 ± 3.86	19.81 ± 1.01
HC	5.88 ± 0.08[a]	79.78 ± 2.66	19.92 ± 0.67
LAS-clear	5.75 ± 0.08[a]	79.35 ± 3.40	20.19 ± 0.98
HO	3.35 ± 0.15[b]	81.45 ± 2.47	20.51 ± 1.15
CL	3.58 ± 0.11[b]	79.69 ± 6.98	19.90 ± 0.55
LAS-opaque	3.69 ± 0.14[b]	80.21 ± 3.30	19.94 ± 0.93

*indicates each column was no significant difference between the groups (N = 10).

The same superscript letter in each column indicates no significant difference between the groups (N = 10).

of the growth media-treated control, DC, HC, HO, LAS-clear, and LAS-opaque groups ($p < 0.05$). There was no significant difference in cell viability between the control, DC, HC, HO, LAS-clear, and LAS-opaque groups ($p > 0.05$).

Discussion

Because of their minimally invasive procedures, safety, and ease in manipulation, light activated dental sealants are widely recommended as a powerful tool in the prevention of pit and fissure caries and/or to prevent the development of incipient caries into invasive caries (Simonsen and Neal, 2011). In the present study, DC and HC were used as clear dental sealant references, and HO and CL were used as opaque dental sealant references. These commercial dental sealants were used to assess the physical properties and cytotoxicity of the experimental resin sealants LAS-clear and LAS-opaque.

Depth of cure is how deep into a material's thickness it can be efficiently polymerized after adequate light activation. In the clinic, there is a maximum practical thickness that a material can be used and be completely cured at the base (Darvell, 2006). Our data revealed that the opaque light activated sealants (HO, CL, and LAS-opaque) had a significantly lower depth of cure compared with the clear light activated sealants (DC, HC, and LAS-clear). These results corresponded to those of Yue et al., 2009 and Borges et al., 2011. Due to its high refractive index and semiconductive property, titanium dioxide, used as a pigment in sealants, reduces or blocks light penetration by scattering and absorption (Yang et al., 2004; Gupta et al, 2002). This may account for the lower depth of cure we observed for the opaque materials in our study. Based on our data, LAS-clear and LAS-opaque both met the ISO depth of cure requirements of a light activated dental sealant.

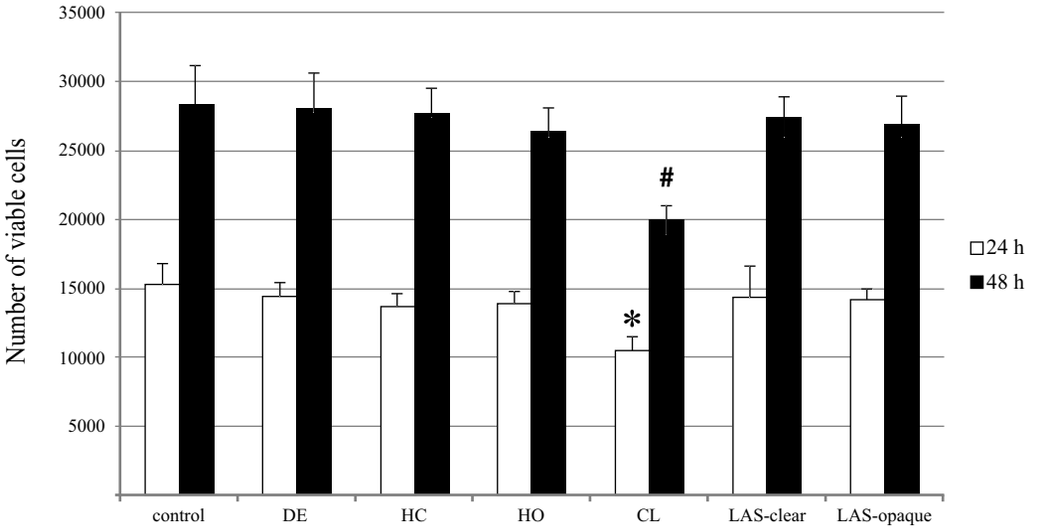

Figure 1 Cytotoxicity of gingival fibroblasts after incubating in conditioned medium from each material (Delton® -Clear, Helioseal®-Clear, Helioseal®-opaque, Clinpro™ Sealant, LAS-clear, or LAS-opaque) for 24 hours and 48 hours. Cells incubated with growth medium were used as control. The data were obtained from at least three independent experiments and shown as mean ± SD. *indicates a significant difference compared to the control group at 24 h ($p < 0.05$). #indicates a significant difference compared to the control group at 48 hours ($p < 0.05$), N = 4.

Although the International Standards Organization 6874:2005 does not require a three-point loading test and microhardness examination to evaluate the strength of a dental sealant, in the present study we evaluated these parameters. Clinically, a material is subjected to a considerable amount of flexural stress and complex forces during mastication. Therefore, flexural strength is considered an important mechanical characteristic for brittle resin polymer-based materials that are much weaker under tension than in compression (Dell Bona et al., 2003; Rodrigues et al., 2008). However, the use of a sealant at its practical thickness presents technical difficulties in evaluating its physical strength. The flexural strength assay employs bar-shaped specimens that are subjected to compressive loading in the midpoint between two lower supports, promoting tensile stress in the lower surface that is associated with fracture initiation. Our findings indicated that LAS-clear and LAS-opaque had flexural strength in between those of the commercial dental sealants.

The Vickers test is a technique for determining the microhardness of resin-based materials, enamel, and dentin (Toledano et al., 2005; Chuenarrom et al., 2009; Poggio et al., 2012; Kim et al., 2002). A square-based pyramid at the tip of a diamond indenter is impressed on the polished surface of the material. The load is divided by the square area of the indentation depression on the surface of the material, and the quotient is referred to as the Vickers hardness number. Therefore, a larger number indicates a harder material. In the present study, there was no significant difference between the clear-and opaque-dental sealants, or within the sealant groups. This finding is corresponded with previous studies of Poggio et al. and Rueggeberg et al. Indeed, our data showed that the dental sealant materials were all close in microhardness value. This suggests that adding titanium dioxide up to 2% does not affect the flexural strength and microhardness of pit and fissure resin polymers.

The gingiva is an oral soft tissue that would be exposed to substances released from a sealant. In the present study, human gingival fibroblasts were used to examine the cytotoxicity of the dental sealants. The indirect contact test was selected to investigate cytotoxicity because, in this assay, the material does not directly contact the gingival cells. According to ISO 19003-5, the MTT assay is an acceptable method to evaluate the cytotoxicity of a dental material. This assay measures the conversion of a yellow water-soluble MTT dye into a purple formazan crystal that is produced by active mitochondria. The MTT test not only evaluates cell-material interaction, but also relates to the number of viable cells. This colorimetric method is recommended as an economic, accurate, and reliable test for the determination of cytotoxicity (Zhang et al., 2013; Freshney, 2005). Our data indicated that CL was cytotoxic to the cells compared with the experimental dental sealants after 24 h of exposure. The red coloring agent in this sealant is an extra component compared with the other sealants. Thus, the increased cytotoxicity of CL likely resulted from this specific component of its formulation leaching into the test medium. In addition, incomplete polymerization of a resin-based material and the leaching of non-polymerized monomers such as TEGDMA, Bis-GMA, and camphoroquinone negatively affect a material's biocompatibility (Geurtsen et al., 1998; Furche et al., 2013; Volk et al., 2009; Yano et al., 2011). The release of these monomers from the resin-based dental materials induce cellular stress, DNA damage, apoptosis, and cytotoxicity.

Proprietary restrictions limit our knowledge of the exact amounts of the components in the commercial dental sealants. Thus, we cannot precisely explain why the depth of cure, flexural strength, microhardness values, and the cytotoxicity of each sealant material were different. The practical explanation is that each dental sealant has a different composition and percentage of each component (Nicholson and Czarnecka, 2008;

Wilson, 1990). Therefore, degree of conversion should be assessed. Identification and quantification of the eluents from CL using high performance liquid chromatography and gas chromatography/mass spectrometry, and evaluation of its cytotoxicity should be also performed in future studies (Furche, et al., 2013; Nicholson and Czarnecka, 2008).

Clinical studies have shown that all commercial resin-based materials are biocompatible with oral tissues (Beauchamp et al., 2009; Nicholson and Czarnecka, 2008; Wells, 2013). Therefore, *in vitro* conditions may not completely simulate the *in vivo* environment. The complex orchestration of the effects of the vascular system, immune system, and inflammation are absent in the *in vitro* environment (Wataha, 2003). Without the buffering, detoxification, and excretion systems present *in vivo,* the accumulation of toxic substances released from the specimens likely occurs. To confirm the cytotoxicity of LAS-clear and LAS-opaque, the biocompatibility of these sealant prototypes should be assessed in an animal model.

It should be noted that the most common failure of sealant is the partial or complete loss from tooth leaving a tooth equally susceptible to caries as an unsealed tooth (Horowitz et al., 1977, Mertz-Fairhurst et al., 1984). The major causes are the insufficient bond strength and retention (Feigal et al., 2000). The assessment of shear bond strength in laboratory and clinical trial of survival should be taken to evaluate the effectiveness of these sealant prototypes.

In conclusion, LAS-clear and LAS-opaque met the ISO 6874:2005 requirements for depth of cure. LAS-clear and LAS-opaque have sufficient flexural strength, and microhardness. All sealants, except CL, are biocompatible with gingival fibroblast at 24 and 48 h exposure.

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