Original article

# Working Time, Depth of Cure, Flexural Strength, and Cytotoxicity of an Experimental Resin Modified Glass Ionomer Cement Prototype

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## Abstract

The objective of this study was to investigate the working time, depth of cure, flexural strength, and cytotoxicity of Research Unit-Herbal Medicine, Biomaterial and Material 1 (RU-HBM1), a resin modified glass ionomer (RMGI) cement prototype, compared with commercial RMGIs. RU-HBM1, GC-Gold Label Light-Cured Universal Restorative (GC), and VitrebondTM (VB) were evaluated for working time, depth of cure, and flexural strength per ISO 9917-2:2010 and ISO 9917-2:1998. Five specimens of each material were incubated in growth medium for 24 h. Primary human dental pulp cells were cultured in a 50 % dilution of the conditioned medium from each specimen, with growth media used as a control. Cytotoxicity was determined using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) based colorimetric assay. One-way analysis of variance and the Scheffe´ multiple comparison test were used to analyze the data. It was found that the three materials met the requirements of ISO 9917-2:2010 and ISO 9917-2:1998 for working time, depth of cure, and flexural strength. GC had the highest depth of cure and flexural strength with VB presenting the lowest values ($p < 0.05$). RU-HBM1 demonstrated a significantly higher depth of cure compared with that of VB, and a significantly lower depth of cure compared with that of GC ($p < 0.05$). RU-HBM1 showed no significant difference in flexural strength compared with those of GC and VB. The MTT assay indicated that VB conditioned media significantly reduced cell viability at 24 and 48 hours ($p < 0.05$). RU-HBM1 and GC conditioned media slightly decreased cell viability at 48 hours. In conclusion, RU-HBM1 met the requirements for working time, depth of cure, and flexural strength per ISO 9917-2:2010 and ISO 9917-2:1998. RU-HBM1 condition media was biocompatible with pulp cells after culturing for 48 hours.

**Key words:** Cytotoxicity; Depth of cure; Flexural strength; RMGIs; Working time

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## Introduction

In 2009, Thailand imported dental materials and related instruments valued at approximately 1.468 billion baht, or 6% of the total imported medical and dental materials and equipment.[1] This expense has been dramatically increasing and will be a burden on the budgets of the government and individuals. The development of low-cost basic dental materials is an important strategy in solving this problem.

Light-cured resin modified glass ionomer (RMGI) cement is commonly used as a liner or base prior to the use of a definitive restorative material.[2-3] RMGI is composed of a powder, which is mainly fluoro-alumino-silicate glass, and a liquid, which is a mixture of light and chemical sensitive polyalkenoic acid and methacrylate monomer. The dual curing reaction involving both light activated polymerization and acid-base reaction gives RMGI more favorable clinical properties such as a longer working time and a shorter setting time compared to conventional glass ionomer cement.

Our research group has developed a light-cured RMGI prototype, Research Unit-Herbal Medicine, Biomaterial and Material 1 (RU-HBM1), to use as a liner or a base. The cost of this material is much lower than that of similar imported products. The working time, depth of cure, and flexural strength of RU-HBM1 was investigated. The cytotoxicity of RU-HBM1 was evaluated using human dental pulp cells. The results for RU-HBM1 were compared to those of two commercial RMGIs.

## Materials and Methods

Two commercial light-cured RMGIs, Vitrebond™ (VB; A3 color) and GC Gold Label Light-Cured Universal Restorative (GC; A3 color), were used as reference materials in this study. The expiration date of both RMGIs was more than 6 months after the completion of the experiments. The powder and liquid components of VB, GC, and RU-HBM1 are shown in Table 1. A light activator (Halogen Curing Light, Elipar™ 2500, 3M ESPE, USA) was used to light-cure the materials at an intensity of 700 mW/cm$^2$.

***Table 1*** *Powder and liquid components of experimental Resin Modified Glass Ionomer Cements (RMGIs)*

| Material | Composition | Recommend light curing duration | Powder/Liquid Ratio (g/g) | Manufacturer |
|---|---|---|---|---|
| GC Gold Label Light-cured Universal Restoration (Lot no. 1211081) | Powder<br>Fluoroaluminosilicate glass<br>Liquid<br>Polyacrylic acid; HEMA; TEGDMA; Photoinitiator | 20 seconds | 3.2/1 | GC, Japan |
| Vitrebond™ (Lot no. N516621) | Powder<br>Fluoroaluminosilicate glass<br>Liquid<br>Polyacrylic acid; HEMA; TEGDMA; Photoinitiator | 30 seconds | 1.4/1 | 3M™ ESPE™, USA |

*Table 1* (Continued)

| Material | Composition | Recommend light curing duration | Powder/Liquid Ratio (g/g) | Manufacturer |
|---|---|---|---|---|
| RU-HBM1 | Powder<br>Fluoroaluminosilicate glass<br>Liquid<br>Polyacrylic acid (29 %);<br>HEMA (33 %);<br>TEGDMA (7 %);<br>Photoinitiator | 20 seconds | 1.6/1 | Research Unit of Herbal Medicine, Biomaterial and Material for Dental Treatment Chulalongkorn University |

### Working time

The powder and liquid of each RGMI (N = 5) was prepared and mixed following the manufacturer's instructions. Briefly, the cement was loaded into a stainless steel mold (5 mm high, 7 mm long, and 10 mm wide). Ten seconds before the end of the working time of each material (160 seconds for VB and 225 seconds for GC), a flat-end indenter (28 g) was lowered onto the surface of the cement and remained there for 5 seconds. According to ISO 9917-2:2010, "the indenter shall make a complete circular mark in the surface of the specimen at 10 seconds before the working time claimed by the manufacturer or at least 90 seconds from the start of mixing".[4]

### Depth of cure

Each RMGI (N = 5) was prepared and mixed per the manufacturer's instructions. Within the working time, the cement was loaded into a customized circular stainless steel mold (6 mm high and 4 mm in diameter) placed on a glass slide. The cement was covered by polyester film and a second glass slide was placed on top of the mold under gentle pressure to extrude excess cement. After removing the excess material, the cement was light-cured for 20 or 40 seconds following the manufacturer's instruction. The specimen was then removed from the mold and the uncured material was immediately and gently removed with a blade. The height of the cured cement was evaluated with a digital caliper (Mitsutoyo Co., Kawasaki, Japan) and the value was divided by two. According to ISO 9917-2:1998, the minimal depth of cure of RMGI used as a liner or as a base is 1.0 mm.

### Flexural strength

Each RMGI (N = 5) was prepared and mixed per the manufacturer's instructions and placed into a customized stainless steel mold (25 x 2 x 2 $mm^3$) within the working time of each material. Polyester film and a glass slide were placed on both sides of the mold. The cement was cured using three overlapping curing-light exposures of 20 or 30 seconds each (based on the manufacturer's instructions). The specimen was cured on the opposite side of the mold in the same fashion. The slides were clamped to the mold and placed in a water bath at 37ºC for 15 minutes. After removing the specimen from the mold, the flash was removed using sandpaper, and the specimen was immersed in distilled water at 37ºC 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 formula[6]: $\delta = 3Fl/2bh^2$, where $\delta$ = flexural strength (MPa),

F = maximum load (N), I = span length between the supports (mm), b = specimen width (mm), and h = specimen height (mm). According to ISO 9917-2:2010, the minimal flexural strength of RMGI used as a liner or as a base is 10 MPa.

### Cell culture

The study protocols were approved by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University (No.16/2007). Human dental pulp cells were explanted from the healthy pulps of impacted third molars extracted from healthy patients as previously described.[7] Briefly, the teeth were decoronated and the pulp tissues were removed. The pulp was minced and the pieces were placed on 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 in a humidified 5 % $CO_2$ atmosphere at 37ºC. When the cells reached confluence, the cells were subcultured using 0.25 % trypsin-EDTA. All experiments were performed using cells from the third to the fifth passage. Cells from three donors were used in the study. All cell culture media were purchased from GibcoBRL™ (Invitrogen™, Grand Island, NY, USA).

### MTT cytotoxicity assay

Five specimens of each material were prepared according to the manufacturer's instructions in an autoclaved 12 mm x 2 mm x 2 mm mold. The cement was cured as described in the flexural strength test. After sterilization by UV exposure for 30 minutes on each side, the specimen was immersed in 1 mL of growth media with gentle agitation for 24 hours at 37ºC. 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). An equal volume of conditioned media and fresh growth media (50 % dilution) was used to treat the cells.

The MTT test was performed as previously described with some modifications.[8] Briefly, 40,000 cells/well were seeded into a 24 well culture plate (Nunc™ cell culture plate, Thermo Scientific, USA). After 24 h, the cells were washed twice with Phosphate Buffer Saline (PBS) and then treated with the conditioned media of each RMGI type for 24 or 48 h. Cells incubated with growth medium were used as control. 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 working time, depth of cure and flexural strength assays, and mean ± SE for the number of cell viability. The results were analyzed by one-way analysis of variance using the SPSS program for Windows, version 11.5 (SPSS, Inc., Chicago, IL, USA). The Scheffe´ multiple comparison test was used for post-hoc analysis. Significance was assumed at a $p < 0.05$.

## Results

### Working time, depth of cure, and flexural strength of the RMGIs

The results of the working time, depth of cure, and flexural strength assays of the RMGIs are presented in Table 2. The working time assay indicated that the RU-HBM1, GC, and VB groups had mean working times of 210 ± 25, 255 ± 11, and 180 ± 18 seconds, respectively. According to ISO 9917-2:2010, an RMGI used as a liner or base should have a working time of more than 90 seconds. Therefore, RU-HBM1 passed the criteria of working time.

The GC group had the highest curing depth, and the VB had the lowest curing depth. The depth of cure of the GC and RU-HBM1 groups was significantly higher than that of the VB group ($p < 0.05$). The depth of cure of the GC group was also significantly higher than that of the RU-HBM1 group ($p < 0.05$). According to ISO 9917-2:1998, a light activated dental cement should have a depth of cure greater than or equal to 1 mm. Therefore, RU-HBM1 passed the depth of cure standard.

The GC group had the highest flexural strength, and the VB group had the lowest flexural strength. The flexural strength of the GC group was significantly higher than that of the VB group ($p < 0.05$), however, the flexural strength of the RU-HBM1 group was not significantly different from those of the GC and VB groups. According to ISO 9917-2:2010, an RMGI used as a liner or base should have the flexural strength greater than 10 MPa. Therefore, RU-HBM1 passed the flexural strength criteria.

### Cytotoxicity of RMGIs to pulp cells

Figure 1 shows, after 24 h incubation, the mean number of viable cells in the control, RU-HBM1, GC, and VB groups were 60,902 ± 3,938, 62,483 ± 4,017, 58,467 ± 3,212, and 25,042 ± 959, respectively. The VB group exhibited significantly decreased cell viability compared with the growth media-treated control, RU-HBM1, and GC groups ($p < 0.05$). There was no significant difference in cell viability between the control, RU-HBM1, and GC groups.

After 48 h treatment, the mean number of viable cells in the control, RU-HBM1, GC, and VB groups were 89,428 ± 4,096, 86,775 ± 5,579, 85,805 ± 2,025, and 24,113 ± 846, respectively. The VB group demonstrated significantly reduced cell viability compared with that of the growth media-treated control, RU-HBM1, and GC groups ($p < 0.05$). There was no significant difference in cell viability between the control, RU-HBM1, and GC groups.

***Table 2*** *Working time, depth of cure, and flexural strength of GC Gold Label Light-Cured Universal Restoration (GC), Vitrebond™ (VB), and RU-HBM1. The data is expressed as Mean ± SD. The same superscript letter in each column indicates no significant difference between the groups (N = 5).*

| RMGIs | Working time* (seconds) | Depth of cure (mm) | Flexural strength (MPa) |
|---|---|---|---|
| GC | 255 ± 11 | 2.14 ± 0.22[a] | 33.51 ± 7.31[a] |
| VB | 180 ± 18 | 1.18 ± 0.02[b] | 19.11 ± 2.59[b] |
| RU-HBM1 | 210 ± 25 | 1.63 ± 0.07[c] | 27.97 ± 7.64[a,b] |

* From the manufacturer's instruction, the working times of GC and VB are equal to or more than 225 and 160 seconds, respectively. The same superscript letter in each column indicates no significant difference between the groups (N = 5).

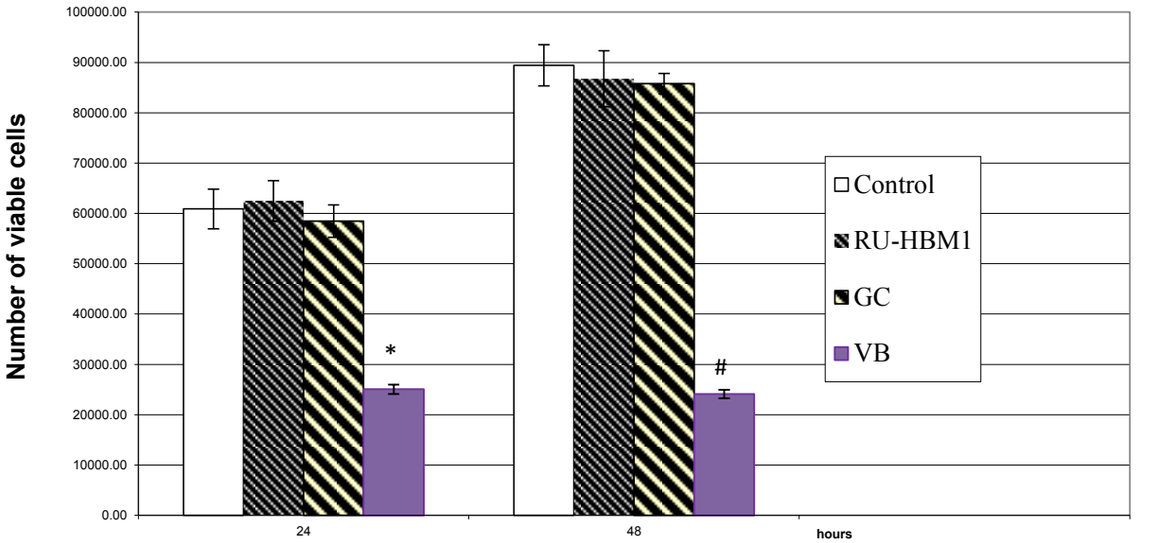


***Figure 1.*** *Cytotoxicity of dental pulp cells after culturing in medium treated with the experimental materials (RU-HBM1, GC Gold Label Light-Cured Universal Restoration, or Vitrebond™) for 24 or 48 hours. Cells incubated with growth medium were used as control. Data were obtained from three independent experiments and shown as mean ± SE.*

** Significant difference compared to the control group at 24 h ($p < 0.05$).*

*# Significant difference compared to the control group at 48 hours ($p < 0.05$), N = 5.*

## Discussion

Resin modified glass ionomer cements (RMGI) are widely used as a dental preparation liner or cement to support an overlying resin or amalgam restoration because of their better physical and mechanical properties compared to traditional glass ionomer cement.[9,10] In the present study, GC and VB were used as reference RMGIs to evaluate the physical properties and cytotoxicity of RU-HBM1 as a liner or base material. Our data revealed that RU-HBM1 met the ISO physical requirements of a RMGI cement for working time, depth of cure, and flexural strength. The cytotoxicity assay indicated that RU-HBM1 was less cytotoxic to the cells compared with the commercial RMGIs.

Depth of cure is how far into a material's thickness it can be efficiently polymerized after adequate irradiation. In the clinic, there is a maximum practical thickness that a material can be applied and completely cured at the bottom.[11] Although our results indicated that the maximum thickness of GC, RU-HBM1, and VB that can be cured are 4.28, 3.25, and 2.36 mm, respectively, there are concerns about the heat generated during the curing reaction and non-polymerized monomer that could damage the pulp tissue.[12] When considering the accessibility of the light to cure the material, the depth of penetration of the light source, light absorption and scattering in the material, and the variable distance between the light source and material which influences on the light power density and degree of conversion, the recommended maximal thickness of RU-HBM1 used in a cavity preparation should be 2 mm, which is the same as for VB and GC.[13]

Clinically, a material is subjected to a considerable amount of flexural stress, and a cement liner or base is subjected to complex forces during mastication. Therefore, flexural strength is considered an important mechanical characteristic for brittle polymer-based materials that are much weaker in tension than in compression.[14] The International Standards Organization requires a three-point loading test to evaluate the strength of a

resin modified cement used as a liner or base. This test 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 more likely related to fracture initiation.[15] Although our findings indicated that RU-HBM1 had a flexural strength in between those of the commercial RMGIs, all the materials had a flexural strength above the ISO requirements.

In the present study, the effect of RMGIs on human dental pulp cell was investigated using the MTT test. The MTT assay is a well-established method for analyzing the cytotoxicity of a substance based on cell viability.[16,17] In the assay, cell viability and proliferation are assessed relative to the functional state of the cells' mitochondria. The mitochondrial dehydrogenases in living cells reduce the yellow tetrazolium salt, MTT, to blue-purple MTT formazan, which is then retained in the cells. The formation of the formazan product has been found to correlate well with the number of viable cells.[8]

Sterilization is a critical factor for the success of cell culture experiments. To achieve this, the mold was autoclaved prior to loading the material. However, based on the instructions of GC and VB, it is not clear if these products were sterile. Therefore, sterilization of the mold would be insufficient to ensure a sterilized specimen. In the present study, the specimens were sterilized by UV radiation before immersion in growth media.

We chose UV sterilization because although UV radiation could affect the degree of polymerization of the material, this method is superior to other available methods. The use of 0.2 μm filter sterilization, steam sterilization, ethylene oxide sterilization, or gamma irradiation are not practical for the sterilization of RMGI glass powder, RMGI liquids, and RMGI specimens. The steam sterilization technique (121°C, 100 kPa for 15 minutes) would change the physical properties of the RMGI liquid and specimens. Silk fibroin membranes underwent the protein content and physical property changes after autoclave or ethylene oxide sterilization, while membranes that underwent sterilization by UV radiation presented properties similar to the nonsterilized membranes.[18] Biodegradable poly (caprolactone-urea) urethane lost all structural morphology and integrity after steam sterilization.[19] When using ethylene oxide sterilization, the residual gas is entrapped in the surface or micropores of the specimen and would be released into the conditioned media making it more toxic. Several studies have shown the cytotoxic effects of bone replacement materials on fibroblasts and changes in cell morphology after ethylene oxide sterilization.[20-22] Gamma radiation-treated nanocomposite biomaterials have been reported to have a cytotoxic effect.[19,23] Based on the dose and exposure time of the irradiation, gamma irradiation can induce cross-linking and break the molecule chains adversely affecting material properties.[23-25] In addition, with a few available agencies, time of documentation, and highly strict operation process, it would take at least 5 working days to gamma radiate the specimens, which is much different than the clinical use situation.

In the present study, the conditioned media was obtained by immersing the specimens in growth media at 37°C for 24 hours. The conditioned media of the control group was obtained by incubating growth media at 37°C for 24 hours. Subsequently, the conditioned media was sterilized using a 0.2 μm sterilized filter prior to treating the cells for 24 or 48 hours. In this method, the media was at 37°C for 48 or 72 hours. Some growth factors, proteins, and essential nutrients in the condition media could possibly be degraded prior to cell incubation. In addition, the soluble substances and unpolymerized materials from the specimens would release and alter the contents and acid-base balance in the media. Therefore, the conditioned media was 50 % diluted with fresh growth media to ensure normal cell growth and activity. Although the 50 % dilution of the specimen extract would lower the concentration of any cytotoxic substances and make the materials less cytotoxic than that of the undiluted extract, it would not affect the relative cytotoxicities of the materials tested. The use of a serial dilution or undiluted conditioned media should be performed to confirm our finding.

When used as a liner or base, the material and the dental pulp are separated from each other by the remaining dentin that is typically more than 0.5 mm thick. Therefore, the indirect contact test was selected to investigate cytotoxicity. The study of Hebling *et al*., Aranha *et al*. and Souza *et al*. reported that VB was highly cytotoxicity to odontoblast cell lines.[26-28] Our results indicated that RU-HBM1, GC, and VB all released toxic products in different amounts during their 24 hours immersion in the conditioned medium. However, RU-HBM1 and GC released less cytotoxic compounds compared with VB, and their cytotoxicities were not significantly different as compared with the control group. The cytotoxicity of VB found in our study most likely resulted from the specific components of their formulation leaching into the test medium. Incomplete polymerization of a resin-based material and the leaching of non-polymerized monomers negatively affect a material's biocompatibility.[29-31] The release of the monomers HEMA,TEGDMA, Bis-GMA and camphoroquinone from resin-based dental materials have been reported.[31-35] HEMA and TEGDMA induce cellular stress via the formation of reactive oxygen species (ROS) that results in cytotoxicity.[32] Non-polymerized camphoroquinone and Bis-GMA stimulate oxidative stress, DNA damage, apoptosis and cytotoxicity.[34,35]

Proprietary restrictions limit our knowledge of the exact amounts of the components in the commercial RMGIs. Thus, we cannot precisely explain why the depth of cure and flexural strength values, and the cytotoxicity of each RMGI materials were different. The practical explanation is that each RMGI has a different composition and percentage of each component.[36] Identification and quantification of the eluents from the cements using high performance liquid chromatography and gas chromatography/mass spectrometry, and cytotoxicity test should be performed.[33,37]

Clinical studies have shown that GC and VB are biocompatible with the dental pulp.[2,3] Therefore, the *in vitro* condition may not completely simulate the *in vivo* pulp tissue environment. The complex orchestration of the effects of the vascular system, immune system, and inflammation are absent in the *in vitro* environment.[38] Without the buffering, detoxification, and excretion systems present *in vivo*, the accumulation of toxic substances released from the specimens is likely increased. To confirm the cytotoxicity of RU-HBM1, the biocompatibility of RU-HBM1 should be investigated in an animal dental pulp cell and tissue model.

## Discussion

Based on the requirements of ISO 9917-2:2010 and ISO 9917-2:1998, the RMGI prototype RU-HBM1 has sufficient working time, depth of cure, and flexural strength. RU-HBM1 and GC Gold Label Light-Cured Universal Restorative are biocompatible with pulp cells, however Vitrebond™ is cytotoxic to pulp cells at 24 and 48 hours exposure.

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