Cytotoxicity Test of Sponge Amnion on BHK-21 Fibroblast Cell

Elly Munadziroh[1]*, Anita Yuliati[1], Annisa Noor Ragilia[2]

1. Department of Dental Material, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.
2. Undergraduate Student, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.

Abstract

Biomaterials that can be used to accelerate wound healing is the amniotic membrane that contains growth hormone. In its use, amniotic membrane tends to be difficult to use because of their sheets form. To make it easier to use, the amniotic membrane is mixed with gelatin as an adhesive material to produce sponge amnion. Sponge amnion which is used as a biomaterial needs to meet several requirements, one of them is biocompatibility. To complete these requirements cytotoxicity testing is necessary to determine the toxic potential that a material may produce. The cytotoxicity test was performed on BHK-21 fibroblast cells.

The aim of this study is to determine the citotoxicity effect of amnion in sponge form on BHK-21 fibroblast cells.

This study was done towards the culture cell line BHK-21 using MTT assay method. In this study used four sample groups. Two groups are the control group (media control and cell control), and the other two groups are samples consisted of amniotic membrane and sponge amnion. Living cells were quantified after treatment by ELISA reader on 620 nm.

The fibroblast cell life percentage after exposure with amniotic membrane and sponge amnion is 98,16% and 98.13%. Based on the ANOVA test result was obtained *P*-value of 0.000 (*P*-value <0.05) so that it can be said that the treatment result gives a significant difference.

Amnion in sponge form has no toxic for BHK-21 fibroblast cell.

Experimental article (J Int Dent Med Res 2018; 11(3): 819-822)

Keywords: BHK-21 cells, Cytotoxicity, Sponge amnion.

Received date: 16 March 2018 **Accept date:** 20 May 2018

Introduction

Alternative ingredients to accelerate the wound healing process is an amniotic membrane containing growth factor.[1] The amniotic membrane is derived from extra-embryonal tissue comprising the fetal component (chorionic plate) and the maternal component (decidua). The amniotic membrane is the inner layer of the fetal component.[2]

Amniotic membranes have specific properties, including anti-inflammatory, anti-bacterial, anti-viral, anti fibrotic, anti-scaring, reducing the occurrence of sikatrik tissue, very high tensile strength,[2] protecting wounds, reducing pain, increasing basal epithelial cell adesion and differentiation of cells also have reepithelization characteristic.[3] In recent years, amniotic membranes are used as biomaterials in the field of dentistry and its use is increasing. This is because the price is affordable and easy to obtain.[4] Amniotic membrane proved safe to use,[5] but its use is limited due to its sheet form. So as to easily its application, gelatin is added to amniotic membrane so as produce sponge form which is known as sponge amnion.

One of the form of amniotic is the sponge amnion. Sponge amnion is a smoothed amniotic membrane with a size of about 250 µm mixed with gelatin as an adhesive material and processed by freeze drying method using room temperature.[6] Amniotic membrane that are converted into sponge forms, are expected to facilitate dentists in their use to make it easier. Sponge amnion as a biomaterial that easy to get, affordable price and easy to apply expected to be used as an alternative material in accelerating the wound healing process.

Fibroblasts are cells that play an important role in the oral mucosa located in the

Corresponding author:
Elly Munadziroh, DDS, M.Sc., Ph.D
Departement of Dental Material, Faculty of Dental Medicine, Universitas Airlangga, Jl. Mayjen Prof. Dr. Moestopo 47, Surabaya, Indonesia 60132
E-mail: elly-m@fkg.unair.ac.id

lamina propria.[7] Sponge amnion as a biomaterial that applied to the oral cavity must meet several conditions, including non-toxic, non-irritating, and must have biocompatibility or materials produced should not harm the biological environment, both local and systemic.[8] To achieve these requirements, cytotoxicity testing is necessary. The cytotoxicity test is useful for determining the toxic potential that a substance may produce in order to determine the appropriate dose of use.[9]

This study was done to determine the citotoxicity effect amnion in sponge form on BHK-21 fibroblast cells. In vitro cytotoxicity test on BHK-21 cell culture using MTT assay method. Cultural cell lines are used because these cells are derived from embryo so that they are easy to grow and are easily reprocessed. Cell lines have been widely used to test the toxicity of dental materials and drugs, including BHK-21 cells derived from fibroblast baby hamster kidney.[10]

Materials and methods

This study is a laboratorial experimental research. The sample used in this study are amniotic membrane and sponge amnion. Amniotic membrane is used as comparator because the form of amnion that is often to used is in the form of a membrane. Also prepared cell control as a positive control which contains BHK-21 fibroblast cells in Eagle's MEM culture medium, is considered 100% living cell percentage and media control as a negative control which contains Eagle's MEM culture media, is considered 0% living cell percentage. In this study, sponge amnion that used was 0.1 mg. The weight of the sponge amnion is based on the previous study that was done by Indrawati (2017)[6] which uses sponge amnion with size 1.5 x 5 mm. Sponge amnion of this size was then weighed using a digital scale and the result is 0.1 mg of sponge amnion. The amniotic membrane weight that used adjusts to the weight of the sponge amnion.

The fibroblast cells were taken from BHK-21 cell culture in cell-line form which is the 57th passage. The fibroblast cells were grown in Eagle's MEM culture medium containing 5% FBS then incubated for 24 hours at 37 °C. Cells were transferred into roux bottles and cultured with a density of 2x10^5 cells/ml. Cells were grown on each well, containing 50 µl Eagle's MEM culture medium, with 2x10^5 cell/ml density of 50 µl cells and incubated for 24 hours at 37.°C. The amniotic membran and sponge amnion are removed from its package and then cut into pieces and weighed in digital scale and then inserted into microplate 96-well containing 50 µl of Eagle's MEM culture media and 50 µl of BHK

-21 fibroblast cells. Each group performed replication of 6 times. Microplate was incubated at 37 °C for 24 hours. The growth media of the cell was then washed with PBS 200 µl twice. The 40 ml Eagle's MEM culture medium was added to the well. The 10 µl MTT reagent was added at each well then incubated for 4 hours at 37 °C. Media in the well was discarded and added 50 µl DMSO to each well. Microplate is stirred using plate shaker for 5 minutes. The absorbance of formazan is read spectophotometrically using ELISA reader with 620 nm wavelength. The more concentrated the color, the higher the absorbance value and the more the number of living cells.

The percentage of living cells is calculated by:

$$\%\ \text{living cell} = \frac{\text{treatment} - \text{media control}}{\text{cell control} - \text{media control}} \times 100\%$$

Description:
% living cell: percentage of the number of living cell after treatment
Treatment: formazan optical density value on each sample after treatment
Media control: formazan optical density average value on media control
Cell control: formazan optical density average value on cell control

The data obtained were analyzed by using ANOVA test to see whether there is significant difference in all groups. In order to perform ANOVA test, it is necessary to perform normality test to determine the data that has been collected were normally distributed or taken from the normal population by using Kolmogorov-Smirnov test. It is also necessary to perform homogenity test to determine whether or not the variance of two or more populations using Levene's test.[11]

Results

Based on observations and calculations of the density of living fibroblast cells on amniotic membrane and sponge amnion expossure, the

results showed that both did not show any toxic effect (Table 1).

Based on Kolmogorov-Smirnov test results showed that all groups had a normality probability value of 0.200 where the value is greater than 0.05 (P>0.05) so it can be concluded that the optical density of the data is normally distributed. Then, continued with homogenity test using Levene's test obtained p-value of 0.129, which means that the data is homogeneous because P>0.05. Then, the data was tested using ANOVA test and got p-value of 0.065, where P value>0.05 (Table 2). Based on these results it can be said that the results of treatment did not give any significant difference.

	Mean	Standard deviation	% living cell
Media Control	0.06150	0.002881	0%
Cell Control	0.40967	0.006713	100%
Amniotic Membrane	0.40333	0.004676	98.16%
Sponge amnion	0.40317	0.002858	98.13%

Table 1. Mean, Standard Deviation and Percentage of Living BHK-21 Fibroblas Cells.

	Sum of Squares	Mean Square	F	Sig.
Between Groups	0.000	0.000	3.291	0.065
Within Groups	0.000	0.000		
Total	0.001			

Tabel 2. ANOVA Test Result.

Discussion

Amniotic membrane that sold on the market are placed above the sterile gauze. In its application, the sheet form and the storage can be said to be quite difficult and takes a long time to apply it because it must release the gauze fiber one by one from the thin amniotic membrane, so the risk of amniotic membrane become rupture is larger. When the amniotic membrane ruptures, the application becomes more difficult. Therefore, new innovations are created by adding gelatin to the amniotic membrane.

Gelatin acts as an adhesive material so that the amniotic membrane can be formed in a spongous form or often called as sponge amnion. In the form of a sponge, it is expected that this biomaterial is more easily applied and does not take a long time in its application. Its tube-like shape can facilitate the application of sponge amnion in tooth extraction socket. Sponge amnion is expected to be an alternative material to accelerate the wound healing in tooth extraction case. Gelatin is known to increase adhesion attachment of cells. Another benefit of gelatin is that it is an ideal material for transporting the proteins needed in the wound healing process.[12] Gelatin has biodegradable and biocompatible properties. Gelatin also has an amino acid arrangement that is almost similar to collagen which is an organic component of the body, so gelatin can be said as a non toxic material and safe to use.[13]

Fibroblasts are major element in the wound healing process. Based on ANOVA test result there is no significant difference between groups. This is because the exposure of amniotic membrane and sponge membrane do not cause cell death in high quantities so that the percentage of living cells after the treatment remains high close to the percentage of living cells in the cell control group.

The common parameter that used to determine cytotoxicity is cytotoxic dilution 50% (CD_{50}). Cytotoxic dilution 50% (CD_{50}) is the standard of a substance said toxic if the percentage of living cells after treatment is less than 50%.[14] The CD_{50} parameter is used when the use of a material is topical in the oral mucosal section.[15]

The result of the study between the groups on the BHK-21 fibroblast cells using the MTT assay method showed that the average percentage of living fibroblast cells after the exposure of amniotic membrane is 98.16% and after the exposure of sponge amnion is 98.13%. These percentages are above the CD_{50} parameter so that it can be said that the amniotic membrane and sponge amnion are not toxic. This is demonstrated by purplish-blue cells after cytotoxicity testing. If the exposed material is not toxic, the dehydrogenase is activated so that the formazane crystals will be produced.[16] When formazane crystals are produced, the fibroblast cells will be colored with purplish-blue colour. Vital cells will be colored, while cells with

membrane damage will not be colored.[17] Absorbance of this colored solution then measured using an ELISA reader at a wavelength of 620 nm.[18] The increase of the number of living cells caused the formazane formed increase so that increase the absorbance, the color can be directly related to the number of living cells.[19] The higher the purplish-blue color of the solution after cytotoxicity testing, indicating that the more number of living cells, so that given material can be said to be a non toxic material.

Based on the result of the study, it is found that amniotic membrane and sponge amnion are not toxic to BHK-21 fibroblas cells. This is consistent with the theory that the growth factors contained in the amniotic membrane and sponge amnion can make the percentage of living cells remain high. In the previous study mentioned that the use of amniotic membranes as biomaterials does not caused immunological reactions so it is safe to use.[5] Research that was done by Islamiah (2006)[20] shows that gelatin is not toxic. Sponge amnion is made up of amniotic membrane and gelatine, both of which are not toxic. Based on the results of the study that was done by the authors, it is proved that sponge amnion is not toxic to BHK-21 fibroblas cells so it can be concluded that sponge amnion is safe to use. Sponge amnion can be used as an alternative material to accelerate the wound healing of tooth extraction case.

Conclusions

Based on the result of cytotoxicity test, it can be concluded that amnion in sponge form is no toxic to BHK-21 fibroblas cells.

Declaration of Interest

The authors report no conflict of interest and the article is not funded or supported by any research grant.

References

1. Dipietro LA, Burns AL. Wound healing method and protocols, method in mollecular medicine. Totawa: Humana Press 2003;89(3):25-33.
2. Niknejad H, Peirovi H, Jorjani M, Ahmadiani A, Ghavani J, Seiflian. Properties of the amniotic membrane for potential use in tissue engineering. Eur Cells Mat. 2008;15(2):88-95.
3. Gupta A, Kedige SD, Jain K. Amnion and chorion membranes: potential stem cell reservoir with wide applications in periodontics. International Journal of Biomaterial, India, Hindawi Publishing Co 2015;2(3):2-7.
4. Yasuyoshi K, Toru H, Akimi U, Liyang W, Michiyo MN, Kenji H, Hirofumi Y, Toshiyuji Y. Zoledronic acid delays wound healing of the tooth extraction socket, inhibits oral epithelial cell migration, and promotes proliferation and adhesion to hydroxyapatite of oral bacteria, without causing osteonecrosis of the jaw, in mice.. J Bone Miner Metab 2010;28(2):165-75.
5. Shimmura S, Shimazaki J, Ohashi Y, Tsubota K. Antiinflamatory effect of amniotic membrane transplantation in ocular surface disorder. Cornes 2012;8(2):408-13.
6. Indrawati DW. Ekspresi IL-6 dan BMP-2 pada luka pencabutan gigi tikus wistar setelah pemberian sponge amnion. Thesis. Surabaya: Universitas Airlangga;2017:60.
7. Nanci, A. Ten cat's oral histology: development, structure and function. 8th ed. USA: Elsevier;2013:84,192-3.
8. Hatrick CD, Eakle S, Bird WF. Dental materials. 2nd ed. Misouri: Senders Elsevier;2011:6.
9. Hodgson, E. A textbook of modern toxicology. 3rd ed. USA: John wiley and Sons, Inc;2004:353.
10. Tamis DA, Nalbantsoy A, Elibol M, Gurhan SI. Cultivation and comparison of BHK-21 anchorage semidependent cell line in different production systems. Turkish Journal of Biochemistry 2012;3(37):281.
11. Corder GW, Foreman DI. Nonparametric statistics: a step-by-step aproach. 2nd e. New Jersey: Wiley;2014:14.
12. Wang J, Wang L, Zhou Z, Lai H, Xu Pan, Liao Lan, Wei J. Biodegradable polymer membrane applied on guided bone/tissue regeneration: a review. Polymer Journal MDPI 2016;8(115):1-20.
13. Khanchan M, Dasgupta S, Kundu B, Bissoyi A. Development of gelatin-chitosan-hydroxyapatite based bioactive bone scaffold with controlled pore size and mechanical strength. Journal of Biomaterials Science, Polymer Edition 2015;26(16):1190-209.
14. Telli C, Serper A, Dogan L, Guc D. Evaluation of the cytotoxicity of calcium phosphate root canal salers by MTT assay. Journal of Endodontics 1999;25(12):6.
15. Samuel D. Toxicity of garlic juice (Allium sativum) in BHK-21 cell. Oral Med Dent Journal 2013;5(2):19.
16. Ariani MD, Yuliati A, Adiarto T. Toxicity testing of chitosan from tiger prawn shell waste on cell culture. Dent J. (Maj. Ked. Gigi) 2009;42(1):16,99-100.
17. Schmalz G, Bindslev DA. Biocompability of dental materials. Germany: Springer;2009:162.
18. Junedy S. Isolasi dan uji sitotoksisitas senyawa alkaloid dari spon koleksi no. MD-02 Cyang. Skripsi. Yogyakarta: Fakultas Farmasi, Universitas Gadjah Mada;2005:26-7.
19. Freshney RI. Culture of animal cells a manual of basic technique and specialized applications. 6th ed. Hoboken, New jersey: John Wiley & Sons, Inc;2010:365-75,402.
20. Islamiah AR. Uji toksisitas akut gelatin babi pada tikus betina galur sprague dawley. Skripsi. Jakarta: UIN Syarif Hidayatullah;2016:46.