

Research Report

Cytotoxicity test of 40, 50 and 60% citric acid as dentin conditioner by using MTT assay on culture cell line

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ABSTRACT

Background: Open dentin is always covered by smear layer, therefore before restoration is performed, cavity or tooth which has been prepared should be clean from dirt. The researchers suggested that clean dentin surface would reach effective adhesion between resin and tooth structure, therefore dentin conditioner like citric acid was used to reach the condition. Even though citric acid is not strong acid but it can be very erosive to oral mucous. Several requirements should be fulfilled for dental product such as non toxic, non irritant, biocompatible and should not have negative effect against local, systemic or biological environment. Cytotoxicity test was apart of biomaterial evaluation and needed for standard screening. **Purpose:** This study was to know the cytotoxicity of 40, 50, 60% citric acid as dentin conditioner using MTT assay. **Method:** This study is an experimental research using the Post-Test Only Control Group Design. Six samples of each 40, 50 and 60% citric acid for citotoxicity test using MTT assay. The density of optic formazan indicated the number of living cells. All data were statistically analyzed by one way ANOVA. **Result:** The percentage of living cells in 40, 50 and 60% citric acid were 95.14%, 93.42% and 93.14%. **Conclusion:** Citric acid is non toxic and safe to be used as dentine conditioner.

Key words: cytotoxicity, citric acid, MTT assay

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INTRODUCTION

The structure of natural tooth is limited structure, therefore it should be maintained and protected as long as possible, consequently, in selecting restorative material, some aspects are necessarily considered in order to obtain the most proper material.¹ Open dentin is always covered by smear layer therefore, before restoration is performed, cavity or tooth which has been prepared should be clean from dirt produced during preparation, bacteria produced by carries or should not be contaminated by environment.² The researchers suggested that clean dentin surface would reach effective adhesion between resin and dentin material, therefore dentin conditioner is used to reach the condition.³

Dentin conditioner is solution which is polished on dentin surface.⁴ The term of dentin conditioner can be meant as an effort to make or to prepare dentin surface

to be able to accept dentin bonding. Dentin conditioner usually consist of weak acid solution and design to modify or clean the smear layer on dentin surface which has been prepared to be able to function well as supporting material for dentin bonding. The material can also be capable to wet the dentin surface, it can decrease constrain of dentin surface. In this way dentin bonding material can penetrate more into dentin tubule and finally the condition of binding each other can be reached.⁵

Dentin conditioner technique for glass ionomer cement bonding in tooth substrate is applied in dentistry field with the aim to increase adhesion between resin and tooth substrate.⁵ Glass ionomer cement is widely applied in class V cavity on cervical tooth which is bordered by gingival, prior to cement application in the cavity, clean surface is conditioner. In vitro study on the surface of the third molar showed that the effect of 40, 50 and 60% citrate acid dentin conditioner was effective.⁶ Citric acid is not strong acid but

it can be very erosive due to the capability to bind metal ion. The other researcher found that influence of citrate acid is necessarily observed if it has surface contact either in enamel or dentin. Enamel surface which is polished by citric acid will be erosive. Smear layer will disappear from dentin and open dentin tubule through the loss dentin perihedral.⁷ Powis *et al.*⁸ suggested that the effect of citric acid is necessarily observed if it gets into contact with the surface either polished enamel or dentin. Enamel surface which is polished by citric acid will be erosive. Optimal adhesion will not be reached using excessive amount of citric acid or citrate acid in excessive high concentration. The loss of smear layer and tubule opening can cause tooth demineralization.⁷

The requirement of dental product applied in dentistry are non toxic, non irritant, biocompatible and should not have negative effect against local, systemic or biological environment.⁹ Cytotoxicity test of a material can be done using enzymatic test MTT [3-(4,5-Dimethylthiazol-2YL); 2,5-diphenyltetrazolium bromide]. MTT test is used to measure the capability of living cell based on mitochondria activity of culture cell which can reduce yellow tetrazolium bromide into insoluble purple blue formazan endapan. The product of formazan is solved by solvent in order to be easily detected. In this way, the number of detected living cell using spectrophotometer as the result of MTT product. The darker the color of purple blue, the higher the absorbent score and the higher the number of living cell this test is widely used to measure quantitatively cellular proliferation or the number of living cell.¹⁰ The advantage of this test is the accuracy and sensitivity measurement because the spectrophotometer which is used can detect clearly metabolism alteration, can easily be manipulated, and available at the laboratory, can save time and energy and it does not use radioactive isotope. Based on the above reasons, in this study MTT test was used to test the cytotoxicity of dental material using fibroblast culture cell of Baby Hamster Kidney-21 (BHK-21) considering the origin of this cell is embryonic cell so it would be easily grow and repeated subculture is easily done, having stable character, sensitive and mutation does not occur.¹¹ Based on the above aspects, the problem arises whether there is different cytotoxic effect of 40, 50 and 60% citrate acid dentin conditioner on MTT test.

The purpose of this study was to prove the cytotoxicity of 40, 50 and 60% citric acid as dentin conditioner using MTT assay. The advantage of this study would inform the safety of citric acid as dentin conditioner, especially for dentistry colleague before filling the cavity and citric acid can be alternative material for dentin conditioner.

MATERIAL AND METHOD

This study is an experimental research using The Post-Test Only Control Group Design. Fibroblast cell culture (Baby Hamster Kidney-21) was used with the number of samples was 8 based on the estimation, material and tools were applied such as: incubator for cell culture, micro-plate reader, micropipet multi channel, roux bottle, yellow tips, laminar flow, MTT reagent (sigma), saline water, dimetil-sulfoksid (DMSO) solution, 40, 50 and 60% citric acid, cell culture of baby hamster kidney (BHK-21), culture media Rosewell Park Memorial Institute-1640 (RPMI-1640) 89%, 1% Penstrep, 10% Fetal Bovine Serum (FBS), 100 until/ml fungizone, Phosphate Buffer Saline (PBS).

Cell culture of BHK-21 in cell-line form was embedded in roux bottle. After it was confluent, the culture was harvested using trypsin versene solution. The result was embedded in media Rosewell Park memorial institute 1640 containing 10% fetal bovine serum albumin incubated for 24 hours at 37° C. the cell was cultured in every microplate 96-well until confluent. Every well contained cell and RPMI media with 2×10^5 cell/ml density in 50 μ then each well was given 20 μ of 40, 50 and 60% citric acid. Each group consisted of 8 samples. Control cell was prepared as positive control, and it was considered the percentage of living cell was 100%. Control media as negative control considered the percentage of living cells was 0%. The microplate was incubated at 37° C, then, removed from incubator, added by 5 mg/ml MTT in PBS 20 μ for every well, incubated again for 4 hours. Next, every well was added by 50 μ l DMSO. Formazan which had been formed was solved by into the solvent and the absorption was measured by a reader using spectrometer with 595 nm wave length. To know the percentage member of living cell, the equation below was used.¹²

Table 1. Mean of formazan optic density, standard deviation, percentage of living cell

Concentration of citric acid	Number of sample	Mean of optic formazan density	Standard Deviation	% living cell
40%	8	0.254	0.008	95.14%
50%	8	0.248	0.007	93.42%
60%	8	0.247	0.008	93.14%
Control cell	8	0.271	0.007	100%
Control media	8	0.079	0.0072	0%

Table 2. The ANOVA result from fibroblast cell on citric acid and control cell

Variation source	Number square	Free level	Mean square	F. account	Probability
Between group	0,201	4	0.05	838.848	0.001
In group	0,002	35	0.001		
Total	0,203	39			

$$\% \text{ living cell} = \frac{\text{Tested group} + \text{media}}{\text{cell} + \text{media}} \times 100$$

Note: % living cell = percentage number of living cell after the test
 Tested group = score of formazan optic density of every sample after the test
 Media = score of formazan optic density in control media
 Cell = score of formazan optic density in control

RESULT

The data tabulated, then, statistical analysis using One-Way ANOVA test with 5% significant rate was done and continued by Tukey HSD test if there was significant difference. The mean of formazan optic density, standard deviation, percentage of living cell could be seen on table 1. In tested group has show significant decrease. Percentage of living cell that is percentage of optic density of dehydrogenase mitochondria enzyme in cell culture of BHK-21 as well as in control media has shown in table 1.

Table 1 shows all distribution group is normal, continued by parametrix test of one way ANOVA to know the difference in the group with 5% significant rate (Table 2).

Table 2 shows there is significant difference in every citric acid group and control, therefore continued by Tukey HSD seen on table 3.

Table 3. The result of Tukey HSD test shows the difference of living cell percentage between control group and 40, 50 and 60% citric acid group

	Control cell	Group 40%	Group 50%	Group 60%
Control cell				
Group 40%	S			
Group 50%	S	NS		
Group 60%	S	NS	NS	

S for significant and NS for non significant

DISCUSSION

Citric acid is weak organic acid, functions as natural preservative substance, cleansing material and anti oxidant, used to add sour taste in food and soft drink, found in fruit especially citrus and vegetable. Lemon and lime have the highest content of citric acid which containing 8 % of the whole fruit weight.¹³

Dentin conditioner is solution which is polished on dentin surface.⁴ The term of dentin conditioner can be meant as an effort to prepare dentin surface so that it can accept dentin bonding. Dentin conditioner usually consist of weak acid solution such as citric acid and designed to clean smear layer on dentin surface which has been prepared so that it can function well as supporting material of dentin bonding. The method is previously wetting dentin surface before polishing by dentin bonding. Then, conditioner is cleansed using water spraying and dried by air blowing. So, dentin conditioner is solution which is polished on dentin surface before being covered by dentin bonding.⁵

The adhesion mechanism has not been exactly exposed, probably, this material could be wet much better the dentin surface. In this way, dentin bonding material can penetrate deeply into dentinal tubule and then bound each other.⁴

In this study, citric acid was used as dentin conditioner material. Citric acid would permanently demineralized dentin surface by erasing smear layer and the most superficial dentin. The acidity of dentin conditioner would open dentinal tubule and making tissue of collagenous fibre due to microporosity which is filled by water in intratubular and peritubular dentin.^{14,15} The use of demineralized material on dentin surface is to improve the bond strength between tooth and restorative material.

There are some controversial opinions on dentin conditioner. Currently, dentin conditioner of third generation is made of weak acid, one of them is citric acid. However, the safety of citric acid in high concentration is still doubtful, considering strong acid such as 37% phosphate acid has proven that dentin bonding containing strong acid is very dangerous for the vitality of pulp tissue. Citric acid as dentin conditioner can wash smear layer by hydroxiapatite dentin reaction. Citric acid will release hydrogen ion so dentin structure would be demineralized. Citric acid will bind calcium ion substrate phosphate ion in hydroxiapatite structure.¹⁶

One of the requirement of dental product to be applied in oral cavity should be biocompatible such as nontoxic substance.⁹ In vitro study of cytotoxicity using MTT assay

on culture cell lines are used due to the advantages such as passage can be done 50–70 times, high rate cell growth, well maintained cell integrity, cell is capable of multiplying in suspension cell lines have been widely applied in toxicity test for dental material and medicine, such as cell of BHK-21.¹⁷ The result using BHK-21 can be applied as accurate basic test.¹⁸ MTT is yellow soluble molecule which can be used to evaluate cellular enzymatic activity based on the capability of living cell to reduce MTT. The mechanism is yellow tetrazolium salt would be reduced in cell which has metabolic activity.^{19,20} Mitochondria are the principle sites for synthesis of adenosine triphosphate, surrounded by two membranes, which are structurally and functionally distinct. Mitochondria of living cell also has important role produces dehydrogenase.^{21–23} If dehydrogenase is not active due to cytotoxicity effect, formazan will not be formed. The number of formed formazan is equal to enzymatic activity of living cell.

The result of this study shows the living cell of 40% citrate acid = 95.14%, group 50% = 93.42%, group 60% = 93.14%. The result of HSD test shows significant difference between control group and citric acid group. Decreasing number of living cell in each tested group shows cytotoxicity of citric acid basically due to the effect of acidity than the content of citrate acid itself. Most common case of dead cell due to citric acid can be prevented by regulating pH of culture media to be 7.5. In the study using sodium citric in 47.6% mmol/L concentration can cause almost no dead cell effect.²⁴ The percentage of living cell in every tested group is almost 100%, meaning dentin conditioner of 40, 50 and 60% citric acid is safe due to good biocompatibility limit for the number of living cell 92.3–100%.¹⁹ This score is higher compared to the limit used by Telli *et al.*,²⁵ suggested that the parameter of toxicity based on CD₅₀ meaning that material which is thought to be toxic if the percentage of living cell is below 50%. The number of living cell after citrate acid application in this study shows citric acid is relatively safe and it is supported by other study indicates that this acid can cause less dentin resorption and less coagulation in blood and tissue compared to other acid materials, reaction on the pulp will not occur and can have reaction with hydroxiapatite dentin.¹⁶ The advantages of using citric acid are: cheap, easily applied and bought. However, the pulp is necessarily protected before the acid is applied. Especially thin dentin in deep caries of the occurrence of channel on root surface.

The conclusion is cytotoxicity test using MTT Assay shows 40, 50 and 60% citric acid is not toxic and safe for dentin conditioner. Further in vivo study is necessarily done to know completely the biocompatibility effect. Citric acid concentration in clinical use is essentially observed considering the acid concentration as dentin conditioner is different from the concentration as material of root canal irrigation.

REFERENCES

1. Nawangsari N. Perbedaan sitotoksitas bahan bonding self etch dan total etch. Surabaya: Karya Tulis Akhir; 2004. p. 1–2.
2. Prijambodo SK. Pengaruh pemberian smear layer pada permeabilitas dentin. *Int Dent* 1996; 3:133.
3. Nordenvale KJ, Brannstrom M. In vivo impregnation dentin tubules. *J Prost Dent* 1980; 76:254–9.
4. Fortin D, Swift EJ, Denehy GE, Reinhardt JW. Bond strength and micro leakage of current dentin adhesives. *Dent Material* 1994; 10:253–8.
5. Attal JP, Asmussen E, Degrange M. Effect of surface energy of dentin. *Dent Material* 1994; 10:159–264.
6. Pramita TP. Pengaruh perbedaan konsentrasi asam sitrat sebagai dentin kondisioner pada permukaan dentin. Surabaya: Karya Tulis Akhir; 2006. p. 4–14.
7. Wahyu A. Perbedaan kekuatan perlekatan tank antara resin komposit dan dentin yang diulas dengan larutan asam laktat 500/0 dan larutan asam sitrat 50%. Surabaya: Skripsi; 2002. p. 2–3.
8. Powis DR, Folleras T, Mearson SA, Wilson AD. Improved adhesion of glass ionomer cement to dentin and enamel. *J Dent Res* 1982; 61:1416–22.
9. Noort VR. Introduction to dental material. 2nd ed. London: CV Mosby Company; 2003. p. 3–5.
10. Fernandez BR, Vetviaeka V. Method in cellular immunology. Boca Raton, New York: CRC Press; 1995. p. 47–52.
11. Freshney IR. Culture of animal cells. 2nd ed. New York: Alan R Liss Inc; 1987 p. 227–45.
12. Titien HA. Pengaruh tegangan listrik dan lama penyinaran pada semen ionomeri gelas modifikasi resin terhadap kekerasan permukaan dan sitotoksitas. Tesis. Surabaya: Pasca Sarjana Universitas Airlangga; 2002. p. 25–35.
13. Wikipedia, Encyclopedia. Wikipedia® is a registered trademark of the wikimedia foundation, Inc. Available at: http://en.wikipedia.org/wiki/Citric_acid. Accessed April 2006.
14. Perdigao J. The effect etching time on dentin demineralization. *Quintessence International* 2001; 32:19–26.
15. Bath MB, Fehrenbach MJ. Dental embryology, histology and anatomy. 2nd ed. Missouri: Elsevier Saunders; 2006. p. 192–5.
16. Soetanto, Dewi, Caecillia. Pemakaian asam sitrat pada perawatan periodontitis marginalis gigi molar dengan cervical enamel projection. Surabaya: Karya Tulis Akhir; 2001. p. 15–20.
17. Siregar F, Hadijono BS. Uji sitotoksitas dengan esei MTT. *JKGUI* 2000; 7:28–32.
18. Craig RG, Powers JM. Restorative dental materials. 6th ed. London: Mosby Co; 2002. p. 135–40.
19. Rubianto M. Biokompatibilitas bahan allograft (human bone powder) dibandingkan dengan bahan alloplast (hydroxylapatite). Kumpulan Naskah Temu Ilmiah Nasional I (TIMNAS I) FKG Unair 1998; p. 507–509.
20. Junqueira LC, Carneiro J, Kelley RO. Basic histology. 9th ed. Appleton and Lange. 2006. p. 14.
21. Nanci A. Oral histology development structure and function. 6th ed. St Louis: Mosby; 2003. p. 341.
22. Burns ER, Cave MD. Histology and cell biology. 2nd ed. Philadelphia: Mosby; 2007. p. 13.
23. Sherwood L. Fisiologi manusia. Edisi 2. Jakarta: Penerbit Buku Kedokteran EGC; 2001. p. 26.
24. Lan WC, Lan WR, Chan CP, Hsieh CC, Chang MC, Jeng JR. The effect of extra cellular citric acid acidosis on the viability, cellular adhesion capacity and protein synthesis of cultured human gingival fibroblast. 1999. p. 25–35.
25. Telli C, Serper A, Dogan AL, Guc D. Evaluation of the citotoxicity of calcium phosphate root canal sealers by MTT assay. *J Endo* 1999; 25:811–3.