

Research Report

Optimum dose of 2-hydroxyethyl methacrylate based bonding material on pulp cells toxicity

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ABSTRACT

Background: 2-hydroxyethyl methacrylate (HEMA), one type of resins commonly used as bonding base material, is commonly used due to its advantageous chemical characteristics. Several preliminary studies indicated that resin is a material capable to induce damage in dentin-pulp complex. It is necessary to perform further investigation related with its biological safety for hard and soft tissues in oral cavity. **Purpose:** The author performed an in vitro test to find optimum dose of HEMA resin monomer that may induce toxicity in pulp fibroblast cells. **Method:** The method of this study was experimental laboratory with post test control group design. Primary cell culture was made from dental pulp fibroblast cells, and was given with HEMA resin bonding material in various concentrations (5 µg/ml–2560 µg/ml), and then subjected to toxicity test (MTT assay). **Result:** HEMA optimum concentration was 320 µg/ml to induce cytotoxicity in pulp fibroblast cells. **Conclusion:** The used of HEMA - base bonding material with the concentration of 200 µg/ml may induced pulp fibroblas cell toxicity.

Key words: Citotoxicity, HEMA, resin, bonding material, fibroblast cells

ABSTRAK

Latar belakang: Keberhasilan suatu bahan bonding secara klinis tergantung pada kandungan fisik, kimia dan keamanan secara biologis. HEMA (2-hydroxyethyl methacrylate) adalah bahan resin yang paling banyak digunakan karena memiliki sifat fisik-kimia yang baik. Beberapa penelitian pendahuluan menyebutkan bahwa resin merupakan bahan yang mampu menyebabkan gangguan pada kompleks dentin pulpa sehingga perlu dilakukan penelitian lebih lanjut menyangkut segi keamanan secara biologis bagi jaringan keras dan jaringan lunak di rongga mulut. **Tujuan:** Penelitian ini akan menguji secara in vitro (pada kultur sel fibroblas pulpa gigi) untuk mengetahui dosis optimal monomer resin HEMA yang dapat menyebabkan toksisitas pada sel fibroblas pulpa. **Metode:** Metode penelitian ini adalah eksperimental laboratoris dengan rancangan penelitian post test control group design. Kultur sel primer dibuat dari sel fibroblas pulpa gigi, dan diberi bahan bonding resin HEMA dengan berbagai konsentrasi (5 µg/ml–2560 µg/ml) kemudian dilakukan uji toksisitas (MTT assay) **Hasil:** Didapatkan konsentrasi optimal HEMA adalah 320µg/ml untuk dapat menginduksi terjadinya sitotoksitas pada sel fibroblas pulpa. **Kesimpulan:** Penggunaan bahan dasar bonding HEMA dengan konsentrasi mulai 320 µg/ml dapat menyebabkan toksisitas pada sel fibrosis pulpa.

Kata kunci: Sitotoksitas, HEMA, resin, bahan bonding, sel fibroblas

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INTRODUCTION

Bonding material, one of the materials commonly applied in dentistry, particularly in the field of dental

conservation, has an important role. The application of adhesive resin material in dentistry may enforce the adherence retention to the teeth and reduce micro leakage.¹ Bonding materials marketed in public are mostly using resin

as the basic material. Resin monomer commonly used as the primary composition of bonding material is 2-hydroxyethyl dimethacrylate (HEMA).²

HEMA (Figure 1) is known to have satisfactory biocompatibility as bonding base material. Maximum binding strength may also be obtained from this material base.² HEMA has several advantages, such as relatively easy production, relatively longer endurance since it is generally added with preservative substances such as hydroquinone and butylated hydroxytoluene (BHT), relatively lower viscosity, and good physical as well as chemical characteristics.^{3,4} HEMA is regarded to have capacity to wet (wettability) dentin surface since HEMA has a character as a hydrophilic humidifier material.² HEMA is a resin that combines composite restoration material with tooth, both in enamel and dentin. The HEMA group has a function either as hydrophilic or hydrophobic group.² Hydrophilic means possessing a predisposition to have strong binding with water or, in other words, having capability to wet the dentin surface. Hydrophobic indicate a predisposition not to bind with water. Hydrophilic group adheres to the dental dentin, while the hydrophobics adhere to upperlying composite resin.⁵

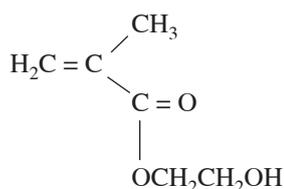


Figure 1. Molecular structure of HEMA (C₆H₁₀O₃).¹⁴

Several authors remark that HEMA is ideal as dentin bonding base material due to its reliable penetration into demineralized dentin.⁴ It was also reported that HEMA had good adherence to caries dentin.⁵ In addition to its ideal characteristics as dentin bonding material, several studies also reported that HEMA have potential to kill cells in vitro.⁶

One requirement of material in dentistry, particularly when the material is going to be applied in oral mouth, is that it should be biocompatible, implying that it can be accepted by the body, non-toxic, no irritative, not carcinogenic, and not inducing allergic reactions.² Toxicity test is conducted to find whether the material can be accepted by the tissue, containing systemic response-inducing substances when it is diffused and absorbed into circulatory system, free from sensitizing agents that may induce allergy, and not carcinogenic.²

Toxicity test of a material is related to the viability of living cells. Quantitative determination of viable cells can be performed using MTT assay.⁷ The frequently employed toxicity test is using in vitro method by means of cell culture.⁷ The source of cultured material is generally a primary cell culture, a culture from materials directly taken from animals or humans, which may present as organs, tissues, or cells. Cell culture is the growth of cells within a

media after being removed from an organ or tissue. Primary cell culture has a short life. Therefore, subculture can be performed only several times and thereafter the cells will die.¹¹ Compared to cell lines, primary cell culture is more sensitive against the toxicity of any chemical materials. Cell lines has an advantage of being able for 50–70 times passage, having rapid cell growth, maintained cell integrity, and able to multiply in suspension.¹¹

One of cell types that can be used for material toxicity test in dentistry is the pulp fibroblast cells. The cells are predominant in dental connective tissue, having a shape like spindle with oval nuclei and a long cytoplasmic process. Fibroblasts are cells that mostly found in the pulp and they originate from undeveloped mesenchymal pulp cells or from the part of fibroblast. The cells are present in the whole pulp, but they tend to be present in cell-dense area, particularly in the corona. Fibroblasts may produce denticle and are able to develop for replacing dead odontoblasts to create reparative dentin.⁸

The toxicity test can be applied as the early stage of a series of deeper studies on resin monomer, the basic of bonding material, and can be used as the preliminary test on biocompatibility of resin monomer material. Therefore, it is necessary to carry out cytotoxicity test on the basic material of dentin bonding, the HEMA monomer, to identify the optimum concentration that may lead to cell death. It was expected that it can be applied as a consideration to choose safe bonding material for the patient.

MATERIALS AND METHODS

This was a laboratory experimental study using post test only controlled group design. Samples in this study were pulp fibroblast cells isolated from the pulp of intact-extracted third molar. This study was performed in Lembaga Pengembangan dan Penelitian Terpadu (LPPT) Universitas Gadjah Mada Yogyakarta. Material used in this study was pulp fibroblast culture. Cells were isolated from intact-extracted third molar. The resin was pure HEMA (Sigma) in concentration of 95%. Pulp fibroblast cell was isolated from pulp tissue of the third molar through odontectomy operation. The pulp tissue was put on petri dish and added with 10% RosewellPark Memorial Institute-1640 (RPMI-1640) media in a volume of 2 ml, then penicillin-streptomycin and gentamycin were added. Cell culture was kept within 5% CO₂ incubator in 37° C for 4 days. Media replacement (followed with penicillin-streptomycin and gentamycin administration) was done every 4 days. After the cells became confluent, trypsinization and centrifugation were performed, and divided into several flasks (cell number about 800,000–1 million in one flask).

Cells were put within 96 culture wells, each containing 2 ml solution, with HEMA concentrations in 5, 10, 20, 40, 80, 160, 320, 640, 1280, 2560 ug/ml. Control group was media culture given with stop solution without the addition of HEMA. Samples were incubated in CO₂ incubator in

37 degree C for 24 hours. Subsequently, toxicity test was carried out to the samples to obtain lethal concentration (LC) of cell culture that had been given with HEMA monomer in various concentrations. Toxicity test was performed according to standard procedure for MTT assay. Prior to toxicity test, the samples were counted and entered into plate as many as 2×10^4 per well. Starvation was done for 2–4 hours in 37° C, and then fetal bovine Serum (FBS) was added in concentration of 0.5%. The media were replaced and added with FBS solution in 10% concentration. HEMA was added in concentration of 5–2560 $\mu\text{g/ml}$. The sample was incubated for 24 hours with CC>2 incubator in 37° C. The reactant MTT {3-(4,5 Dimethylthiazol-2-yl) 2-5 diphenyltetrazolin bromide} was added as much as 5 mg/ml in phosphat buffer saline (PBS) as much as 20 μl in each well and re-incubated for 4-6 hours in 37° C. Furthermore, stop solution (SDS) was added in each well and incubated overnight (12–18 hours). Reading was done with Elisa Reader (wavelength of 550 nm). The result was presented in optical density/absorbent and the magnitude of absorbent in each well indicated viable cell count in media culture.

RESULT

Toxicity levels measurable from the application of HEMA bonding material in fibroblast cell culture can be seen in Table 1. Prior to performing toxicity comparative test between HEMA monomer concentration groups, we performed data distribution test using Kolmogorov-Smirnov test, and followed by variance homogeneity test with Levene test.

Data distribution test with Kolmogorov Smirnov revealed $p > 0.05$, indicating that sample groups in various HEMA concentrations had normal data distribution. Data homogeneity test using Levene statistical test revealed $p < 0.05$, indicating that in sample groups (HEMA and control) the variance was homogeneous. To analyze the difference of HEMA concentration in one group, we performed One-Way ANOVA statistical test, which revealed $p = 0.374$ ($p < 0.05$), indicating the influence of different concentration on cell death between cell culture groups receiving HEMA in concentrations varied from 5 $\mu\text{g/ml}$ to 2560 $\mu\text{g/ml}$. Subsequently, Tukey's HSD test was performed to identify the different group. The result of Tukey's HSD test revealed $p < 0.05$, indicating significant difference between sample groups, showing that HEMA administration had effect on pulp fibroblast cells apoptosis.

DISCUSSION

The use of resin monomer as bonding material has been significant in dentistry. A reliability of dental material cannot be evaluated from their physical and chemical characteristics only, but its biological safety

also becomes a primary consideration.¹³ It is reported that resin monomer may induce various biological damages.¹⁴ It was also disclosed that there are cellular metabolism alterations due to induction of resin material.¹⁵ Resin monomer with imperfect polymerization may produce reactive chemical compounds that impairs cell balance within dental tissues.¹⁶ Toxicity test using MTT assay in this study used samples of pulp fibroblast culture provided with resin material 2 hydroxyethyl dimethacrylate (HEMA) in various concentrations, which was determined serially from 5 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$, 20 $\mu\text{g/ml}$, 40 $\mu\text{g/ml}$, 80 $\mu\text{g/ml}$, 180 $\mu\text{g/ml}$, 320 $\mu\text{g/ml}$, 640 $\mu\text{g/ml}$, 1280 $\mu\text{g/ml}$ and 2560 $\mu\text{g/ml}$. Statistical test using One-Way ANOVA with significance of 5% indicated that HEMA addition concentrations from 5 $\mu\text{g/ml}$ –180 $\mu\text{g/ml}$ did not induce toxicity in pulp fibroblast cell culture, since HEMA concentrations in that range did not influence cluster function and structure that may result in toxicity. This study shows that in a concentration as much as 320 $\mu\text{g/ml}$, the comparison between living and dead cells was each 50%.

MTT assay was performed based on the capability of living cells to reduce MTT salt. The principle of this examination was to breakdown MTT tetrazolium ring (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide) that has a yellow color due to dehydrogenation in active mitochondria and produces insoluble purplish blue formazan crystal.⁸ Reduction mechanism of yellow tetrazolium salt occurs in cells with metabolism activity. In this mechanism, mitochondria in viable cells have an important role in producing dehydrogenation. When the dehydrogenation process does not take place actively due to cytotoxic effect, formazan may not be formed. Formazan production can be measured by solving it and the optic density of produced solution is assessed. The lower the optic density percentage, the lower the number of active metabolic cells that are able to reduce MTT. Detected cell count from MTT reduction can be measured using spectrophotometer or Elisa reader. The darker the color, the higher the absorption value, indicating that the number of living cells are also higher.¹² This toxicity test method showed difference in living and dead cells due to damaged cell wall marked by the entry of trypan blue into the cells. Living cells is round and have bright color, while dead cells look enlarged and have blue color before they finally break down.¹¹ One-way ANOVA statistical test with significance level of 50% demonstrates that increased concentration affects formazan crystal optic density. Whereas, the result of Tukey's HSD value in this study showed the effect of different concentrations on cell death between cell cultures receiving HEMA in concentrations varied between 5 $\mu\text{g/ml}$ to 2560 $\mu\text{g/ml}$. In the addition of HEMA monomer in concentrations of 640 $\mu\text{g/ml}$, 320 $\mu\text{g/ml}$, 160 $\mu\text{g/ml}$ and 80 $\mu\text{g/ml}$. Significant difference was found with $p < 0.05$.

The effect of concentration on toxicity indicates that the higher the HEMA monomer concentration, the higher the toxicity. A study by Thimbrell¹² revealed that all materials are toxic, but it is the difference of concentration level in one material that differentiates the degree of toxicity of that

material. Toxicity test in this study showed the occurrence of optimum cell death with HEMA administration in a concentration of $320\ \mu\text{g/ml}$. The cause of the cell death may be the presence of free radicals possessed by the HEMA. HEMA has two hydroxyl groups in each of its carbon chains. Hydroxyl group is a free radical that has potentials to damage cell membrane.¹⁷ Cell membrane contains fatty acid elements that are responsive to free radicals. Cell membrane damage may alter osmotic balance, which relates to the damage of protein, enzyme, co-enzyme and ribonucleic acid that induce cell death. Therefore, when there is reaction of the fatty acid in membrane cell due to the presence of free radicals, the damage of function and structure of cell membrane will lead to cell death.

Table 1. Mean and standard deviation of pulp fibroblast cells in toxicity test with HEMA addition ($\mu\text{g/ml}$)

Monomer	Concentrations	\bar{X}	SD
HEMA	320	175.600	6.465
	160	166.400	7.765
	80	63.400	8.561
	40	42.000	8.944
	20	17.000	4.159
Control		2.000	1.870

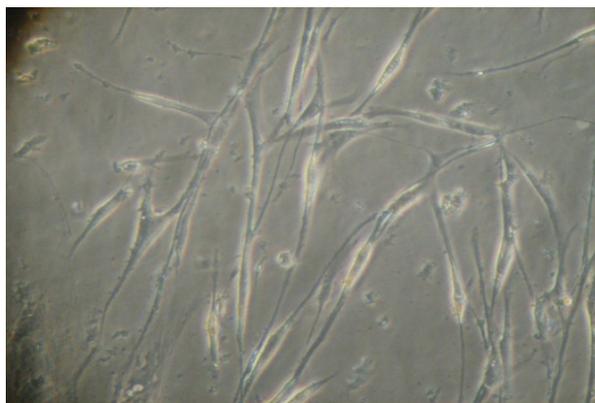


Figure 2. Pulp fibroblast primary cell culture before treatment. Note: (→) confluent pulp fibroblast cells

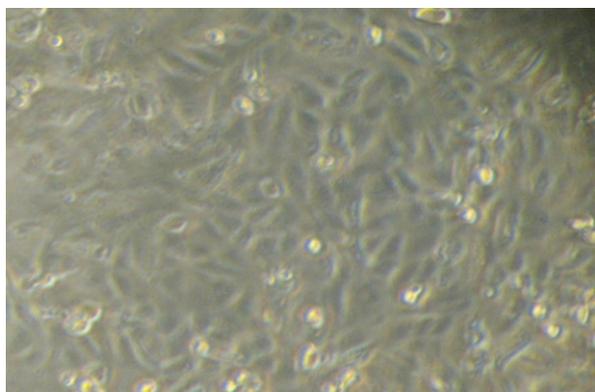


Figure 3. Profile of pulp fibroblast cell culture after HEMA administration in $320\ \mu\text{g/ml}$, cells appear to be round with bluish nuclei.

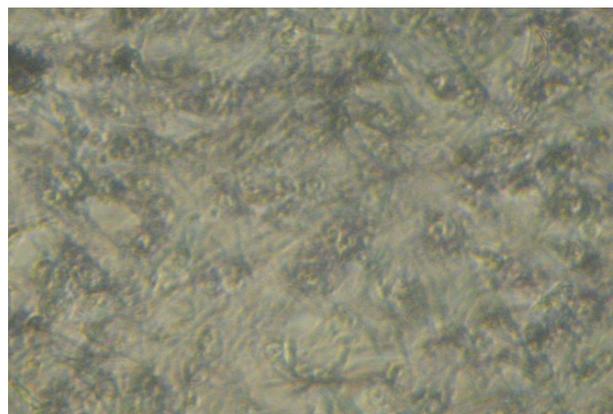


Figure 4. Profile of pulp fibroblast cell culture after HEMA administration in $1280\ \mu\text{g/ml}$, cells appear to be irregular with the absence of nuclei.

One of the requirements for a reliable material in dentistry is the presence of physical and biological safety.¹⁸ To obtain such reliability, one of the early actions that should be taken is testing material toxicity. Based on the result of this study, it can be concluded that pure HEMA material (95%) in concentration of $320\ \mu\text{g/ml}$ is an optimum concentration that may induce toxicity in pulp fibroblast cells. The result of this study can be used as the basis for further research on HEMA material, one of the materials applied in dentistry.

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