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Pulp Fibroblast Cell Apoptosis After Application of Hema Dentine Bonding Material with Ethanol and Water Solvent

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The most common main materials for dentin bonding for composite resin restoration is 2-hydroxyethyl methacrylate (HEMA). HEMA has beneficial physical and chemical properties, and stable, yet toxic. The addition of ethanol or water, may reduce the toxic effect of HEMA. Ethanol solvent has lower H-bonding capacity compared to water solvent, so it can bind less free radicals from the residual monomer. This study aimed to analyze apoptosis due to dentine bonding application with ethanol and water solvent. Fibroblast culture cells were obtained from extracted third molar, by means of tripsinasion method. The cells were divided into 4 groups as reached confluent: cell culture without treatment as control, cell culture with scaffold chitosan, cell culture with scaffold and polymerized dentin bonding with ethanol or water solvent. Apoptosis observation was conducted using immunohistochemistry method with ethidium bromide acridin orange staining, under fluorescent microscope with 40× magnification. There was a significant difference among groups (p=0.0001), yet no differences found between different solvent. Apoptosis rate in fibroblast cells culture exposed to HEMA bonding with ethanol solvent was 67%, while the cells exposed to HEMA bonding with water solvent was 44%. The effect of dentin bonding with ethanol solvent and water solvent towards apoptosis rate of pulp fibroblast cells is not different.

Key Words: dentine bonding, apoptosis, 2-hydroxyethyl methacrylate, ethanol solvent, water solvent.

Introduction

Composite resin is restoration material that is commonly used in conservative dentistry for its esthetic value is better compared to other restoration material. Composite resin binds to teeth structure through adhesive material called dentin bonding. Most of various dentin bonding that is currently available, thus far, containing 2-hydroxyethyl methacrylate (HEMA) as basic material (1). HEMA is widely used since have several advantages, such as having good physical-chemical properties, that is stable, both as base material bonding and when mixed with other materials (1). HEMA is one of the derivatives of methacrylate resin, that can be hydrolyzed into two compounds namely methacrylic acid and ethylene glycol, which has a toxic effect (2). Based on in vitro study, HEMA can stimulate apoptosis after 24 h (3). HEMA can also induce the production of Reactive Oxygen Species (ROS) in fibroblast cells that cause DNA fragmentation, characterized by apoptosis due to oxidative stress (4).

HEMA monomer is toxic to mammalian cells because it contains hydroxyl groups which are free radicals that can induce oxidative stress (5). According to Anusavice (1), free radicals are negatively charged ions that have no pairs in an atomic orbit, so to fill the void of the ion, it will attract other free ions which are nearby. According to Kitamura et al. (6), the diffusion of HEMA into dentin may lead to pulp irritation. An in vitro study discovered that HEMA can induce apoptosis after 24 h (7). Other study by Bakopolou et al (8) stated that HEMA could diffuse through dentin, toward pulp chamber in a significant high concentration, while other study stated that HEMA could affect human lungs epithelial cell, by inducing the formation of ROS, leading to DNA fragmentation, indicated by apoptosis (9).

Cytotoxic activity of chemical compounds can be observed from its ability to stimulate cell death (8). According to Krifka (10), monomer-induced apoptosis is an active cell response to ROS levels that exceed the cells ability to maintain reduction and oxidation homeostasis. Cells activate different enzymatic cellular antioxidant tissue balance to control intracellular oxidative states after exposure to HEMA monomers. It has also been shown that HEMA may increase ROS production which further lead to inflammation and delay the cell cycle process (11). Biochemical materials as well as resin-based dentin bonding materials can produce ROS in polymerization process (1).

The solvent contained in dentin bonding plays role in adequate bond formation between dentin and resin. Besides, bonding also act as the carrier of resin monomer into the dentin after etch. However, the residual solvent may also affect the polymerization process, impair the

bond strength, and causing inteface degradation (12). The solvents that is frequently used in adhesive systems are water, ethanol and acetone (13). According to Bianci (14) in his study suggested that the ratio of cell death both apoptosis and necrosis in the dentin-self-etch group of dentine-saturated-ethanol ranged between 3% -10% and 12% -47%. Indirectly, transdentinal diffusion of monomers occurs in the presence of ethanol, so that the presence of monomers diffusing into the pulp can induce oxidative stress and cytotoxicity in the pulp (14). The induction of apoptosis associated with the production of ROS is a major incident of direct cell response to monomers exposure.

Apoptosis is programmed cell death, active, requires energy and not accompanied by inflammation. Changes in cell morphology with apoptosis include cell shrinkage, chromatin segregation, cytoplasmic condensation and apoptotic bodies. The process of apoptosis through intrinsic and extrinsic pathways (15).

Dentin bonding in this study will be tested on pulp fibroblast human fibrous cell culture because fibroblast is the largest component of the pulp, periodontal ligament and gingiva. Fibroblast is the main cell in pulp tissue that can produce collagen fibers and differentiate into odontoblast like cells. Pulp fibroblasts are capable of producing growth factors and cytokines that act as growth control and response to injury (16). The purpose of this study was to investigate the effect of apoptosis on fibroblast pulp cells after dentin bonding with HEMA based on ethanol and water.

Material and Methods

This study has obtained ethical approval from Health Research Ethical Clearance Commission (No: 154/HRECC. FODM/VIII/2017).

Pulp Fibroblast Cell Isolation

Pulp fibroblast cell culture was obtained from mandibular third molar of 18-years-old female patient that were extracted for orthodontic purpose, by means of explant method. The extracted teeth were washed with Phosphate Buffer Saline (PBS), then split into two part vertically using a low-speed tapered round diamond bur (Intensiv, Switzerland) (0.84 mm in diameter), and the pulp tissues were extracted subsequently. The pulp tissues were cut using scissors, then chopped using scalpel blade no 3 (Sellaco, Germany). Hereafter, the pulp tissues were cultured into petridisc, covered with deckglass, in complete medium (DMEM 10%, FBS 10%, Trypsin 1%, Fungizone 0.5µg/ml, Penstreep 1-2%) (Gibco, Germany), and incubated in atmosphere of 5% and at a temperature of 37 ^{0}C. The medium was replaced every 3-4 days, until confluent. Fibroblast cells were identified in spindle shape (17).

Apoptosis Examination

The cell cultures were divided into 4 groups, fibroblast cell culture without treatment as control group, culture with chitosan scaffold, culture with chitosan scaffold that treated using dentin bonding with ethanol solvent, and culture with chitosan scaffold that treated using dentin bonding with water solvent. For treatment group, the scaffolds were added with dentin bonding and polymerized for 10 s, then put into the cell culture. Scaffolds in this study acts as matrices that hold and pass the bonding agent to the cell.

Immunohistochemical staining method was used to visualize the difference of apoptotic cells and normal cells, by means of double staining Ethidium Bromide Acridin Orange (EBAO). EBAO detected the apoptotic cells as fluorescent yellow, reddish orange. The cells were observed using fluorescent microscope with 40x magnification. The cell was counted using cell counting chamber (Neubauer, Germany), observed under light microscope (Olympus, Japan). The observation was done by 3 histology expert, which were blind to the group allocation.

Statistical Analysis

The obtained data was analyzed using Kruskal Wallis to find the difference among groups, and comparison between apoptosis rate in both dentin bonding material was done using Pairwise Comparisons. Statistical analysis was conducted using SPSS for windows.

Result

Based on the observations using a fluorescence microscope, the cultured cells from all groups was showing the characteristics of pulp fibroblast cell (Fig.1) Based on the acquired data, the mean and standard deviation of apoptotic fibroblast cells are shown in Table 1.

The acquired data were not normally distributed, therefore, Kruskal-Wallis was used to find the differences among groups. A significant difference was found among groups, with p value of 0.0001. Chitosan scaffold control group showed 0.9% apoptotic human fibroblast cells, pulp fibroblast cultures exposed to ethanol-based HEMA dentin bonding showed apoptosis of 67%, exposure to water-based HEMA dentin bonding showed the apoptotic rate of 44%. To determine the degree of apoptosis of human fibroblas pulp cells after exposure to ethanol-based HEMA and water, the data was analyzed by a pairwise comparative test (Table 2).

According to the result of different Pairwise Comparisons test, the apoptotic fibroblast cell in control group were significantly lower compared both to the water solvent group and ethanol solvent group ($p<0,05$). As for the comparison between the two treatment groups, showed that the apoptotic fibroblast cell of group treated

Table 1. Mean and standard deviation values of pulp fibroblast cell apoptosis

Group	Mean
Scaffold control	0.9 ± 0.316
Apoptosis of ethanol solvent	67 ± 26,895
Apoptosis of water solvent	44 ±19,692

Table 2. Results of Kruskall-Wallis test

	Scaffold control	Apoptosis of water solvent	Apoptosis of ethanol solvent	p value
Scaffold control				
Apoptosis of water solvent	p = 0.000*			0.0001*
Apoptosis of ethanol solvent	p = 0.000*	p = 0.058		

*Significant difference at p<0.05.

with ethanol solvent was higher than water solvent, yet there was no significant differences of apoptotic fibroblast cells (p>0.05).

Discussion

One of the basic ingredients that commonly found in the composite resin restoration and dentin bonding in dentistry is HEMA (2-hydroxyethyl methacrylate). The hydrolysis reaction of HEMA may produce methacrylic acid compounds and ethylene glycol. Besides, the residual monomer, as the result of unpolymerized HEMA, also contain hydroxyl group (-OH). Hydroxyl groups denote free radical molecule, which presence is allegedly causing cell wall damage, since the hydroxyl group denotes free radical molecule. The damage caused by free radicals can be chronic, by taking electrons from the body that may cause changes of DNA structure, and produce mutant cells (1).

One of the additive agent in dentin bonding materials is the solvent, that occupy 80% of the total weight. Besides act as the conductor of the monomer to diffuse into the

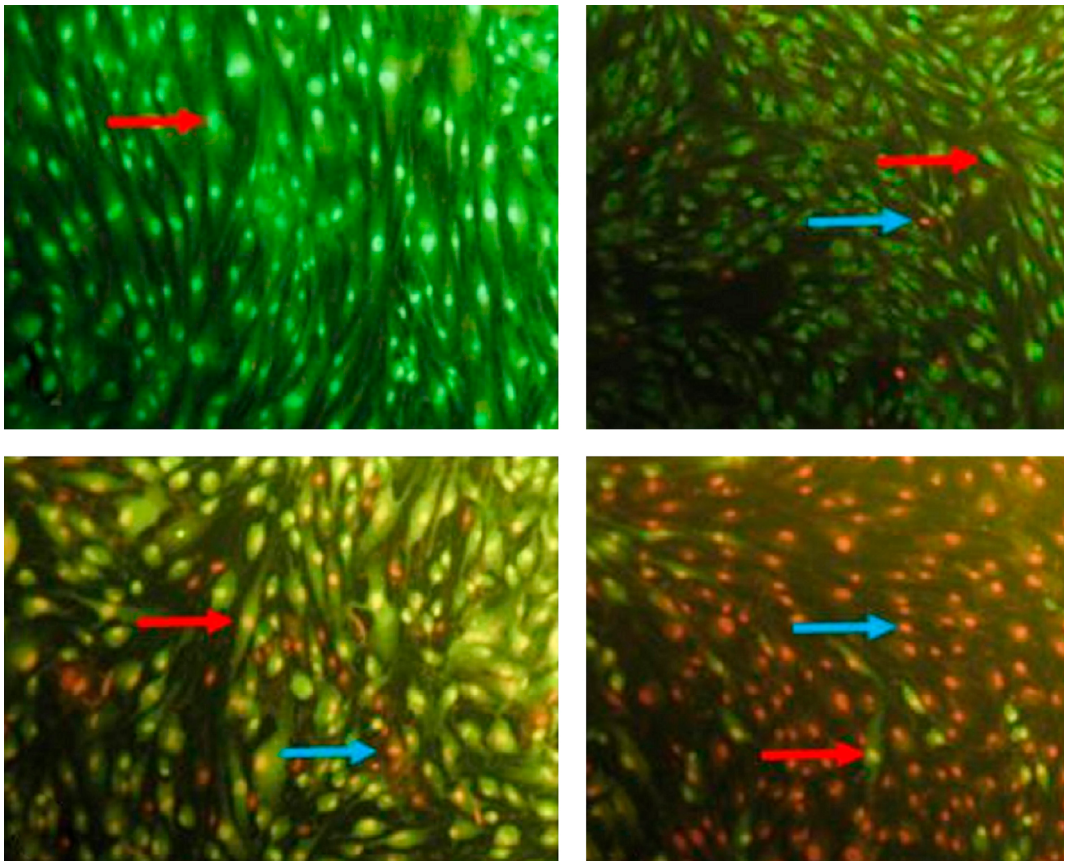

Figure 1. Apoptosis of pulp fibroblast cell culture. Red arrow indicates normal fibroblast cells, blue arrows indicates apoptotic fibroblast cells. (A) Normal pulp fibroblast cell culture; (B) Pulp fibroblast cell culture with scaffold chitosan; (C) Pulp fibroblast cell culture with scaffold chitosan and addition of HEMA dentin bonding with water solvent, (D) Pulp fibroblast cell culture with scaffold chitosan and addition of HEMA dentin bonding with ethanol-solvent. Original magnification 40×.

demineralised dentine, the solvent also minimize the toxic effects of its monomers, such as HEMA (1).

Prior conducting the apoptosis test, the Lethal Concentration 50% (LC 50) toxicity test was done towards dentin bonding material with water and ethanol solvent. The results showed that the LC 50 of dentine bonding with ethanol solvent on concentration of 0.625 µL/mL and dentine bonding with water solvent on concentration of 0.3125 µL/mL. This study used LC 50 concentration because it is the toxic limit of a chemical substance, that is characterised by the death of 50% of the cell population (18,19).

Based on the apoptotic test, the apoptotic fibroblast cells in group treated with bonding with ethanol solvent was higher than water solvent, yet interestingly, there was no significant difference recorded ($p>0.05$). Besides, according to the toxicity test (LC 50), it was recorded that ethanol solvent is more toxic on higher concentration, while water solvent is more toxic on low concentration. This proves that LC 50 alone does not lead to apoptosis, because of the present of cell cycle as a repair mechanism. In cell cycle, to ensure that DNA duplicates accurately and the separation of chromosomes occurs correctly, there is a checkpoint mechanism. The checkpoint mechanism plays role in detecting DNA damage, and will trigger the cell cycle temporary arrest in attempt to repair DNA damage or a permanent cell cycle arrest so that the cell enters the senescent phase. If the cell cycle arrest mechanism failed in preventing the duplication of damaged DNA, then the cell will be eliminated by apoptosis (18).

Based on the results of this study, the mean of pulp fibroblast apoptosis cell treated using dentin bonding with ethanol solvent recorded was greater than pulp fibroblast apoptosis treated using dentin bonding with water solvent. The H-bonding capacity of ethanol solvent is 19.4 J/cm^3, that is smaller than the water solvent H-bonding capacity of 42.3 J/cm^3, that may affect the free radical binding. Therefore, dentin bonding with water solvent has the ability to bind more free radicals. Besides, H-bonding capacity also affects the re-expansion of collapsed collagen fibrils (13).

The surface tension of dentin bonding with ethanol solvent is 22.27 dyn/cm, while dentin bonding with water solvent is 72.8 dyn/cm. The lower the surface tension that the solvent has, the easier it is to penetrate a cell surface layer, so that the monomer can diffuse further into the cell and may induce higher oxidative stress and, therefore, it has higher cytotoxic effects towards the pulp fibroblast cell (13).

HEMA has the ability to induce apoptosis in vitro after 24 h. Monomer-induced apoptosis is an active cell response towards ROS level that beyond the cell ability to maintain redox homeostasis (18). Pulp fibroblast apoptosis due to dentin bonding materials exposure is caused by the high level of ROS that leads to oxidative stress conditions, thus trigger apoptosis via intrinsic pathway. The increase of ROS level is caused by imbalance between free radicals from residual monomers (OH-) with the H-bonding capacity of the solvent. SOD (Super Oxyde Dismutase) that acts as an antioxidant, plays the same role as the solvent's H-bonding capacity. If the amount of ROS is greater than SOD, the damage of pulp fibroblast cell wall will occur, that subsequently activate the proapoptotic protein in cytosol. Proapoptotic protein activation will induce the opening of Mitochondrion Permeability Transition Pores (MPTPs), and release cytochrome c that contained in the mitochondria to the cytosol (18).

Cytochrome c is a heme protein that acts as a water-soluble electron carrier in mitochondrial oxidative phosphorylation. When a coil of electrons occurs through cytochrome c oxidase or the IV complex, a change of ionic force causes a matrix wave. When the inner mitochondrial membrane has a wider surface than the outer membrane, the matrix wave causes nonspecific inner membrane permeability transition pore open so that cytochrome c exits to the cytoplasm. The cytochrome c that exits into the cytoplasm then binds to Apaf-1 forming the CARD (Caspase Recruitment Domain). Some CARDs combine to form an apoptosome complex and then bind to pro-caspase 9 and activate it into caspase 9 (initiator caspase) (19).

Caspase 9 is one of the proteases derived from aspartic acid residues. The activation of one kaspase causes the activation of another caspase and leads to proteolytic activity causing the digestion of the protein structure in the cytoplasm, the degradation of the chromosomal DNA and finally the cell will be phagocyted. Caspase 9 will activate pro-kaspase-3 into caspase 3 which is a kaspase effector that carries out apoptosis. Apoptosis is characterized by DNA fragmentation, shrinkage cells, blistered membranes (18).

Previous research suggested that oxidative stress is involved in mutagenic processes and apoptosis induced by monomeric resin materials in vitro (4). While the study by Chang (20) suggested that the cessation of the cell cycle of the pulp and the gingival epithelium and the formation of ROS due to HEMA application. The HEMA monomer diffusion with fewer water solvents, the water solvent also has an H-bonding capacity of 42.3 J/cm^3. The amount of H-bonding capacity can bind the number of free radicals is more supported by the presence of SOD that can reduce the number of free radicals, so the number of free radicals can be reduced and apoptosis becomes less.

Based on the result and within the limitation of this study, it is concluded in this study that the effect of dentin bonding with ethanol solvent and water solvent towards apoptosis rate of pulp fibroblast cells is not different.

Resumo

Os principais materiais para adesão dentinária em restaurações de resina composta são o 2-hidroxietil metacrilato (HEMA). O HEMA possui propriedades físicas e químicas benéficas e estáveis, ainda que tóxicas. A adição de etanol ou água pode reduzir o efeito tóxico do HEMA. O solvente etanol possui uma menor capacidade de ligação H em comparação com o solvente água, de modo que pode ligar menos radicais livres do monômero residual. Este trabalho teve como objetivo analisar a apoptose pela aplicação de adesivos dentinários com solventes etanol e água. Células de cultura de fibroblastos foram obtidas a partir do terceiro molar extraído, por meio do método de tripsinaion. As células foram divididas em 4 grupos como confluentes: cultura celular sem tratamento como controle, cultura celular com arcabouço de quitosana, cultura celular com arcabouço e adesivo dentinário polimerizado com solvente etanol ou água. A observação da apoptose foi realizada utilizando o método imunohistoquímico com coloração com brometo de etídio e acridina laranja, sob microscópio de fluorescência com aumento de 40×. Houve uma diferença significativa entre os grupos (p = 0,0001), mas não houve diferenças entre os solventes. A taxa de apoptose em cultura de células de fibroblastos expostos à adesão baseada em HEMA com solvente etanol foi de 67%, enquanto as células expostas à adesão baseada em HEMA com solvente de água foi de 44%. O efeito da adesão dentinária com solvente etanol e solvente água sobre a taxa de apoptose de células de fibroblastos de polpa não é diferente.

References

1. Anusavice KJ, Shen C, Rawls HR. Physical properties of dental materials. phillips' science of dental materials. 12th ed. St. Louis: Elsevier Inc 2012. p.57.
2. Durner J, Spahl W, Zaspel J, Schweikl H, Hickel R, Reichl FX. Eluted substances from unpolymerized and polymerized dental restorative materials and their Nernst partition coefficient. Dent Mater 2010;26:91-99.
3. Gallorini M, Cataldi A, Giacomo V. HEMA-induced cytotoxicity: oxidative stress, genotoxicity and apoptosis. Int endod J 2014;47:813-818.
4. Morisbak E, Ansteinsson V, Samuelsen JT, Cell toxicity of 2-hydroxyethyl methacrylate (HEMA): the role of oxidative stress. Eur J Oral Sci 2015;123:282-287.
5. Stephanie K, 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;33:5177-5186.
6. Kitamura C, Ogawa Y, Morotomi T, Terashita M. Differential induction of apoptosis by capping agents during pulp wound healing. J of Endod 2003;29:41-43.
7. Gallorini M, Cataldi A, Giacomo V. HEMA-induced cytotoxicity: oxidative stress, genotoxicity and apoptosis. Int Endod J 2014;47:813-818.
8. Bakopoulou A, Papadopoulos T, Garefis P. Molecular toxicology of substances released from resin-based dental restorative materials. Int J of Mol Sci 2009;10:3861–3899.
9. Morisbak E, Ansteinsson V, Samuelsen JT. Cell toxicity of 2-hydroxyethyl methacrylate (HEMA): the role of oxidative stress. Eur J Oral Sci 2015;123:282–287.
10. 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;33:5177–5186.
11. Di Nisio C, Zara S, Cataldi A, di Giacomo V. 2-Hydroxyethyl methacrylate inflammatory effects in human gingival fibroblasts. Int Endod J 2013;46:466–476.
12. Georges G, Karen BPF, Fernanda CPG, Paulo HPD, Nádia da RS, Linda W. Solvent retention of contemporary commercial dentin bonding agents in a demineralized dentin matrix. Eur J Dent 2010;4:293-297.
13. Ekambara, M, Yiu CKY, Matinlinna JP. An overview of solvents in resin–dentin bonding. Int J of Adhesion and Adhesives 2015;57:22-33.
14. Bianci Craig RG, Powers JM, Wataha J. C. Direct esthetic restorative materials. In: Dental Materials Properties and Manipulation, 11th ed.;2015. pp. 57-79.
15. Pollard JW. Macrophages define the invasive microenvironment in breast cancer. J leukoc biol 2008;84:623-630.
16. Nanci A. Dentin-pulp complex. In: Ten Cate's Oral Histology: Development, Structure and Function. 8th ed.;2012:191-238.
17. Tucci VMV, Bernardo H, Jerônimo PF, Lydia MF. 'Keloid explant culture: a model for keloid fibroblasts isolation and cultivation based on the biological differences of its specific regions'. Int Wound J 2010;7:339-348.
18. Gad. LD50/LC50 (Lethal Dosage 50/Lethal Concentration 50). Encyclopedia of Toxicology, 3rd ed.;2014. pp. 58-60.
19. Fan TJ, Han LH, Cong RS, Liang J. Caspase family proteases and apoptosis. Acta biochim biophys Sin 2005;37:719-727.
20. Chang HH, Guo MK, Kasten FH, Chang MC, Huang GF, Wang YL, et al. Stimulation of glutathione depletion, ROS production and cell cycle arrest of dental pulp cells and gingival epithelial cells by HEMA. Biomaterials 2005;26:745-753.

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