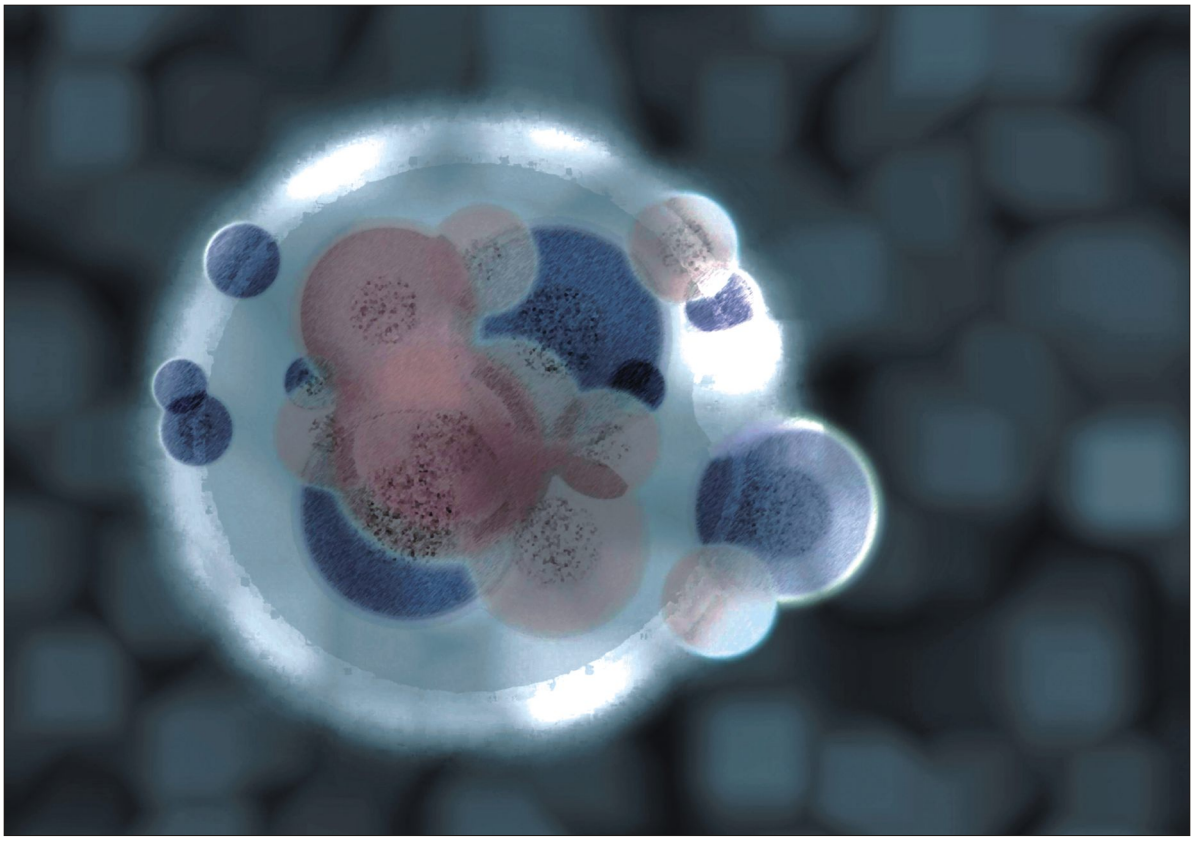

Journal of Biological Sciences

ISSN 1727-3048

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OPEN ACCESS Journal of Biological Sciences

ISSN 1727-3048
DOI: 10.3923/jbs.2019.201.209

Research Article
Morphology and Osteogenic Capability of MC3T3-E1 Cells on Granular Hydroxyapatite Scaffold

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Abstract

Background and Objective: Effectiveness of bone tissue regeneration utilizing cells and scaffold mainly determined by initial seeding density. Initial seeding density controlled cell-cell interaction which greatly influenced cell osteoblast differentiation. Therefore, the objective of this study was to determine the effect of different initial seeding densities on the morphology and osteoblast capability of MC3T3-E1 cells in hydroxyapatite granular scaffold. **Materials and Methods:** MC3T3-E1 cells at different seeding density, mainly 5×10^5 and 1×10^6 cells cm^{-2} were cultured in the 2-dimensional flask and 3-dimensional granular hydroxyapatite scaffold. Morphology of the cells in both cultures was analyzed using CellB software while biochemical activity was assessed via alkaline phosphatase (ALP) analysis. **Results:** At both culture conditions, MC3T3-E1 showed a mononucleated, fibroblast-like shape cell with extended cytoplasmic projection. Cells at higher seeding density reached confluency faster within 6 days of culture. Variations in cell seeding density significantly influenced the cell osteodifferentiation as lower initial seeding density resulted in higher ALP activity. This study has shown that the seeded cell population in the 3-dimensional scaffolds clearly affected the degree of osteoblast cell differentiation in which a higher seeding density was not necessarily better. **Conclusion:** The seeding density played an important role in influencing the corresponding cell differentiation. Therefore, it is preferable to seed cells onto scaffold at optimal lower seeding density as it influenced the corresponding cell osteoblast differentiation.

Key words: Alkaline phosphatase, osteodifferentiation, pre-osteoblastic cells, seeding density, bone tissue regeneration, granular scaffold

Citation: Farinawati Yazid, Amy Ng May Kay, Wong Yik Qin, Nur Atmaliya Luchman, Rohaya Megat Abdul Wahab and Shahrul Hisham Zainal Ariffin, 2019. Morphology and osteogenic capability of MC3T3-E1 cells on granular hydroxyapatite scaffold. J. Biol. Sci., 19: 201-209.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

There is an increase in research trends of using stem cells to increase the quality and quantity of regenerative materials and sufficient *in vitro* stem cells cultures that can ensure effective utilization in the medical field[1]. One example of an application in dentistry is the cleft palate, whereby the current treatment includes alveolar bone grafting, in which the bone is harvested from patient's iliac crest[2]. It is uncomfortable and unpractical for the patient to have two surgical site areas and the healing of both surgical areas may take up more time for the whole treatment to progress. In this developing world, tissue engineering using stem cell will be an upgraded choice of option in medical practice, consequently applicable in alveolar bone grafting[3,4]. The fundamental in stem cell research includes discovering the feasible source of stem cells by regulating certain conditions for differentiation.

Progenitor cell is a type of unipotent proliferative cells that can only develop into one type of cell. The MC3T3-E1 cells, which can be obtained from the cranium of the mouse (*Mus musculus*) is an example of a unipotent cell which has proven its capability to differentiate into osteoblast with fibroblast morphology[5]. Due to the ability to form bone mineral *in vitro*, the cells are commonly used for studies of osteoblast differentiation. In order to initiate the formation of extracellular matrix and synthesis of osteoblast-related protein, ascorbic acid played an important role in increasing alkaline phosphatase when MC3T3-E1 is treated by it[6]. A study by Quinlan *et al.*[7] has highlighted the importance of ascorbic acid and β-glycerophosphate being essential for expression of osteoblast phenotype assessed by alkaline phosphatase (ALP) activity and mineralization of extracellular matrix.

Scaffold is a solid framework that provides support to the cells for desirable cellular interactions. It is a 3-dimensional structure, which later supports the cell to grow in a 3-dimensional environment for the formation of new functional tissues[8]. Its porosity enables the diffusion of nutrients and proteins into the structure to support the growth of the cells[9]. There are many types of scaffolds, which are ceramic based, titanium, collagen, hydroxyapatite, hyaluronic acid and polymer scaffold. Hydroxyapatite scaffold is widely recognized as a bioactive material for guided bone regeneration and studies showed that a 3-dimensional interconnected porous structure is necessary to allow the cell to attach, proliferate, differentiate and provide a flowable pathway for biofluids[10,11]. Bone-mimetic scaffold by hydroxyapatite has shown a good ability in adhesion, integrin-related signaling and proliferation of mesenchymal stem cells. The hydroxyapatite matrices serve as degradable structures for osteogeneration. However, the pore sizes of scaffolds might influence the tissue growth *in vivo* and cell infiltration *in vitro* and this will significantly have an impact on the clinical usage[12].

The difference in cell seeding densities dramatically influences the cell proliferation rate, differentiation and cell-matrix interaction. The previous study has shown that a lower initial seeding density will give a higher proliferation rate on a specific time period *in vitro*[13]. Cells in lower densities may show a constant proliferation up to a specific time period. Vice versa, if higher seeding densities are used initially, there will be no significant increase in cell numbers by the same period. However, the level of ALP was shown to be dependent on cell density. The cell-cell interactions and induction of cell differentiation can be controlled by the seeding density[14].

To take full advantage of osteoblast differentiation potential of MC3T3-E1 cells, it would be highly advantageous to analyze the effect by using Alkaline Phosphatase (ALP) activity. The availability of the cells would also aid in the characterization of MC3T3-E1 cell's morphology of on hydroxyapatite scaffold. Following that, the relationship between the cell seeding densities on hydroxyapatite scaffold during osteoblast differentiation can also be determined. This study, therefore, investigated whether seeding density affects the osteogenic performance of MC3T3-E1 cells during 2-dimensional and 3-dimensional cultures.

MATERIALS AND METHODS

Cell cultures: This study had obtained approval from the Research Ethics Secretariat, Universiti Kebangsaan Malaysia (UKM) with ethics code UKM PP1/111/8/JEP-2017-272. This research has been conducted in Cell and Tissue Culture Laboratory, Faculty of Dentistry, UKM, from April, 2017 to March, 2018. The MC3T3-E1 cells (ATCC, USA) were cultured in complete medium consist of α-Minimal Essential Medium (α-MEM) (Gibco, Grand Island, NY, USA), 1% (v/v) penicillin/streptomycin (Himedia, India), 10% (v/v) Fetal Bovine Serum (FBS) (Gibco, Grand Island, NY, USA) and 1 mM sodium pyruvate (Hyclone, USA) and incubated at 37°C, 95% (v/v) humidity and 5% (v/v) CO_2 humidified incubator. The complete medium was changed every 3 days until cells reached a confluency of approximately 80% and accutase (Himidia, India) was used to detach cells from the surface of the plate during the subculturing process. Cells at the 7th-8th passage were used to differentiate into an osteoblast.

Cell seeding on Granumas®: The type of scaffold used was Granumas® (GranuLab, Malaysia), which consists of calcium phosphate bioceramic constituents in the form of mineral hydroxyapatite. The scaffolds were placed in 96-well plates and incubated with complete medium for 2 h. The cells amounting to 5×10^5 cells cm^{-2} and 1×10^6 cells cm^{-2} was resuspended, respectively in 100 µL complete medium. The scaffold incubation mediums were removed and the resuspended cells were gently pipetted onto the scaffold. Then, the scaffold was placed in a humidified incubator at 37°C for 24 h for cells attachment.

Osteoblast differentiation: For 2D and 3D osteoblast differentiation, the complete medium was added with differentiation factors consist of 50 mg mL^{-1} ascorbic acid (Sigma, USA) and 10 mM (w/v) β-glycerophosphate (Sigma, USA) as described by Kermani *et al.*[1]. Osteoblast differentiation medium was changed every 3 days throughout 21 days of induction. Cells in complete medium without differentiation factors were used as a negative control for osteoblast differentiation. Osteoblast potential of cells were evaluated in term of morphology and alkaline phosphatase (ALP) activity.

Cell morphology: For morphological analysis, MC3T3-E1 cells in 2-dimensional culture were placed into a 6 well-plate, while cells in 3-dimensional culture were placed into a 96-well plate. Both culture conditions were treated with complete medium and differentiation medium, respectively, up to their confluency. The CellB software from the inverted microscope (IX71 inverted microscope, Olympus) was used to record the morphology of the cells throughout the culture.

Alkaline phosphatase (ALP) assay: Alkaline phosphatase assay was done on cells that have been differentiated into osteoblast cells as described by Yazid *et al.*[15]. Each cell sample was washed with Tris-buffered saline (TBS) and 30 µL lysis buffer formed by 0.1% (v/v) Triton-X 100 (Sigma, USA) and cold TBS was added in each well. The harvested cells lysate was freeze-thawed three times for 10 min at 1600 g. Supernatant obtained was incubated in 0.1 M sodium carbonate-bicarbonate buffer (pH 10) (MERCK, Germany) consisting of 2 mM (v/w) magnesium sulphate (Sigma, USA) and 6 mM (w/v) p-nitrophenyl phosphate (pNPP) for 30 min at 37°C.

In order to stop the enzyme-substrate reaction, 1.5 M sodium hydroxide (MERCK, Germany) was placed into each well. Measurement of optical density was then taken and recorded at a wavelength of 405 nm with a spectrophotometer (Varioskan Flash, Thermo). On the other hand, Bradford analysis was also performed using the remaining cell lysate and the measurement of optical density was taken at 450 and 595 nm. Bradford analysis was used to measure protein quantity in a cell lysate.

Statistical analysis: All the experiments were performed in triplicate (n = 3) and the results were expressed as the mean±standard deviation and t-test was used to make statistical comparisons in between the seeding densities within the same day of osteoblast induction. Two-way analysis of variance (ANOVA) was also conducted to determine the overall difference between the two seeding densities. The p-value of less than 0.05 will be considered statistically significant.

RESULTS

Morphology of MC3T3-E1 cells on 2-dimensional culture: The morphology of MC3T3-E1 cells at different density was examined with an inverted microscope. The MC3T3-E1 cells at the density of 5×10^5 and 1×10^6 cells cm^{-2} were observed and it can be seen that at day 0, the cells manifest as rounded morphology following 4 h of seeding (Fig. 1a, 2a). Cells at both densities begin forming a monolayer with fibroblast shape after day 1 of culture (Fig. 1b, 2b). At day 3, cells at lower seeding density started to become semi-confluent at about 50% confluency while cells at higher seeding density began to become confluent at about 90% confluency (Fig. 1c, 2c). However, there is a difference of confluency between difference density at day 6 in which cells at higher seeding density remained at 90% confluent compared to 70% confluent reached by cells at lower seeding density (Fig. 1d, 2d).

Bone morphology in 3-dimensional granular hydroxyapatite scaffold: The comparison of bone morphology in 3-dimensional culture shown that there is a presence of bone formation in both seeding densities of 5×10^5 and 1×10^6 cells cm^{-2} throughout 21 days of osteoblast induction. For both seeding densities, there is no bone formation observed during the initial culture, with the cells being seen in the background (Fig. 3a, 4a). However, there is bone formation from as early as day 7 of culture with a reduction in empty space between the scaffolds (Fig. 3b, 4b). On day 14, it is shown that bone formation is intense in the cells at a seeding density of 1×10^6 cells cm^{-2} when compared to 5×10^5 cells cm^{-2} (Fig. 3c, 4c), which shows more prominent bone growth on day 21 (Fig. 3d, 4d). Moreover, degradation of Granumas® can be notified in both conditions with the increase of bone formation surrounding the targeted area and reduction of space between scaffolds.

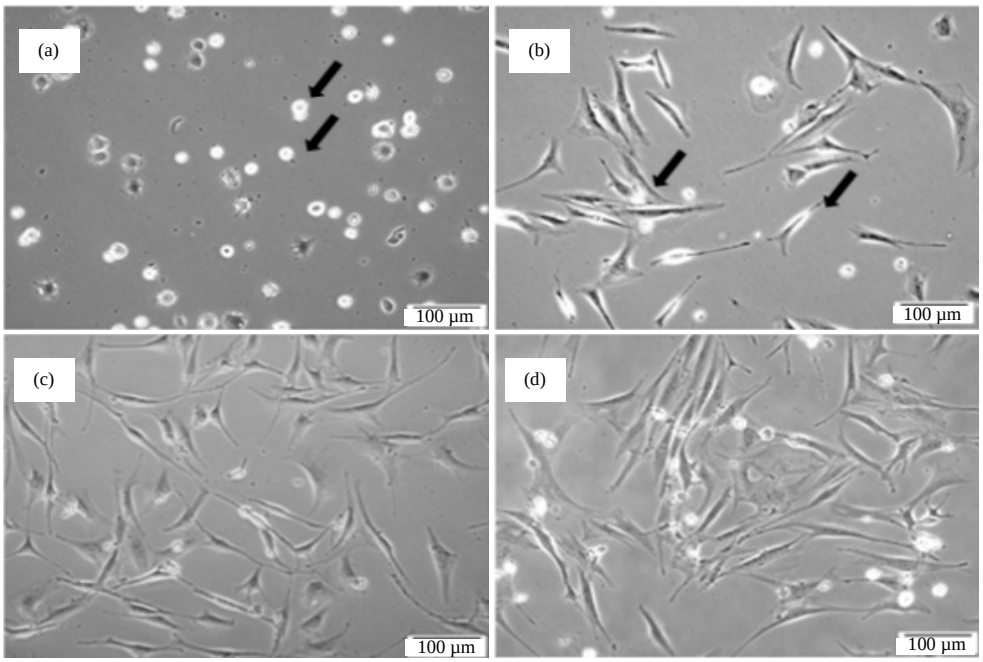

Fig. 1(a-d): Morphology of MC3T3-E1 cells at initial seeding density of 5×10^5 cells cm^{-2} in 2-dimensional culture condition up to confluency (magnification 10×) (a) At day 0, the cells at 4 h of seeding, (b) Cells begin to manifest fibroblast morphology at day 1 of culture, (c) 50% confluent of cells at day 3 of culture and (d) 70% confluent of cells at day 6 of culture. Arrows indicate rounded and fibroblast morphology of cells during culture

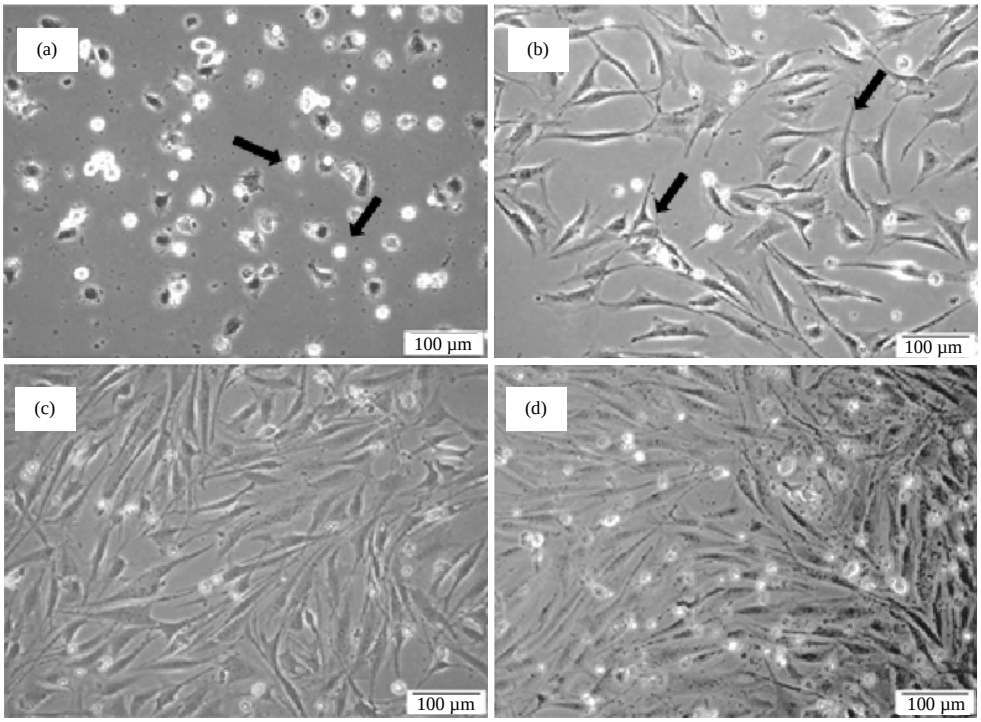

Fig. 2(a-d): Morphology of MC3T3-E1 cells of initial seeding density 1×10^6 cells cm^{-2} in 2-dimensional culture condition up to confluency (magnification 10x). (a) At day 0, the cells at 4 h of seeding, (b) Cells begin to manifest fibroblast morphology at day 1 of culture, (c) 90% confluent of cells at day 3 of culture and (d) Cells remain 90% confluent at day 6 of culture. Arrows indicate rounded and fibroblast morphology of cells during culture

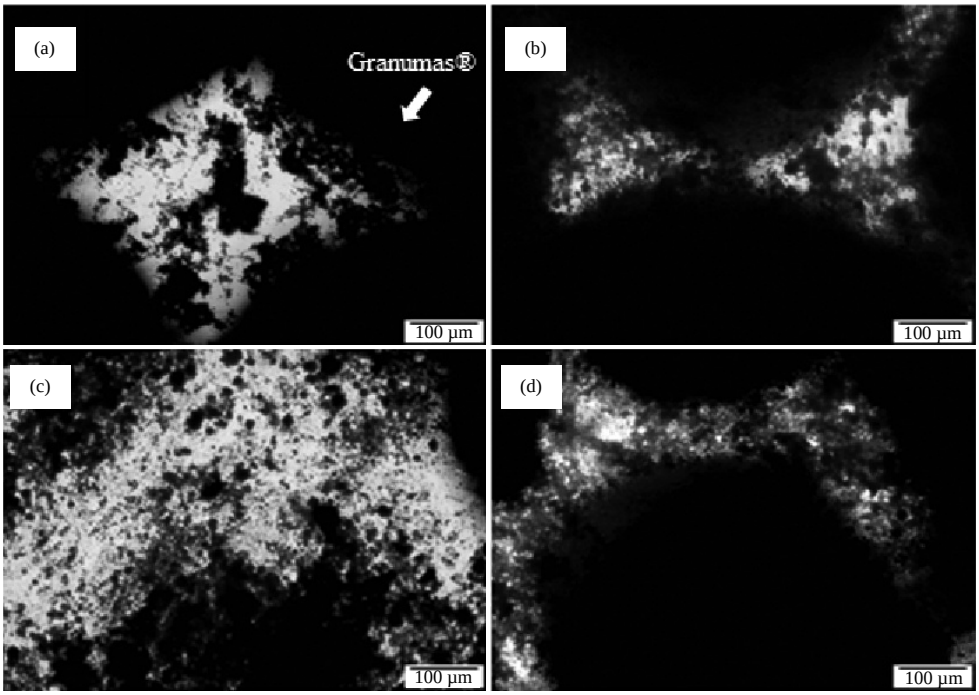

Fig. 3(a-d): Morphology of MC3T3-E1 cells at seeding density 5×10^5 cells cm^{-2} on Granumas® scaffold throughout 21 days of osteoblast induction (magnification of 10x) (a) Cell at initial culture, (b) Cells attachment and reduced in space between scaffold at day 7 of culture, (c) Degradation of scaffold parallel to the bone formation at day 14 and (d) Intense volume of bone formation at day 21

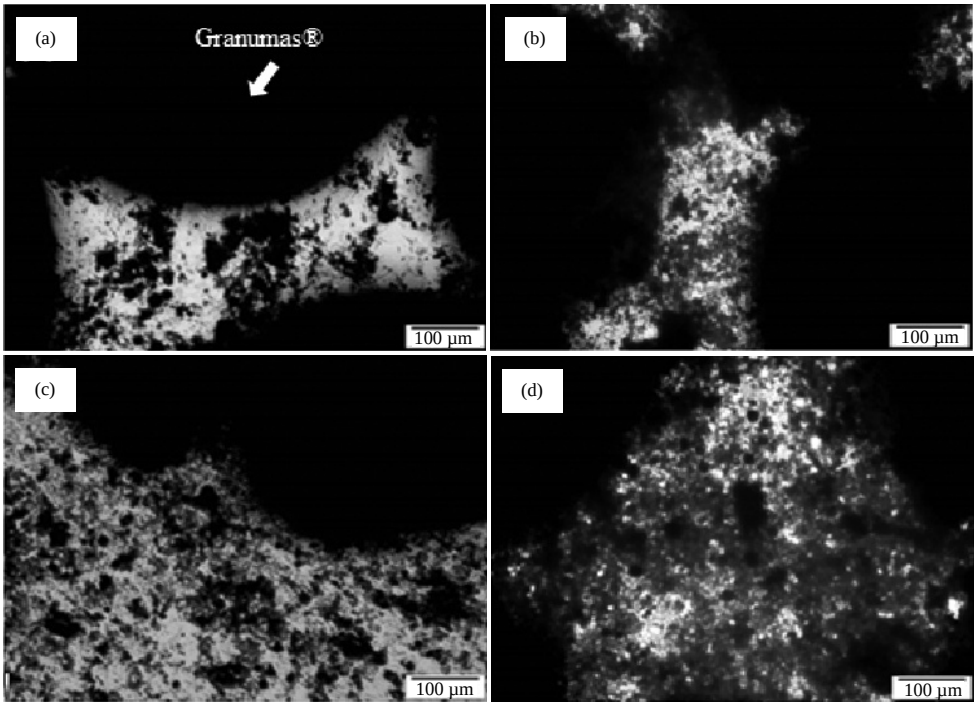

Fig. 4(a-d): Morphology of MC3T3-E1 cells at seeding density of 1×10^6 cells cm^{-2} on Granumas® scaffold throughout 21 days of osteoblast induction (magnification of 10x). (a) Day 0 of cells seeded on scaffold, (b) Cells attachment and reduced in space between scaffold at day 7 of culture, (c) Day 14 of culture with bone formation and (d) Day 21 of culture with significant intense volume of the bone formation

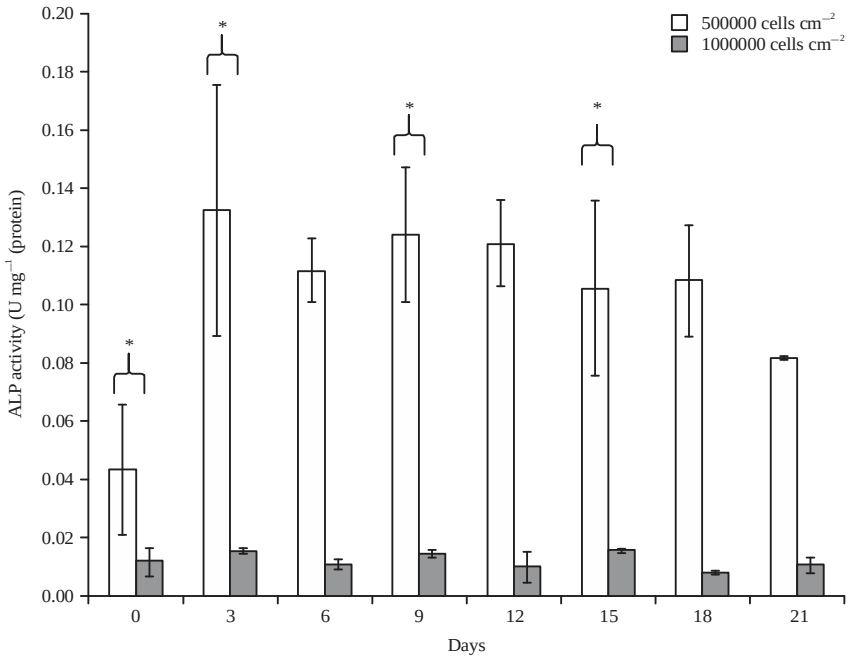

Fig. 5: ALP activities for MC3T3-E1 cells with density of 5×10^5 cells cm^{-2} and 1×10^6 cells cm^{-2} in 2-dimensional culture with osteoblast differentiation medium for 21 days of induction

*t-test ($p<0.05$) indicate a significant difference between two different seeding densities on differentiated MC3T3-E1 cells

ALP activity of MC3T3-E1 in 2-dimensional culture: Cell at both seeding density capable to differentiate toward osteoblast. Comparison of ALP activity between cells at initial culture and during 21 days of osteoblast induction was conducted for each seeding densities. However, cells at seeding density of 5×10^5 and 1×10^6 cells cm^{-2} showed significant difference throughout 21 days of analysis when compared to control cells. Comparison of ALP activity between two seeding densities showed a statistically significant difference on day 0, 3, 9 and 15 in favour of cells at lower seeding density ($n = 3$, $p<0.05$) (Fig. 5). Although there is a significant difference between the two densities in certain days, two-way ANOVA test showed that the overall ALP activities were not significant between these two densities.

ALP activity of MC3T3-E1 cells in 3-dimensional culture: The comparison of ALP activity in 3-dimensional (Granumas®) was examined between MC3T3-E1 cells in densities of 5×10^5 cells cm^{-2} and 1×10^6 cells cm^{-2}. Throughout 21 days of osteoblast induction, the scaffold seeded with cells at density of 5×10^5 cells cm^{-2} has higher ALP activity compared with cell at density of 1×10^6 cells cm^{-2}. Cells at higher seeding density showed peaked ALP activity after day 14 while cells at lower seeding density reached peaked ALP activity at day 21. Comparison of ALP activity between two seeding densities was evaluated using t-test and it shows statistically significant difference on day 0, 7 and 14 which favor cells at density of 5×10^5 cells cm^{-2} (Fig. 6). Although there is a significant difference between the two densities in certain days, two-way ANOVA test showed that the overall ALP activities were not significant between these two densities.

DISCUSSION

Suitable micro-environment involving cell-cell interaction within compatible scaffold may greatly influence the seeded population behavior and the eventual structure and properties of the engineered tissue. The cell-cell interactions may be controlled by the seeding density and eventually induce a specific cell differentiation needed for each tissue type. As shown in the microscopic records of the cells at a higher density, the replication decreases until the cells become quiescent after reaching 90% confluent. Meanwhile, it takes a longer period for the cells at lower seeding density to express similar event. Contact inhibition was apparent in this study, as cell ability to proliferate were proportional to their seeding densities. Spitzer *et al.*[16] showed that an increase in mesenchymal stem cells seeding density will cause an increase in viable density of cells. Seeding of cells at higher density may result in the inhibition of cell proliferation and reduction of cell function during culture.

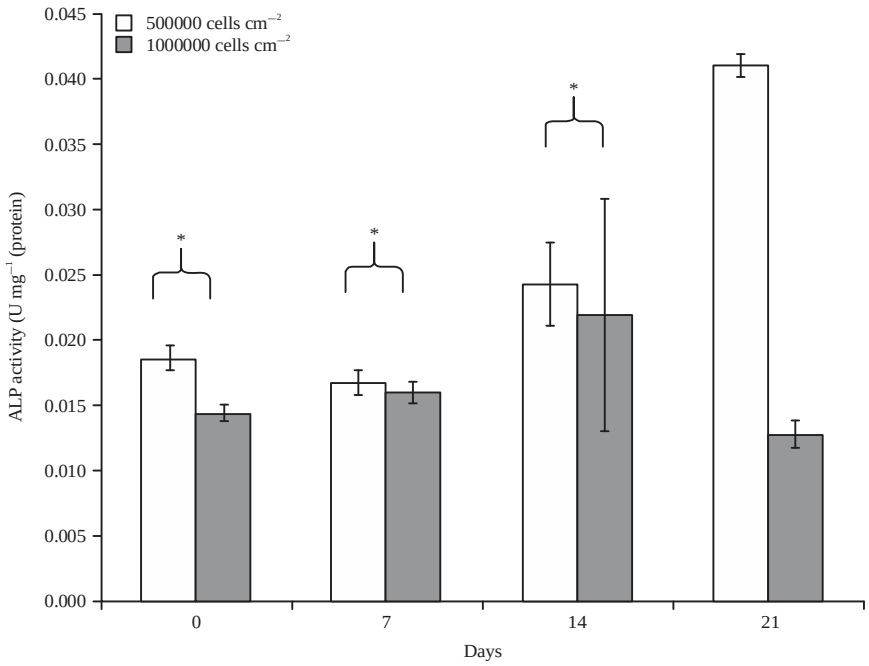

Fig. 6: ALP activities of MC3T3-E1 cells of seeding density of 5×10^5 cells cm^{-2} and 1×10^6 cells cm^{-2}, which is seeded in 3-dimensional Granumas® scaffolds for 21 days of osteoblast induction

*t-test ($p<0.05$) indicate a significant difference between two different seeding densities on differentiated MC3T3-E1 cells

The osteogenic performance of MC3T3-E1 cells in hydroxyapatite scaffold can be measured by bone regeneration. In this study, granular hydroxyapatite scaffold used was Granumas® which is similar to the human bone material. Current results demonstrated that the MC3T3-E1 cells are capable of forming bone on this bioceramic scaffold. A study by Baudequin *et al.*[17] suggested that osteoblastic MC3T3-E1 cells capable of regenerate bone when seeded on a bioceramic granular scaffold with promising cellular and mechanical properties. Higher osteoblast cell distribution with increased survival and proliferation rate has been documented in a study by Pourmollaabbassi *et al.*[18]. Moreover, differentiated MC3T3-E1 cells on granular hydroxyapatite scaffold demonstrated a characteristic of a matrix and minerals phases in which the structure and composition observed are highly similar to mouse bone by Addison *et al.*[19]. Cells at a higher seeding density generate more bone at a faster rate which is within 14 days of culture while cells at lower seeding density required at least 21 days to generate the same results. Increase in bone formation results from higher seeding density might due to the increase of cell-cell interaction and subsequently cells proliferation ability.

Alkaline phosphatase (ALP) is an early biochemical marker for osteoblast differentiation as it is vital for bone matrix mineralization as supported by Yazid *et al.*[5]. Tripathi and Basu[20] have proven that the ALP assay is a sensitive and reproducible method for identifying ALP activity of isolated osteoblast. The result from this study has shown that MC3T3-E1 cells have the ability to differentiate into osteoblast cells and the ALP activity is greater in lower seeding density for both culture conditions. In the 2-dimensional analysis, MC3T3-E1 cells at lower seeding density showed statistically significant difference in ALP activity when compared to cells at higher seeding density. This result also significant to 3-dimensional analysis. The MC3T3-E1 cells with lower seeding densities seeded in Granumas® reached peaked ALP activity at day 21 while 14 days was required for higher seeding density to manifest the same result. Through this study, initial seeding density affects the osteoblast differentiation potential of cells in which higher seeding density was not necessarily promoted better bone formation. A study by Kruyt *et al.*[21] has demonstrated a logarithmic relationship between cell density and bone formation. This indicated that increasing cell numbers above optimal cell density did not stimulate more bone formation. One of the reasons for the decrease in ALP activity of higher seeding density may due to diffusional problems from too many cells as the seeded cells cannot obtain the necessary nutrients to survive and eventually will die or leave the scaffold.

CONCLUSION

This study had observed that the seeded cell population in the three-dimensional granular hydroxyapatite scaffolds clearly affected the degree of osteoblast cell differentiation in which a higher seeding density was not necessarily better. The seeding density played a major role in influencing the corresponding cell differentiation. This study suggests that different cell density can be investigated in tissue engineering in other types of scaffolds.

SIGNIFICANCE STATEMENT

This study discovered the osteogenic effect of different cell seeding densities of MC3T3-E1 in two different culture conditions that can be beneficial for determining the optimal initial number of cells required in the 3-dimensional culture of scaffold. Optimized cell seeding density is important in bone tissue regeneration study as it can control the outcome of cells seeded onto scaffold. This present study will help related researchers to uncover the critical area for efficient delivery of a sufficient number of cells onto the 3-dimensional scaffold that many researchers were not able to explore. Thus, a new theory on successfully bone tissue regeneration utilizing cells and scaffold may be arrived at.

ACKNOWLEDGMENT

The authors gratefully acknowledge Universiti Kebangsaan Malaysia through Fundamental Research Grant Scheme (FRGS/1/2015/SG05/UKM/02/2) from the Ministry of Higher Education Malaysia for providing an opportunity to work on this research.

REFERENCES

1. Kermani, S., R.M.A. Wahab, I.Z.Z. Abidin, Z.Z. Ariffin, S. Senafi and S.H.Z. Ariffin, 2014. Differentiation capacity of mouse dental pulp stem cells into osteoblasts and osteoclasts. Cell J., 16: 31-42.
2. Ong, J.L. and T. Guda, 2017. Translating Biomaterials for Bone Graft: Bench-top to Clinical Applications. CRC Press, Boca Raton, FL., USA., ISBN-13: 9781315363516, Pages: 263.
3. Han, J., D. Menicanin, S. Gronthos and P.M. Bartold, 2014. Stem cells, tissue engineering and periodontal regeneration. Aust. Dent. J., 59: 117-130.
4. Ude, C.C., A. Miskon, R.B.H. Idrus and M.B. Abu Bakar, 2018. Application of stem cells in tissue engineering for defense medicine. Military Med. Res., Vol. 5. 10.1186/s40779-018-0154-9.
5. Yazid, M.D., S.H.Z. Ariffin, S. Senafi, M.A. Razak and R.M.A. Wahab, 2010. Determination of the differentiation capacities of murines' primary mononucleated cells and MC3T3-E1 cells. Cancer Cell Int., Vol. 10. 10.1186/1475-2867-10-42
6. Kim, M.B., Y. Song and J.K. Hwang, 2014. Kirenol stimulates osteoblast differentiation through activation of the BMP and Wnt/β-catenin signaling pathways in MC3T3-E1 cells. Fitoterapia, 98: 59-65.
7. Quinlan, E., A. Lopez-Noriega, E. Thompson, H.M. Kelly, S.A. Cryan and F.J. O'Brien, 2015. Development of collagen-hydroxyapatite scaffolds incorporating PLGA and alginate microparticles for the controlled delivery of rhBMP-2 for bone tissue engineering. J. Controlled Release, 198: 71-79.
8. Knight, E. and S. Przyborski, 2015. Advances in 3D cell culture technologies enabling tissue-like structures to be created *in vitro*. J. Anat., 227: 746-756.
9. Loh, Q.L. and C. Choong, 2013. Three-dimensional scaffolds for tissue engineering applications: Role of porosity and pore size. Tissue Eng. Part B: Rev., 19: 485-502.
10. Ramay, H.R. and M. Zhang, 2003. Preparation of porous hydroxyapatite scaffolds by combination of the gel-casting and polymer sponge methods. Biomaterials, 24: 3293-3302.
11. Sulaiman, S.B., T.K. Keong, C.H. Cheng, A.B. Saim and R.B.H. Idrus, 2013. Tricalcium Phosphate/Hydroxyapatite (TCP-HA) bone scaffold as potential candidate for the formation of tissue engineered bone. Indian J. Med. Res., 137: 1093-1101.
12. Phipps, M.C., W.C. Clem, J.M. Grunda, G.A. Clines and S.L. Bellis, 2012. Increasing the pore sizes of bone-mimetic electrospun scaffolds comprised of polycaprolactone, collagen I and hydroxyapatite to enhance cell infiltration. Biomaterials, 33: 524-534.
13. Grayson, W.L., S. Bhumiratana, C. Cannizzaro, P.H.G. Chao, D.P. Lennon, A.I. Caplan and G. Vunjak-Novakovic, 2008. Effects of initial seeding density and fluid perfusion rate on formation of tissue-engineered bone. Tissue Eng. Part A, 14: 1809-1820.
14. Buxton, P.G., M. Bitar, K. Gellynck, M. Parkar and R.A. Brown *et al*., 2008. Dense collagen matrix accelerates osteogenic differentiation and rescues the apoptotic response to MMP inhibition. Bone, 43: 377-385.
15. Yazid, F., N.A. Luchman, R.M.A. Wahab, S.H.Z. Ariffin and S. Senafi, 2018. Proliferation and osteoblast differentiation mice dental pulp stem cells between enzyme digestion and outgrowth method. Sains Malaysiana, 47: 691-698.

16. Spitzer, T.L., A. Rojas, Z. Zelenko, L. Aghajanova and D.W. Erikson *et al*., 2012. Perivascular human endometrial mesenchymal stem cells express pathways relevant to self-renewal, lineage specification and functional phenotype. Biol. Reprod., Vol. 86, No. 2. 10.1095/biolreprod.111.095885.
17. Baudequin, T., F. Bedoui, M. Dufresne, P. Paullier and C. Legallais, 2015. Towards the development and characterization of an easy handling sheet-like biohybrid bone substitute. Tissue Eng. Part A, 21: 1895-1905.
18. Pourmollaabbassi, B., S. Karbasi and B. Hashemibeni, 2016. Evaluate the growth and adhesion of osteoblast cells on nanocomposite scaffold of hydroxyapatite/titania coated with poly hydroxybutyrate. Adv. Biomed. Res., Vol. 5. 10.4103/2277-9175.188486.
19. Addison, W.N., V. Nelea, F. Chicatun, Y.C. Chien and N. Tran-Khanh *et al*., 2015. Extracellular matrix mineralization in murine MC3T3-E1 osteoblast cultures: An ultrastructural, compositional and comparative analysis with mouse bone. Bone, 71: 244-256.
20. Tripathi, G. and B. Basu, 2012. A porous hydroxyapatite scaffold for bone tissue engineering: Physico-mechanical and biological evaluations. Ceram. Int., 38: 341-349.
21. Kruyt, M., J. de Bruijn, J. Rouwkema, C. van Blitterswijk, C. Oner, A. Verbout and W. Dhert, 2008. Analysis of the dynamics of bone formation, effect of cell seeding density and potential of allogeneic cells in cell-based bone tissue engineering in goats. Tissue Eng. Part A, 14: 1081-1088.