© Universiti Tun Hussein Onn Malaysia Publisher's Office

IJIE

Journal homepage: http://penerbit.uthm.edu.my/ojs/index.php/ijie
ISSN : 2229-838X e-ISSN : 2600-7916

The International Journal of Integrated Engineering

Cytotoxicity of HA-TCP Scaffold on Human Umbilical Cord Mesenchymal Stem Cells using MTT Assay

Vivin Ariestania[1*], Nike Hendrijantini[2], Chiquita Prahasanti[2], Eric Prasetyo[2] Mefina Kuntjoro[2], Rima Parwati Sari[1], Aulia Dwi Maharani[1]

[1]Faculty of Dental Medicine,
Hang Tuah University, JI. Arief Rahman Hakim, No.150, Surabaya, 60111, INDONESIA

[2]Faculty of Dental Medicine,
Airlangga University, JI. Mayjen Prof. Dr. Moestopo No.47, Surabaya, 60132, INDONESIA

*Corresponding Author

DOI: https://doi.org/10.30880/ijie.2022.14.02.012
Received 30 April 2021; Accepted 30 September 2021; Available online 02 June 2022

Abstract: Hydroxyapatite-tricalcium phosphate (HA-TCP) scaffold is a three-dimensional structure used to support bone regeneration. Ideally, the scaffold should be biocompatible, biodegradable, and non-toxic. The tissue engineering technique uses a combined stem cell and scaffold to repair the bone defect. To prove the non-toxic properties of the scaffold, a cytotoxicity test is needed for the human umbilical cord mesenchymal stem cell (HUCMSCs). In this study, 27 samples were divided into eight groups with a variant of scaffold HA-TCP doses ranging from 5–1000 µg. Each treatment group of scaffolds was covered in HUCMSCs. The samples were added by methyl-thiazol-tetrazolium (MTT) colorimetric assay, counted using an optical density (OD) formula, and observed by a microplate reader. The viability of the cells was observed by an inverted TMS microscope with 100x magnification. The MTT assay's test shows that the HUCMSCs cell viability shed each variant of HA-TCP scaffold dose, did not show any toxic effect. The higher the OD value, the higher the viability. It has been found that there is no significant difference between the variable scaffold doses to the percentage viability of umbilical cord cells.

Keywords: Scaffold, hydroxyapatite tricalcium phosphate, umbilical cord mesenchymal stem cells, methyl-thiazol-tetrazolium assay, cytotoxicity

1. Introduction

A bone defect is a pathological process resulting from bone component destruction [1]. In dentistry, 90% of bone defects are caused by tooth extraction, trauma, and jaw disease such as a cyst or jaw tumor, especially those with no further treatment [2]. In some bone defect cases, such as bone cancer, periodontitis, and dental implants, a bone graft is needed to replace a bone defect [3]. A bone graft is part of tissue engineering, whose function is to induce new bone formation and wound healing [4].

A scaffold is a three-dimensional structure used as framework material to support bone regeneration and new bone formation. The scaffold should have a biocompatible feature to ensure interaction with the host without rejection [5]. Ideally, biomaterial scaffold material is already biocompatible, biodegradable, and non-toxic materials [6].

Scaffold HA-TCP material is a combination of hydroxyapatite (HA) and tricalcium phosphate (TCP). Hydroxyapatite with the chemical formula $Ca_{10}(PO_4)_6(OH)_2$ is a compound of calcium phosphate that poses as the main inorganic material of bone formation [7]. Hydroxyapatite has features such as biocompatibility, is osteoconductive, and can bond with bone easily [8]. HA is an apatite mineral compound that has similarities with monoclinic and hexagonal crystal structure minerals in the bone. The utilization of HA in the medical field, especially in dentistry, keeps being

*Corresponding author: vivin.ariestania@hangtuah.ac.id

developed by current research [9]. Hydroxyapatite is a stable material with a small absorption ability. To balance this stable characteristic, TCP is added that has a higher absorption ability because TCP has biodegradable and osteoconductive features [10]. Compared to HA, TCP is more bioresorbable but less osteoinductive. TCP also has an increased ability for biodegradation and incorporation when combined 11 with HA [11].

Tissue engineering using stem cells is recommended in bone regeneration. Based on tissue engineering theory, it is possible to create the desired new tissue by combining these three components: stem cells, scaffold matrix extracellular, and growth factors, known as the Triad of Tissue Engineering concept [12].

Human umbilical cord mesenchymal stem cells (HUCMSCs) are one of the sources of mesenchymal stem cells (MSCs), other than bone marrow (BMMSCs) that comes from the umbilical cord tissue that surrounds blood vessels [13]. HUCMSCs excel not only because they are easy to get but also because they come in large numbers and are easy to duplicate quickly [14]. They also have good immuno-compatibility. The source of the stem cells is discarded and new biological waste, so there will be no ethical issues raised [15]. HUCMSCs have a more primitive cell structure than bone marrow, so they can differentiate into a variety of multipotent non-hematopoietic cells with the ability to self-repair and differentiate into other cells such as osteoblasts, fatty tissue, and chondroblasts [16]. HUCMSC has a similar morphology to fibroblast cells. Even if isolated from various umbilicus compartments, these cells are MSC with multipotent potential. As they are from an extra-embryonic source, these cells can proliferate quickly and have an increased doubling time ability compared with adult stem cells [14].

An ideal characteristic for a biomaterial to be clinically applicable is to have non-toxic features. One of the in-vitro tests for research is a cytotoxicity test where the material is cultured with an MTT assay test using fibroblast cells [17]. Evelyn (2018) [18] described that the cytotoxicity of hydroxyapatite obtained from the synthetic Anadara granosa clamshell using a hydrothermal method shows high cell viability on fibroblast cell BHK-21. The viability of HUCMSCs culture also shows that HUCMSCs are viable in gelatin and its medium α-MEM [19]. Based on the above, the researcher wants to know the toxicity property of scaffold HA-TCP in various doses using the MTT assay method.

2. Material and Method

This research was done experimentally in the laboratory, with a control group only being used post-test. The 27 samples scaffold of HA-TCP were divided into eight plus one control group with three replicate groups of HUCMSCs as the cell control with α-MEM medium. The eight treatment groups where HUCMSCs were distributed had scaffolds of HA-TCP with the following doses 5, 10, 50, 75, 100, 300, 500, and 1000 µg.

2.1 Human Umbilical Cord Mesenchymal Stem Cell (HUCMSCs) Culture

An umbilical cord 10 cm long was cut from a human placenta and washed with phosphate-buffered saline (PBS) solution to eliminate any remaining blood. The cord was then cut into 1 cm lengths to get at 1 mm^3 of *Wharton's jelly.* This isolation procedure was conducted at the stem cell laboratory at the Tropical Disease Institute of Airlangga University.

Trypsinase was conducted on the *Wharton's jelly* to separate the supernatant from its pellets. Pellets were then taken after the centrifuge process to get to the cell. DMEM/F12 was added to the pellets and then re-suspended. The medium containing a single cell was then moved to a petri dish and incubated with 5% CO_2 at 37 °C.

Cells were observed continuously with a microscope until there was 80% confluence. The medium was changed every three days. When the cells reached 80% confluence, they were separated by discarding the medium, adding trypsin then incubating them for 5 mins until the cells were released. More growth medium was added, and the cells were re-suspended. The resuspension was centrifuged for 5 mins. The supernatants were disposed after centrifugation, and the pellets were planted and incubated with 5% of CO_2 at 37 °C.

This research used HUCMSCs 4 stage passage, which was confirmed with flow-cytometry using Human Analysis KIT (BD Stemflow™, BD Biosciences, San Jose, USA) by adding primary antibodies mouse anti-human CD73, CD90, and CD105, and negative cocktail containing CD45, CD34, and CD19 for 40 mins. The results showed the cell being positive at CD73, CD90, and CD105 examination and negative at CD45 and CD34. The confirmed cells were evaluated using a TMS inverted microscope (Nikon, Minato, Japan) and were ready for further treatment.

2.2 Scaffold HA-TCP

HA-TCP is generated from the synthesis of Andara granosa blood clamshells that form a scaffold. The scaffold originally comes from powdered Andara granosa blood clamshells. Shells were first washed and cleaned and then boiled for 30 mins. The shells were then crushed with a mortar and pestle until smooth. The powder was sifted through 100 mesh filter paper to get a smoother particle form. One molar concentration of that smooth shell was dissolved into 0.6 molars of $NH_4H_2PO_4$ liquid concentration and then stirred using a magnetic stirrer for 30 mins and placed into a reactor. The reactor was placed into the electric oven and heated to 200 °C for 12 h. The reactor was then rested at room temperature. Next, the powder was washed using distilled water and then placed on the magnetic stirrer again until the water and powder were separated with liquid and the pH indicator was back to normal at 7. Methanol was used for the

powder's final wash until it formed HA particles during the dry process. The powder sample was then dried again using the electric oven at a temperature of 50°C for four hours. The sample was then sintered at 900°C for 3 h to eliminate dirt and optimize crystallization [20].

The HA powder that resulted was then formed into a scaffold with a freeze-drying method. First, 5 g HA blood clamshell powder was dissolved with 50 mL distilled water (10 w/v), and the polymer was made by dissolving 10 g gelatin in 50 mL aqua distillate (20 w/v) [20]. The scaffold doses being used in this research were 5, 10, 50, 75, 100, 300, 500, and 1000 µg with three replications for each dose.

2.3 HUCMSCs Viability

The Methyl-thiazol-tetrazolium (MTT) colorimetric assay was conducted to measure cytotoxicity by counting HUCMSCs cell's viability on the scaffold HA+TCP. Five thousand cells and 10µl HUCMSCs at the 5th passage were placed into a 96-well culture plate (Iwaki, Asahi, Japan); each well was then applied to a scaffold of HA-TCP.

After incubation, MTT solution was put into the cell that got treatment and incubated again for 3 h. This process was stopped by using 50 mL of DMSO on each well. Cell proliferation was then examined using a microplate reader (GloMax®Explorer, Promega Corporation, Fitchburg, WI, USA) with a wavelength 595 nm and counted using the optical density (OD) formula. Cell viability was then estimated using an inverted microscope TMS (Nikon Corp, Tokyo, Japan) with 100x enlargement to examine cell distribution and morphology.

2.4 Statistical Analysis

This research sample used various scaffold HA-TCP doses with three sample replications in each group, following previous research using HUCMSCs [21]. Data being counted was based on standard deviation and average. Statistical analysis used was SPSS 20.0. All data were normally distributed. One Way ANOVA test was used for a small number of the sample group. Post Hoc LSD was used to see the result comparison in each different dose group. Significant differences between groups were considered when $p > 0.05$.

3. Result and Discussion

Elisa Reader result reading was observed and counted with OD formula. Average results were: Table 1 confirmed that the percentage of viable cells of HUCMSCs on the HA-TCP scaffold has no significant difference. HA-TCP scaffold was made in many doses from 1000–5 µg had above 50% viability, which means there is no toxic effect on the cell (Fig. 1).

Table 1 shows the data was normally distributed (sig > 0.05) for any further homogeneity test. The Levene's Test's homogeneity test result showed a significance value of 0.56, or it can be interpreted as $p > 0.05$, so the data obtained had a homogeneous variant. Cell viability was seen using inverted microscope TMS (TMS Nikon Corp, Tokyo, Japan) with 100x enlargement seen in Fig. 2.

Table 1 - The mean ± standard deviation and the ANOVA test result of cytotoxicity assay scaffold HA-TCP to culture in HUCMSCs passage 5

Groups (Dose) (µg)	Viability Percentage (%) of HUCMSCs	Normality test	Homogeneity Test	ANOVA
Control	0.47 ± 0.02			
1000	69.11 ± 9.39	0.05		
500	73.93 ± 8.47	0.31		
300	70.31 ± 0.16	0.05		
100	71.33 ± 1.00	0.53	0.56	0.00
75	73.56 ± 0.27	1.00		
50	75.88 ± 0.42	0.63		
10	97.12 ± 1.85	0.58		
5	104.45 ± 5.68	0.42		

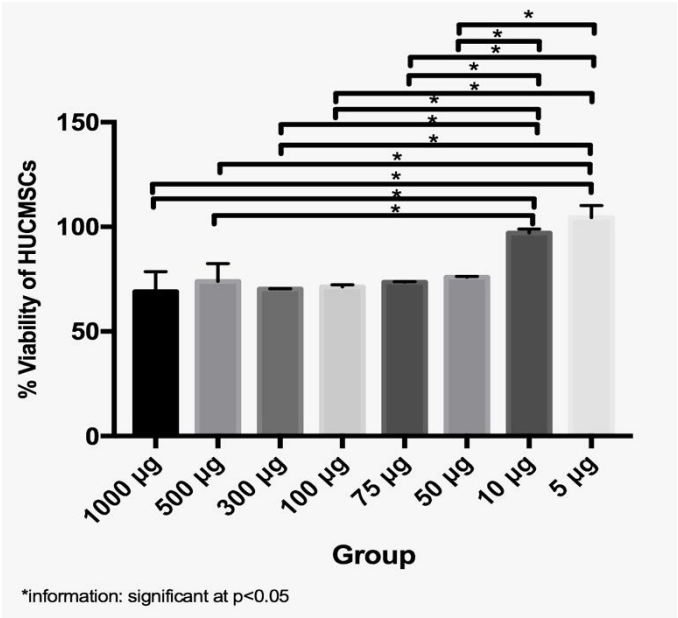

Fig. 1 - Cytotoxicity assay HA-TCP scaffold on HUCMSCs

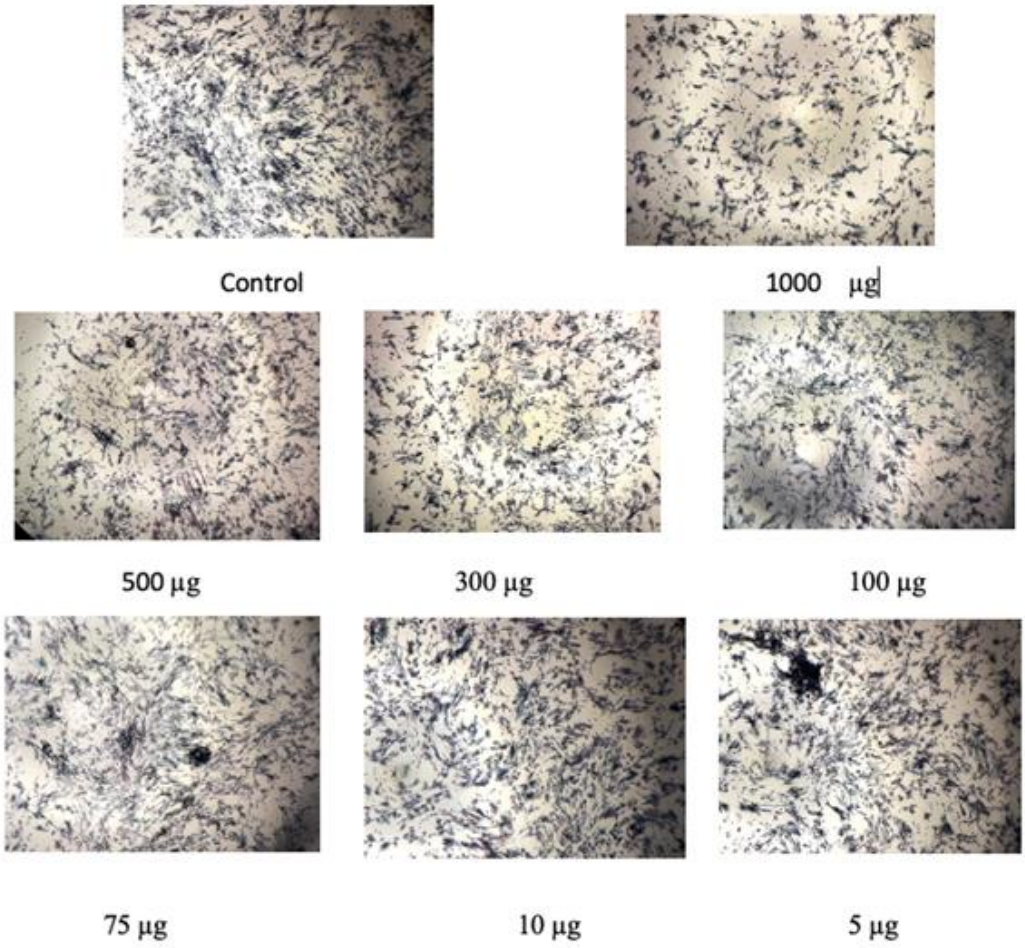

Fig. 2 - Cell viability on various doses

One of specifications of dentistry materials in order to be known as biocompatible the material must not cause a toxic reaction or any allergy [22]. The scaffold is a three-dimensional structure used as a temporary framework to support bone regeneration. The pores in the scaffold were used as a space provider for cells to attach and form new bone [5].

Ideally, the scaffold has biocompatible, biodegradable, and non-toxic features. In addition, the scaffold can support attachment, migration, proliferation, and differentiation of cells, and synthesis of new matrix [6].

Toxicity test is one of the standard specifications in development in medicine [23]. The toxicity method used for this test in this research is the MTT assay method. MTT assay is the first cell viability test developed to use 96-well microplates compatible with the High Throughput Screening (HTS) system, which is a way to select and find the potential of a microorganism in a large number in a short amount of time. The basic principle of MTT assay is to measure cellular activities based on cell metabolism ability, which is done by reducing dissolved yellow tetrazolium dye to become undissolved purple formazan products. That color-changing became the parameter for counting cell viability [24].

HUCMSCs were used in this research because these cells originate from the umbilical cord tissue that surrounds blood vessels [13]. HUCMSCs advantage, other than its ease to obtain, is that there are many and replicate quickly. HUC-MSC has similar morphology to fibroblast, which is why MSC is called fibroblast-like cells. Even when isolated from various umbilicus compartments, all the cells remain MSC with multipotent character [14].

Fibroblast is used as a parameter because this cell is easy to proliferate and subculture [25]. Furthermore, the fibroblast cell is the main element for the repair process to form a structural protein that has a role in tissue repair [26]. The fibroblast can grow in a wound area quickly and stay alive by itself, which is why the fibroblast is the favorite subject in biological research [24].

In this research, there was no difference between HUCMSCs cell viability using scaffold HA+TCP ingredients with various doses 5, 10, 50, 75, 100, 300, 500, and 1000 µg, which means that the scaffold dose did not result in a toxic effect in the cell.

Kasai, et al. [27], confirmed that hydroxyapatite increased α5β1 integrin activity. Integrin is an attachment receptor for extracellular matrix protein to transduce signal line through FAK phospholiration and towards ERK activation [27].

HUCMSCs can expose osteogenic differentiation after getting chemical exposure and osteoinductive biomaterial. Osteogenic differentiation can be proved with the increase of Runx2 and mineralization. A sample of 3D biomaterial that was frequently used was polycaprolactone-collagen-hydroxyapatite nanofibrous scaffold [14]. The 3D biomaterial is required to maintain cell morphology and biologic activity [28].

4. Conclusion

This result showed that scaffold HA-TCP does not cause a toxic effect on HUCMSCs with various doses used. It could be seen with the ANOVA data that there was no significant difference between doses of HUCMSCs. In fact, HA-TCP's scaffold from 1000 µg until 5 µg doses could be used for bone defect therapy without any toxicity effect to the human body because of using HUCMSCs as the target cell. However, the doses used are limited for particular treatments. Therefore, it needs to try more significant doses to prove that the HA-TCP scaffold is safe to use with a major bone defect. Furthermore, further researches are still required if the dose used exceeds dose variants that were already researched.

Acknowledgement

This work was done with a grant from Faculty Dental Medicine, Hang Tuah University Surabaya.

References

[1] Smrke, D., Rozman, P., Veselke, M., & Gubina, B. (2013). Treatment of bone defect - Allogenic platelet gel and autologous bone technique. Regenerative Medicine and Tissue Engineering, 325–340.
[2] Angelina, C. (2013). Penatalaksanaan kerusakan tulang pasca ekstraksi gigi dengan menggunakan bone graft. Jakarta, Indonesia: FKG-Usakti.
[3] Darmawan, D., & Yessy, W. (2008). Sintesis dan karakterisasi komposit hidroksiapatit (Ha) sebagai graft tulang sintetik. Jurnal Ilmiah Aplikasi Isotop dan Radiasi, 4(2), 143–153.
[4] Asakura, T. (2011). Bone regeneration on the epicondyle of the femur supported by silk fibroin-based scaffold: A model system for dental surgery. Journal of Insect Biotechnology and Semicolon, 80, 25–30.
[5] Laurencin, C. T., & Nair, L. S. (2008). Nanotechnology and tissue engineering: The scaffold. Boca Raton: CRC Press.
[6] Patil, A. S., Merchant, Y., & Nagarajan, P. (2003). Tissue engineering of craniofacial tissue: A review. Regenerative Medical Tissue Engineering, 2(6), 1–19.
[7] Vaccaro, A. R. (2002). The role of the osteoconductive scaffold in synthetic bone graft. Orthobluejournal, 25(5), 571-572.
[8] Simon, C. G., Antonucci, J. M., Liu, D. W., & Skrtic, D. (2005). In vitro cytotoxicity of amorphous calcium phosphate composite. Journal of Bioactive and Compatible Polimers, 3, 279-295.
[9] Rahmaniah, R. (2019). Sintesis dan karakterisasi hidroksiapatit dari cangkang kerang darah (anadara granosa) sebagai bahan baku semen tambal gigi. Teknosains: Media Informasi Sains Dan Teknologi, 13(1), 27–32.

[10] Lane, J. M., Muschler, G. F., & Kurz, L. T. (1999). Spinal fusion. Rothman RH, Simeone FA (eds): The Spine, Philadelphia.
[11] Sunil, P. S. C., & Goel, R. A. (2008). Incorporation and biodegradation of hydroxyapatite-tricalcium phosphate implanted in large metaphyseal defect-An animal study. Indian Journal of Experimental Biology, 46, 836–841.
[12] Reddi, A. H. (2007), Morphogenesis and tissue engineering. Principles of tissue engineering 3rd ed. Elsevier Academic Press: Burlington, MA. P.117.
[13] Ding, D. C., Chang, Y. H., Shyu, W. C., & Lin, S. Z. (2015). Human umbilical cord mesenchymal stem cell: A new era for stem cell therapy. Cell Transplantation, 24(3), 339–347.
[14] Wang, L., Ott, L., Seshareddy, K., Weiss, M. & Dretamore, M. (2011). Musculoskeletal tissue engineering with human umbilical cord mesenchymal stromal cells. Regenerative Medicine, 6(1), 95–109.
[15] Lidenmair, A., Hatlapatka, T., Kollwig, G., Hennerbichler, S., Gabriel, C., Wolbank, S., Redl, H., & Kasper, C. (2012), Mesenchymal stem or stromal cells from amnion and umbilical cord tissue and their potential for clinical applications. Cells, 1(4), 1061–1088.
[16] Zarrabi, M., Mousavi, S. H., Abroun, S., & Sadeghi, B. (2014). Potential uses for cord blood mesenchyimal stem cells. Cell Journal, 15(4), 274–281.
[17] Istifarah, (2012). Sintesis dan karakterisasi komposit hidroksiapatit dari tulang sotong (Sepia sp.)-Kitosan untuk kandidat aplikasi bone filler. Skripsi Sarjana Teknik: Universitas Airlangga, Surabaya.
[18] Evelyn, J., Prananingrum, W., & Ariestania V, (2018). Toksisitas hidroksiapatit hasil sintesis cangkang kerang daraha (anadara granosa) menggunakan metode hidrotermal terhadap viabilitas sel. Skripsi: Universitas Hang Tuah, Surabaya.
[19] Hendrijantini, N., Kresnoadi, U., Salim, S., Agustono, B., Retnowati, E., Rantam, F, A., Syahrial, I., Mulawardhana, P., Wardhana, M. P., & Pramono C. (2015). Study biocompatibilty and osteogenic differentiation potential of human umbilical cord mesenchymal stem cells (HUCMSCs) with gelatin solvent. Journal of Biomedical Science and Engineering, 8, 420–428.
[20] Sari, R. M., Revianti, S., Prananingrum, W. (2021). The effect of anadara granosa shell's-stichopus hermani scafold on CD44 and IL-10 expression to decrease osteoclasts in socket healing. European Journal of Dentistry, 2(2), 228-235.
[21] Kuntjoro, M., Prasetyo, E. P., Cahyani, F., Kamadjaja, M. J. K., Hendrijantini, N., Laksono, H., et al. (2020). Lipopolysaccharides cytotoxicity on human umbilical cord mesenchymal stem cell. Pesquisa Brasileira Odontopediatria e Clinica Integrade, 20, 1–7.
[22] Anusavice, K. J. (2003). Phillips: Buku ajar ilmu bahan kedokteran gigi. EGC: Jakarta, Indonesia.
[23] Niles. A. L., Moravec, R. A., & Riss, T. L. (2008). Update on in vitro cytotoxicity assays for drug development. Expert Opinion on Drug Discovery, 3(6), 70-80
[24] Rovani, C. A., & Kamizar, U. M. (2008). Perbandingan sitotoksisitas endomethasone, AH plus, dan apexit plus terhadap sel fibroblas dengan teknik root dipping. Dentofasial, 7(2), 71-72.
[25] Ayuningtyas, N. (2012). Uji sitotoksisitas ekstrak holothuria atra (teripang keling) terhadap kultur sel fibroblas. Skripsi: Universitas Hang Tuah, Surabaya.
[26] Kusyati, E. (2010). Keterampilan dan prosedur laboratorium. EGC: Jakarta, Indonesia.
[27] Kasaj, A., Willerhausen, B., Reichert, C., Kasaj, A. G., Zafiropoulos, G. G., & Schmidt, M. (2008). Human periodontal fibroblast response to a nanostructured hydroxyapatite bone replacement graft in vitro. Archives of Oral Biology, 53(7), 683–689.
[28] Zhu, S., Zhang, T., Sun, C., Yu, A., Qi, B., & Cheng, H. (2013). Bone marrow mesenchymal stem cells combined with calcium alginate gel modified by hTGF-B1 for the construction of tissue-engineered cartilage in three-dimensional condition. Experimental and Therapeutic Medicine, 5(1), 95-101.