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RESEARCH ARTICLE

Hydroxyapatite (HA) scaffold supplemented with VEGF and BMP-2 growth factors enhanced osteogenic proliferation and differentiation of MC3T3-E1 cells

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Abstract

Background: Engineered bone tissue, made up of hydroxyapatite (HA) scaffold is the current alternative in bone transplant. The scaffold needs to mimic the extracellular matrix (ECM) to support bone growth and repair. Vascular endothelial growth factor (VEGF) and bone morphogenetic protein 2 (BMP-2) are vital in producing a suitable ECM environment to promote bone regeneration. **Objective:** To elucidate the synergistic effect of VEGF and BMP-2 growth factors on MC3T3-E1 osteoblast cells. **Method:** MC3T3-E1 cells were cultured *in vitro* and seeded onto a HA scaffold supplied with VEGF and BMP-2 growth factors. The characterisation of its mineralisation was evaluated on Day three and Day seven using the Alizarin Red staining (ARS) technique. **Result:** Positive ARS staining was observed in the HA scaffold supplied with VEGF and BMP-2 growth factors *in vitro*, thus indicating that a high level of calcium was deposited. These results suggest that the combined use of VEGF and BMP-2 will increase cell proliferation and osteoblast differentiation *in vitro*. **Conclusion:** Therefore, the synergistic effects of VEGF and BMP-2-loaded HA scaffold confirm the process as a promising approach to enhance the osteogenic effect and support cellular functions of osteoblastic cells in bone tissue engineering applications.

Introduction

The percentage of ageing individuals above 65 years old is expected to increase by 2040 as speculated by the Department of Statistics, Malaysia (DOSM) (Rashid *et al*, 2016). The elderly population in Malaysia is expected to be 15% of the total population (Tengku, 2015). Hence, an osteoporotic fracture will be a significant threat due to the increasing number of ageing populations.

The osteoporosis-associated bone disorder caused by trauma, severe infection, tumour resection, and skeleton abnormalities may cause the formation of critical-size bone defects. These may require bone grafting transplantation as an intervention technique. Autograft involves harvesting the patient's bone and transplanting it to the fracture sites while allograft involves harvesting bone from one individual and transplanting it into another individual within the same species. Both techniques pose some limitations such as a high risk of immunological reactions, the transmission of infection, and donor-site injury (Oryan *et al*., 2014).

This limitation has led to the development of engineered bone tissue as a potential alternative to the conventional use of bone grafts due to their unlimited supply and their ability in hindering disease transmission. Engineered bone tissue involves the development of new functional bone to induce regeneration via a synergistic combination of the biomaterial scaffold, cells, and biochemical factors (Bouet *et al*, 2015).

A scaffold acts as a vital part of engineered bone tissue to mimic the structure and function of the natural bone

ECM and subsequently promote cell adhesion, proliferation, and differentiation for bone tissue repair. HA is a popular calcium phosphate bio-ceramic scaffold that has been used widely in bone tissue engineering due to its favourable osteoinductivity and biocompatibility properties (Mohammad *et al*, 2020).

Bioactive molecules such as VEGF and BMP-2 are vital in producing a suitable ECM environment in bones. VEGF plays a role in stimulating osteogenesis (Kaigler, Silva, & Mooney, 2013) and acts as an effective regulator of vasculogenesis (Coultas, Chawengsaksophak & Rossant, 2005). In addition, VEGF also plays an important role in improving angiogenesis which is vital for bone repair (Tan *et al.*, 2010).

On the other hand, BMP-2 is an effective regulator of osteoblast proliferation and differentiation that promotes bone formation (Reddi, 1998). In 2017, a study by Park and his teams reported that BMP-2 enhances the osteogenic differentiation of stem cells and promotes bone regeneration (Park *et al*., 2017). Moreover, research has reported that BMP-2 increases proliferation and differentiation when integrated with an HA scaffold *in vitro* (Zhu *et al*., 2017).

A previous study showed that the use of VEGF or BMP-2 could be a promising approach to promote bone regeneration (Hu & Olsen, 2016; Lowery & Rosen, 2018). Yet, there is currently very limited study on the combined effect of VEGF and BMP-2 incorporated with HA scaffold. Hence, this study is expected to elucidate the synergistic effect of applying both the VEGF and BMP-2 on bone regeneration in comparison to the application of a single factor alone.

Methods

In vitro cell culture

Mouse embryo osteoblast precursor cells (MC3T3-E1) subclone 14 were cultured in a complete medium consisting of α-MEM (Gibco) supplemented with 10% foetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco) cultured in a flask at 37°C in a humidified chamber with 5% CO^2. The cell culture condition was maintained in a sterile environment.

Engineered bone model

HA bone scaffold was purchased from GranuLab, Malaysia, and was incubated with a complete media in a six-well cultured plate for one to two hours before cell seeding. The scaffold incubation medium was removed, and 1×10^6 cells were gently pipetted into the bone scaffold. The model of the engineered bone was incorporated with VEGF (10 ng/mL) alone or in combination with BMP-2 (50 ng/mL) as shown in Table I.

Table I: Treatment groups

Growth factor	Groups			
	Control	V	B	BV
VEGF (10 ng/mL)	–	+	–	+
BMP-2 (50 ng/mL)	–	–	+	+

Alizarin Red S staining

Alizarin Red S staining (ARS) was performed on Day Three and Day Seven after treatment to evaluate the calcium deposition. The engineered bone scaffold in the six-well plate was rinsed twice with phosphate buffer saline (PBS) (ThermoFisher Scientific). Then, the cells were fixed by adding 4% formalin and incubated for 15 minutes at room temperature. The fixative reagent was discarded and the cells in each well were washed twice with distilled water. A total of 1.0 mL of ARS working solution was added to each well and the plate was incubated at room temperature for 20 minutes.

The ARS dye solution was prepared by dissolving 0.7 g of ARS powder (Abcam) in 50 ml of distilled water. The pH of the solution was adjusted to 4.1-4.3 by adding Sodium hydroxide. The stained cell was visualised using an inverted microscope (Olympus CKX31) and the images were documented.

Quantification of mineralization

The stained cells were washed with deionized water and incubated in 1.0 mL of de-staining solution (20% methanol and 10% acetic acid in deionized water) for 15-20 minutes. The absorbance values were then measured using a microplate reader at 405 nm.

Statistical analysis

All experiments were performed in triplicate (n=3) and the results were expressed as mean ± standard deviation. Statistical differences were analysed using One-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered to be significant.

Results

ARS staining was performed on Day three and Day seven of incubation and positive ARS staining demonstrated calcium deposits. Calcium deposits were viewed using an inverted microscope at 4X

magnification and the images were captured using a microscope with a dino-eye piece camera. The calcium deposits appeared as a red-orange spot under the light inverted microscope.

Calcium deposition was observed in the engineered bone model *in vitro* treated with control, VEGF (V group), BMP-2 (B group), and BMP-2 **+** VEGF (BV group). V groups showed the deepest staining on Day three while on Day seven, there was an increase of mineralisation in the BV group compared to other treatment groups (Figure 1).

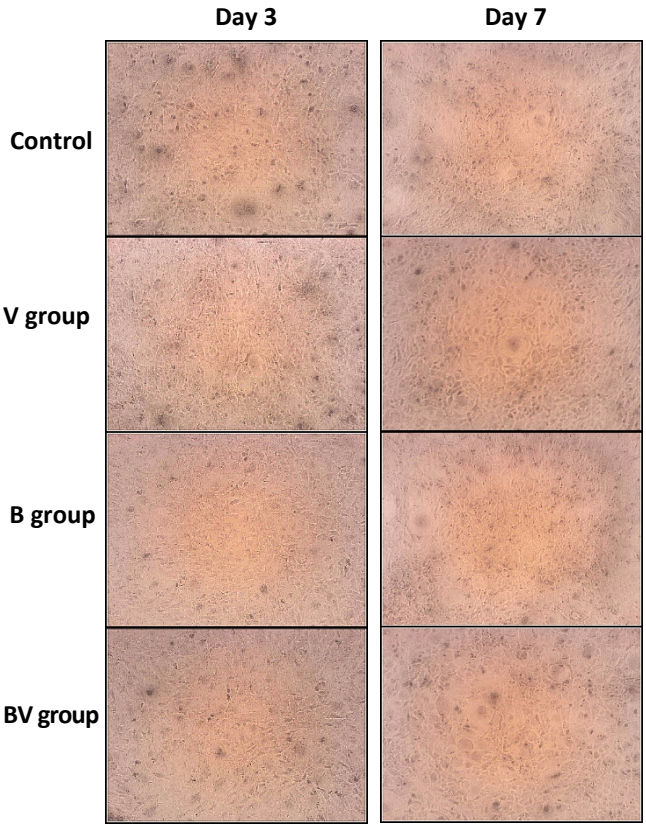

Figure 1: Alizarin Red S staining of the engineered bone model on Day 3 and Day 7 visualised under a light inverted microscope

A semi-quantitative analysis was performed to compare the rate of calcium deposition by comparing the mean absorbance values as shown by the graph in Figure 2. The data were tabulated in Table II. The rate of calcium deposition on Day Three was higher in the engineered bone scaffold incorporated with VEGF. Meanwhile, on Day Seven, the results indicated the highest calcium deposition for the combination of VEGF and BMP-2 incorporated with HA scaffold compared to the use of a single factor of VEGF or BMP-2 alone.

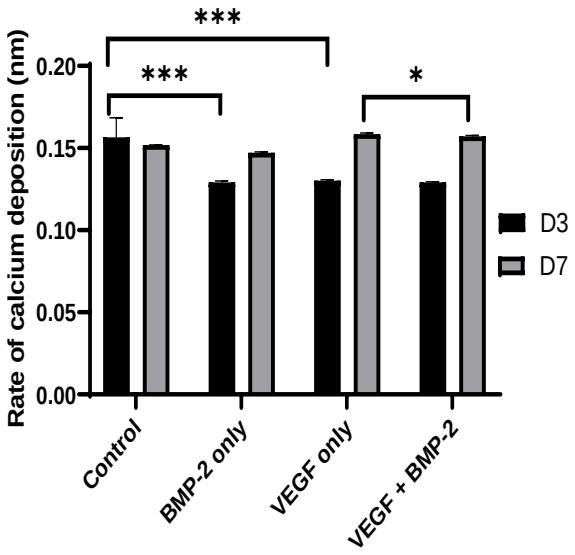

Figure 2: Calcium deposition on Day 3 and Day 7 measured after the stained cell was visualised using light microscopy. The values of the graph bar were expressed as mean ± standard deviation. (n=3, *< 0.05, ***p < 0.0001)

Table II: Rate of calcium deposition (nm)

	Groups		
	V	**B**	**BV**
Rate of mineralisation (nm)	12.0	20.0	21.0

Discussion

In this study, the HA bone scaffold used has a similar component to the human bone in which it consists of calcium phosphate bio-ceramic. A study by Baudequin et al. stated that MC3T3-E1 cells combined with bio-ceramic HA scaffold can promote bone regeneration as it showed propitious cellular and mechanical properties (Baudequin *et al*., 2015).

Incorporation of the VEGF and BMP-2 growth factors into the engineered bone construct was done to create more stable models that can mimic bone natural ECM and subsequently promotes bone growth and repair. To explore this, characterisation of the mineralisation was performed. Raines et al reported that the deposition of calcium is a key major for the late differentiation of osteoblast-like cells (Raines *et al*., 2019). Calcium deposits in the engineered matrix were demonstrated by Alizarin Red S staining.

Further quantification of the calcium deposits confirmed that the combination of VEGF and BMP-2

has the highest absorbance reading reflecting a high amount of calcium deposition. The localisation of calcium deposits suggests that there is a direct role in promoting osteogenic differentiation that will enhance bone regeneration. Zhang et al also showed evidence that the combination of VEGF and BMP-2 not only promotes angiogenesis but also helps in the formation of new bone which could be a favourable approach for bone regeneration (Zhang *et al*., 2014).

Next, it was reported that the combination of VEGF and BMP-2 exhibits a strong synergistic effect and effectively promotes angiogenesis and its proliferation rather than the application of a single agent (Yan *et al*., 2014). Wang and his team also found that the combination of VEGF and BMP-2 can help to enhance osteogenic differentiation (Wang *et al*., 2020). Finally, Liu et al reported that the use of the combined factor of VEGF and BMP-2 showed a positive synergistic effect on bone formation and vascularisation (Liu *et al*., 2020).

In summary, the combination of VEGF and BMP-2 growth factors showed an overall synergistic effect, and this could be a novel strategy to enhance osteogenic capability *in vitro*. This finding provides an insight that scaffold-loaded growth factors can be a promising approach to improve osteogenic proliferation and differentiation thereafter helps in bone tissue engineering.

Conclusion

The results showed positive ARS staining in the HA scaffold supplied with VEGF and BMP-2 growth factors *in vitro*. These results suggest that the combined use of VEGF and BMP-2 will increase cell proliferation and osteoblast differentiation *in vitro* compared to the application of a single factor alone. Thus, the synergistic effect of VEGF and BMP-2-loaded HA scaffold is a promising approach to enhance the osteogenic effect and support the cellular functions of osteoblastic cells in bone tissue engineering applications.

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References

Baudequin, T., Bedoui, F., Dufresne, M., Paullier, P. & Legallais, C. (2015). Towards the Development and Characterization of an Easy Handling Sheet-Like Biohybrid Bone Substitute. *Tissue Engineering - Part A*, **21**(11–12):1895–1905 https://doi.org/10.1089/ten.tea.2014.0580

Bouet, G., Marchat, D., Cruel, M., Malaval, L. & Vico, L. (2015). In vitro three-dimensional bone tissue models: From cells to controlled and dynamic environment. *Tissue Engineering - Part B: Reviews*, **21**(1):133–156 https://doi.org/10.1089/ten.teb.2013.0682

Coultas, L., Chawengsaksophak, K. & Rossant, J. (2005). Endothelial cells and VEGF in vascular development. *Nature*, **438**(7070):937–945 https://doi.org/10.1038/nature04479

Kaigler, D., Silva, E. A. & Mooney, D. J. (2013). Guided Bone Regeneration Using Injectable Vascular Endothelial Growth Factor Delivery Gel. *Journal of Periodontology*, **84**(2):230–238 https://doi.org/10.1902/jop.2012.110684

Liu K., Meng, C.X., Lv, Z.Y., Zhang, Y.J., Li, J., Li, K.Y., Liu, F.Z., Zhang, B. & Cui, F.Z. (2020). Enhancement of BMP-2 and VEGF carried by mineralized collagen for mandibular bone regeneration. *Regenerative Biomaterials*, **7**(4):435–440 https://doi.org/10.1093/RB/RBAA022

Mohammad Shariful Islam., Abdulla-Al-Mamun, M., Khan, A. & Todo, M. (2020). Excellency of Hydroxyapatite Composite Scaffolds for Bone Tissue Engineering. *Biomaterials*, **10**:1–22 https://doi.org/10.5772/intechopen.92900

Oryan, A., Alidadi, S., Moshiri, A. & Maffulli, N. (2014). Bone regenerative medicine: Classic options, novel strategies, and future directions. *Journal of Orthopaedic Surgery and Research*, **9**(1):1–27 https://doi.org/10.1186/1749-799X-9-18

Park, S., Heo, H.A., Lee, K.B., Kim, H.G. & Pyo, S.W. (2017). Improved Bone Regeneration with Multiporous PLGA Scaffold and BMP-2-Transduced Human Adipose-Derived Stem Cells by Cell-Permeable Peptide. *Implant Dentistry*, **26**(1):4–11 https://doi.org/10.1097/ID.0000000000000523

Raines, A.L., Berger, M.B., Patel, N., Hyzy, S.L., Boyan, B.D. & Schwartz, Z. (2019). VEGF-A regulates angiogenesis during osseointegration of Ti implants via paracrine/autocrine regulation of osteoblast response to hierarchical microstructure of the surface. *Journal of Biomedical Materials Research - Part A*, **107**(2):423–433 https://doi.org/10.1002/jbm.a.36559

Rashid, S. A., Daud, N., Norhuda, S. & Syed, N. (2016). Regional Conference on Science, Technology and Social Sciences (RCSTSS 2014). *Regional Conference on Science, Technology and Social Sciences (RCSTSS 2014)*, (January). https://doi.org/10.1007/978-981-10-1458-1

Reddi, A.H. (1998). *1998 Nature Publishing Group http://www.nature.com/naturebiotechnology*

Samadian, H., Khastar, H., Ehterami, A. & Salehi, M. (2021). Bioengineered 3D nanocomposite based on gold nanoparticles and gelatin nanofibers for bone regeneration: in vitro and in vivo study. *Scientific Reports*, **11**(1):1–11 https://doi.org/10.1038/s41598-021-93367-6

Tan, Y.Y., Yang, Y.Q., Chai, L., Wong, R.W.K. & Rabie, A.B.M. (2010). Effects of vascular endothelial growth factor (VEGF) on MC3T3-E1. *Orthodontics and Craniofacial Research*, **13**(4):223–228 https://doi.org/10.1111/j.1601-6343.2010.01498.x

Tengku A.H. (2015). Population Ageing in Malaysia. *A Mosaic of Issues, Challenges and Prospects*, 92

Wang, Z., Sun, J., Li, Y., Chen, C., Xu, Y., Zang, X., Li, L. & Meng, K. (2020). Experimental study of the synergistic effect and network regulation mechanisms of an applied combination of BMP-2, VEGF, and TGF-β1 on osteogenic differentiation. *Journal of Cellular Biochemistry*, **121**(3):2394–2405 https://doi.org/10.1002/jcb.29462

Bai Y., Leng Y., Yin G., Pu X., Huang Z., Liao X., Chen X. & Yao. Y. (2014). Effects of combinations of BMP-2 with FGF-2 and/or VEGF on HUVECs angiogenesis in vitro and CAM angiogenesis in vivo. *Cell and Tissue Research*, **356**(1):109–121 https://doi.org/10.1007/s00441-013-1781-9

Zhang, W., Zhu, C., Wu, Y., Ye, D., Wang, S., Zou, D. & Jiang, X. (2014). VEGF and BMP-2 promote bone regeneration by facilitating bone marrow stem cell homing and differentiation. *European Cells and Materials*, **27**:1–12 https://doi.org/10.22203/eCM.v027a01

Zhu, X., Zhang, H., Zhang, X., Ning, C. & Wang, Y. (2017). In vitro study on the osteogenesis enhancement effect of BMP-2 incorporated biomimetic apatite coating on titanium surfaces. *Dental Materials Journal*, **36**(5):677–685 https://doi.org/10.4012/dmj.2016-189