No Recombinant EGF and bFGF is Required on HUVECs Culture Supplemented with Human Platelet Lysate

Lisa Rinanda Amir[1,3], Ria Puspitawati[1], Hazriani R[2], Shafira Imanina[2], Harvi Damayanti[2], Nadira Dwiyana[2], Afridayanti Nurwulan[2], Mindya Yuniastuti[1], Erik Idrus[1]*

1. Department of Oral Biology, Faculty of Dentistry, Universitas Indonesia.
2. Undergraduate Program, Faculty of Dentistry, Universitas Indonesia.
3. Oral Sciences Research Center Universitas Indonesia.

Abstract

The potential of Human Platelet Lysate (hPL) as animal serum replacement in various cell type has been reported. In HUVECs culture system, exogenous growth factors are routinely added to the media. It is currently unknown whether the presence of hPL in the medium could eliminate the need for exogenous growth factors in HUVECs culture system. This study aimed to evaluate the effect of hPL in HUVECs culture without additional EGF and bFGF, two commonly uses growth factors in HUVECs culture medium. HUVECs proliferation and protein profile was tested with MTT assay and SDS PAGE, respectively. The expression of CD34, CD106 proteins were analyzed by FACS. HUVECs cell proliferation was significantly decreased in the 2% hPL group without additional EGF and bFGF ($p<0.05$). However, no significant difference was found in the control group with 5% hPL without additional EGF and bFGF. Protein profile, the expression of CD34 and CD106 were comparable in all group tested. In the absence of routinely used growth factors in the HUVECs media, hPL could still support the growth of HUVECs. In addition to hPL role as serum replacement in the cell culture system, hPL can be used as an alternate of exogenous growth factors in HUVECs culture.

Experimental article (J Int Dent Med Res 2017; 10(3): pp. 958-963)

Keywords: Human Platelet Lysate (hPL), Human Umbilical Vein Endothelial Cells (HUVECs), Epidermal Growth Factor (EGF), basic Fibroblast Growth Factor (bFGF), CD34, CD106 Expression.

Received date: 18 August 2017 **Accept date:** 20 September 2017

Introduction

The rapidly expanding field of tissue engineering and cell therapy requires the development of good laboratory practices to prepare the cells or cell-tissue construct. According to Good Manufacturing Practice, one of the approaches is to utilize non-animal derived serum in culture medium, thus eliminating the risk of animal pathogen transmission during the transplantation.[1,2] Culture medium supplies all the essential nutrients for cell metabolism, growth and proliferation, and frequently supplemented with animal serum, such as fetal bovine serum. In recent years, human platelet lysate (hPL) has been tested to serve as an alternative to animal serum for clinical-grade cell expansion.[3-9] hPL is prepared from human platelet units by disrupting the platelet membrane through a repeated freeze and thaw cycles. Studies show hPL as an effective medium supplement to support the growth of various cell types.[3-9] Faster proliferation of adipose tissue-derived mesenchymal stromal cells (MSCs) has been reported in the presence of hPL in the culture medium while the effect was less pronounced in bone marrow-derived MSCs.[6] The finding indicates differences exist in nutritional requirements between cell types.

The use of hPL as serum replacement has also been tested in endothelial cells.[7-10] We recently showed the outdated hPL prepared from platelet-rich concentrates beyond its expiry date of 10 days from blood collection could still have the capacity to support HUVECs cell growth comparable to those of fresh hPL prepared within its expiry date of 5 days.[10] The optimum HUVECs isolation and culture protocols requires adequate culture conditions and culture media to support the cell metabolism, growth and proliferation.

Corresponding author:
Lisa Rinanda Amir
Department of Oral Biology
Faculty of Dentistry, Universitas Indonesia
Jl. Salemba Raya No.4, Jakarta 10430, Indonesia
E-mail: lisa.amir@ui.ac.id

Basal media are regularly supplemented with animal serum, various recombinant growth factors and hormone to promotes endothelial cell growth and proliferation.[7-9] Basic Fibroblast Growth Factor (bFGF), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) are known for their potent pro-angiogenic properties and frequently added to endothelial cells culture medium.[7,9] The development of serum-free medium for vascular ECs has been reported to induce comparable cell growth, whereas the cellular mitogenic activity of endothelial cells is supported by the presence of exogenous recombinant growth factors. However, serum-fee medium require higher cell seeding densities than serum-containing medium for long-term cultivation.[11]

Physiologically, activated platelets are known to secrete a broad spectrum of growth factors and other active molecules at the site of injury.[12,13] The growth factors released from alpha granules include platelet-derived growth factor (PDGF), EGF, bFGF, VEGF, insulin growth factor-1 (IGF-1), and transforming growth factor-β (TGF-β).[12,13] These growth factors have been shown to increase cell growth *in vitro* and the concentrations are reported to be higher in hPL compared to those in serum.[14] Although hPL is believed to be a cost-effective source of various growth factors, it is currently unknown whether the use of hPL in ECs culture could replace the need of exogenous recombinant growth factors to support cells growth. As the composition of hPL includes wide range of growth factors, it is hypothesized that the application of hPL as animal serum replacement in endothelial cell culture could also serve as the source of angiogenic growth factors, thus eliminating the need for various exogenous recombinant growth factors. The present study was aimed to examine the effect of hPL without additional EGF and bFGF in HUVECs culture system.

Materials and methods

Human Platelet Lysate (hPL) preparation

The study protocol was approved by the Ethical Review Committee, Faculty of Dentistry, Universitas Indonesia. (57 / Ethical Clearence / FKGUI / VII / 2013). hPL was prepared as previously reported.[15] Briefly, hPL was obtained from platelet-rich concentrates (10^9 per mL) prepared within 5 days from peripheral blood collection. The pooled concentrates were centrifuged at 340 *g* for 6 minutes at 22ºC, the supernatant immediately stored in -20ºC. Next, frozen pooled concentrates were placed in room temperature for 10 minutes and subsequently incubated at 37°C for 2 hours in a water bath for freezing-thawing procedure. This procedure was repeated three times to increase the rate of platelet fragmentation and the amount of released growth factors and resulted in platelet lysis (hPL).[15] hPL was centrifuged at 4000 *g* for 15 minutes to remove platelet fragments and the supernatant was filtered through a 0.22-µm filter (BD Falcon, NJ, USA). Aliquots were stored in -20ºC. Five thawed hPL pool was used for HUVEC culture experiments to minimize variations between donors.

HUVECs Isolation

Human Umbilical Vein Endothelial Cells (HUVECs) isolation was performed according to protocol developed by Baudin et al with some modification.[16] Signed informed consents were obtained prior to umbilical cord collection. Umbilical cords of approximately 15 cm were collected from deliveries with caesarean section and kept in cord buffer transport medium containing 2% glucose (sigma, USA) in PBS with 1% penicillin-streptomycin (Gibco, USA) and processed within 2 hours. Umbilical veins were incubated with 0.2% collagenease I (Gibco, USA) for 7 minutes in 37°C incubator. HUVECs were cultured in 6 well-plate (Nunc, Denmark) in M200 medium supplemented with Low Serum Growth Supplement (LSGS kit, Gibco, USA) for 7 days until it reached 80% confluency. LSGS kit contained fetal bovine serum, 2% v/v; hydrocortisone, 1 mg/mL; human epidermal growth factor, 10 ng/mL; basic fibroblast growth factor, 3 ng/mL; and 10 mg/mL heparin. $3x10^3$ HUVECs were plated in 6 well-plate (Nunclon™ Surface, Roskilde, Denmark) and cultured with 4 types of media, M200 supplemented with 2% FBS, 1 mg/mL hydrocortisone; 10 ng/mL hEGF and 3 ng/mL bFGF and 10 mg/mL heparin (control group 1), M200 supplemented with 2% hPL, 1 mg/mL hydrocortisone; 10 ng/mL hEGF and 3 ng/mL bFGF and 10 mg/mL heparin (control group 2), M200 supplemented with hydrocortisone, 10 mg/mL heparin 2% hPL and 5% hPL without additional EGF and bFGF (Experiment groups 1 and 2, respectively) for 7 days. HUVECs were identified microscopically by their polygonal phenotype (Figure 1) and by the

expression of CD34, VCAM-1 proteins for positive markers and CD45 negative expression (BD Biosciences, USA). Samples were examined in quadruplicate and the experiments were repeated two times.

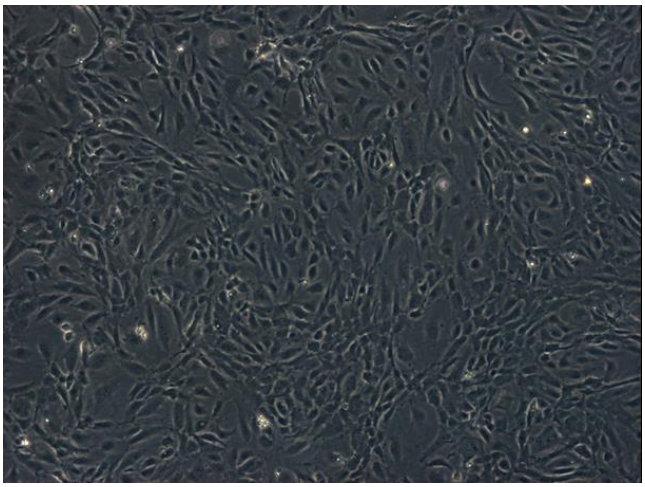


Figure 1. HUVECs Culture Day 5.

MTT Assay

Cell metabolism as an indirect measure of the proliferation of the cells was analyzed by using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Sigma, USA) assay as previously described.[17] Briefly, $3x10^3$ HUVECs were plated in 96-well plates (Nunc, Roskilde, Denmark) and cultured with 2% FBS, 2% or 5% hPL in the presence or absence of EGF and bFGF for 7 days. MTT solution was added to the medium and incubated for 3 hours at 37°C with 5% CO_2. Formazan crystals were dissolved with isopropopanol and incubated for 1 hour on shaker at room temperature. The optical densities (OD) of the samples were determined using micro-plate reader (Benchmark, Biorad, USA) at 655 nm after normalized with blank.

Protein analysis

For protein profiles analysis, $1x10^4$ HUVECs were plated in 24-well plates. Supernatant was collected and the total protein concentration was calculated using Bradford Protein Assay (Bio-Rad protein assay kit, Bio-Rad, USA). Protein concentration was standardized at 500 µg/ml. Samples were electrophoresed through SDS-PAGE 10% of acrylamide gel (Biorad, USA) and stained with non-hazardous Coomassie Blue (Invitrogen, USA) for 60 minutes on orbital shaker. See BluePlus2 (Thermo Scientific, USA) pre-stained standard was used for protein standard. Protein profile was analyzed semi-quantitatively through its band intensity and thickness using ImageJ 1.45r (National Institute of Health, Bethesda, Md, USA).

Flow Cytometric Analysis

$1x10^4$ HUVECs were plated in 24-well plates and were cultured in the presence or absence of exogenous EGF and bFGF. Cells were harvested at day 7, directly stained and analyzed for phenotypic expression of surface markers using preconjugated anti-human monoclonal antibodyies (MAbs). The anti-human MAbs used included anti-CD34-PE, anti-CD106-FITC, anti-CD45-APC and their matched-isotype controls (all from BD Pharmingen, USA).

Statistical Analysis.

Data were presented as mean and standard deviation and were analyzed in Graphpad Prism 6 for Mac OS X. The mean values were analyzed for their normality using Shapiro-Wilk normality test. Kruskal-Wallis test were used, the significance were accepted when $p<0.05$.

Results

Exogenous Growth Factors are dispensable for HUVECs Cell Proliferation in the presence of hPL

When we examined the effect of exogenous growth factors on HUVECs cell proliferation and compared with standard medium of 2% FBS and additional EGF and bFGF, we found significantly faster HUVECs cell proliferation cultured with 2% and 5% hPL without additional growth factors of EGF and bFGF ($p<0.0001$, Figure 2A). The absence of additional growth factors significantly reduced HUVECs cell proliferation when compared with 2% hPL and additional EGF and bFGF ($p=0.012$, Figure 2B). However, HUVEC cells proliferation became comparable when culture with 5% hPL. In this group, the absence exogenous growth factors did not have any effect in HUVECs cell proliferation ($p=0.2$, Figure 2B).

Exogenous Growth Factors are dispensable for HUVECs Total Protein, Protein Prolife and Endothelial Cell Markers in the presence of hPL

The results on HUVECs total protein between standard medium of 2% FBS with additional EGF and bFGF with hPL groups with no exogenous growth factors was in line with the results of HUVECs cell proliferation (Figure 3A).

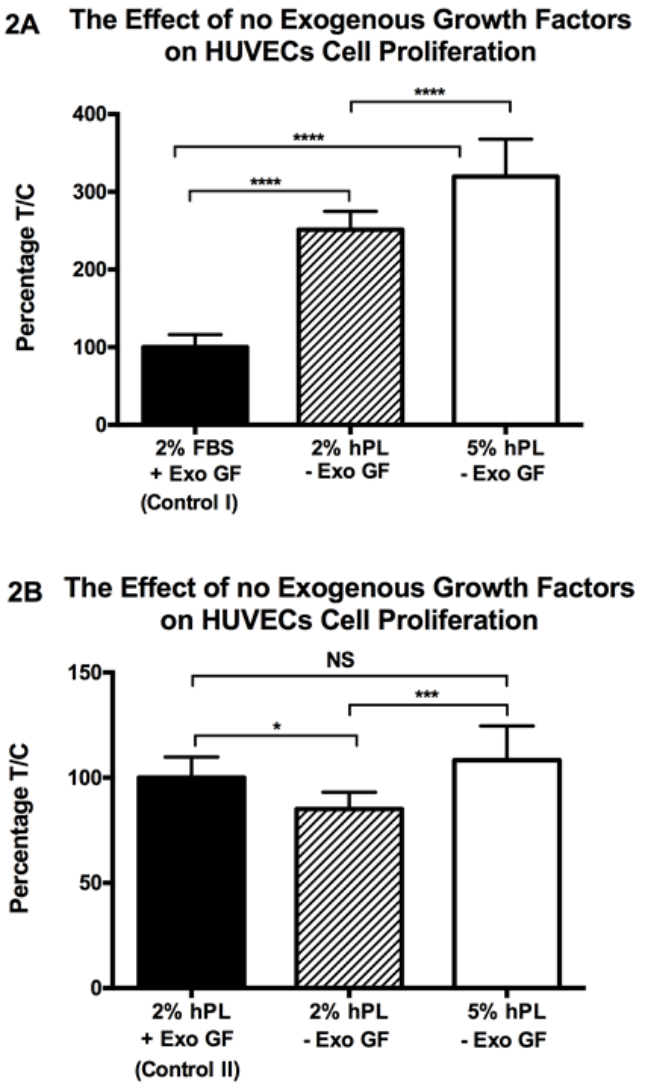


Figure.2. The Effect of no Exogenous Growth Factors on HUVECs Cell Proliferation. (A) In comparison to 2% FBS with EGF and bFGF; (B) In comparison to 2% hPL with EGF and bFGF. ****P<0.0001.

Higher HUVECs total protein was observed in hPL group even in the absence of exogenous EGF and bFGF. When HUVECs total protein in hPL group was compared, higher HUVECs total protein was still detected in the absence of exogenous growth factors (Figure 3B). Next, we examined the HUVECs protein profiles in SDS PAGE gels (Figure 4) and semi-quantified the band intensity and thickness. The absence of exogenous growth factors did not have any effect on the band intensity and thickness. The expression of endothelial cells markers of CD34 and CD106 did not alter in the absence of exogenous EGF and bFGF (p=0.06, Figure 5A-D). No CD45 protein expressions was observed in HUVECs cultured in medium supplemented with FBS, hPL with or without additional growth factors.

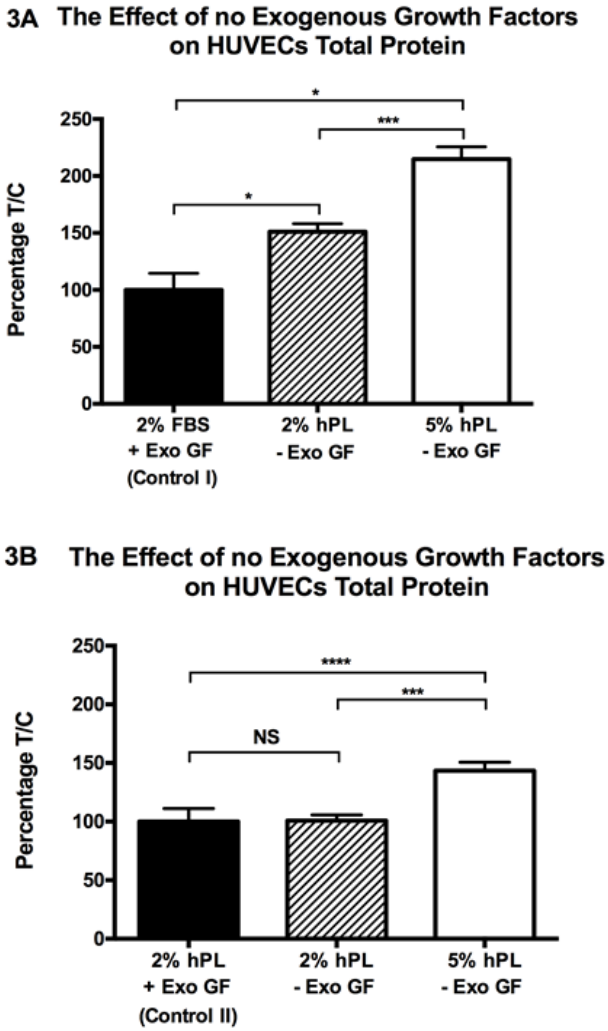


Figure.3. The Effect of no Exogenous Growth Factors on HUVECs Total Protein. (A) In comparison to 2% FBS with EGF and bFGF; (B) In comparison to 2% hPL with EGF and bFGF. *P=0.012, ***P=0.0001, NS= Not Significant.

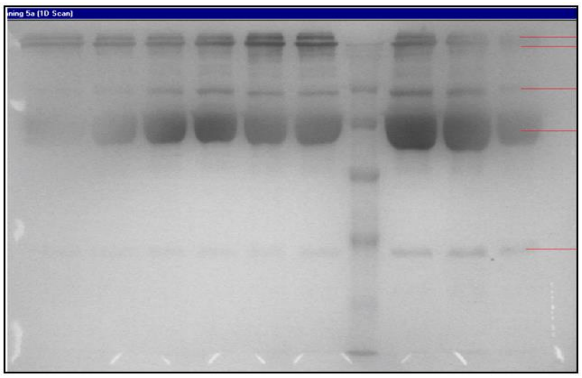
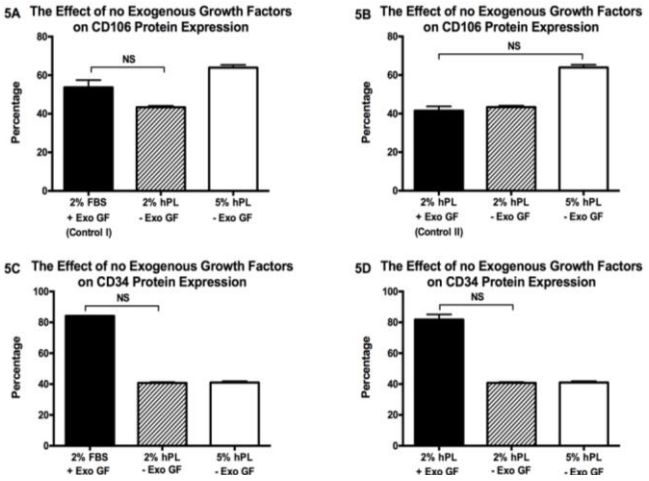


Figure.4. SDS PAGE of HUVECs protein Cultured with and without Exogenous Recombinant Growth Factors.



Discussion

Human platelet lysate is frequently used in cell culture as medium supplement in recent years so as to avoid the risk of xenogenic immunoreactions or transmission of animal pathogens. The potential of hPL as an alternative animal serum replacement have been shown in various cell types.[3-9]

It is believed that the effect of platelet lysate on various cell types attributes to their growth factor released. As exogenous recombinant growth factors are normally added in endothelial cells (ECs) culture media, this study presents evidence that faster proliferation of endothelial cell cultured in medium supplemented with hPL could still be achieved even in the absence of exogenous growth factors supplements. A number of studies have shown the growth factors content released from platelets during hPL preparation. PDGF, a strong mitogenic factor is found to be the major constituent of α-granules. Higher concentrations of PDGF-AB, VEGF, EGF, bFGF, TGF-β1 are found in hPL related to their concentrations in platelet poor plasma, in human serum or FCS, and are evident in the present study.[8,9,14] hPL alone supports higher HUVECs growth higher than those cultured in standard medium consisting of FBS and exogenous recombinant EGF and bFGF. Aside from the role of hPL as an animal serum replacement in cell culture, the present study indicates hPL could effectively serve as the substitute of recombinant growth factors in endothelial cell culture.

Previous studies have reported the effect of hPL on endothelial cells growth.[7-9] A decreased in metabolic activity of lymphatic ECs (LECs) and outgrowth ECs (OECs) was demonstrated while comparable HUVECs proliferation was observed compared to FCS group.[9] Barsotti and colleagues reported an increased in ECs proliferation and migration were observed at higher concentration of hPL (10% and 20% v/v).[7] While a decreased in ECs proliferation and the number of migrated ECs were seen at low hPL concentrations (1% and 5% v/v). Similar results were reported by Chuo and colleagues on which the higher corneal endothelial cells growth was observed of high hPL concentration.[8] However, these studies tested the effect of hPL in the presence of various growth factors such as VEGF, hFGF, IGF and EGF. Our study is the first to report the role of hPL as the substitute of exogenous recombinant growth factors in endothelial cell culture.

The identification of endothelial cells can be developed by its cobblestone appearance and by using endothelial cell markers such as CD34 and CD106 and the negative expression of CD45.[15,18-20] CD34 is a highly glycosylated transmembrane cell surface glycoprotein found in endothelial cells. Recent study suggests CD34 as a novel marker for the selection of endothelial cells with a tip phenotype that play a role in vascular network expansion and sprouting angiogenesis.[20] In this study, we showed the absence of EGF and bFGF in HUVEC media did not change the expression of CD34 and CD106, indicating HUVECs phenotypes were maintained during the course of these experiments. The negative expression of CD45 found in this study might indicate the presence of mature endothelial cells. It is known that CD45 expression is related to the maturity of endothelial cells. Early endothelial progenitor cells (EPC) while late EPCs and mature endothelial cells do not express CD45.[21]

Angiogenesis process is crucial for the success of tissue engineering strategy. Lack of functional microvasculature has been identified as a major challenge in tissue engineering.[22,23] To overcome this challenge, new strategy of vascularized bone tissue engineering has been proposed. Numerous studies have reported the co-seeding of endothelial cells with osteoblasts in the scaffold constructs give rise to an increased in the sprouting of new capillaries from pre-existing blood vessels.[24,25] Microvascular networks could unite with the host vascular

systems, thereby support the survival of the implanted cell tissue construct.

Conclusions

In summary, in the absence of routinely used recombinant growth factors in the endothelial cells media, hPL could still support the growth of HUVECs and the expression of endothelial cells markers remain comparable with those cultured in standard medium. The study suggests in addition to hPL role as serum replacement in the cell culture system, hPL could eliminate the need for exogenous recombinant growth factors in endothelial cell culture.

Acknowledgements

This study has been financially supported by Universitas Indonesia Research Grant (DRPM/R/385/RUUI/2012)(RP, LA) and Indonesia Toray Science Foundation Science and Technology Research Grant (LA, EI)

Declaration of Interest

The authors report no conflict of interest.

References

1. Muschler GF, Nakamoto C, Griffith LG. Engineering principles of clinical cell-based tissue engineering. J Bone Joint Surg Am. 2004; 86: 1541-58.
2. Bieback K, Kinzebach S, Karagianni M. Translating research into clinical scale manufacturing of mesenchymal stromal cells. Stem Cells Int. doi: 10.4061/2010/193519. 2011.
3. Doucet C, Ernou I, Zhang Y, Llense JR, Begot L, Holy X, Lataillade JJ. Platelet lysates promote mesenchymal stem cell expansion: A safety substitute for animal serum in cell-based therapy applications. J Cell Physiol. 2005; 205: 228-36.
4. Prins, HJ, Rozemuller, H, Vonk-Griffioen, S, Verweij, VGM, Dhert, WJ, Slaper-Cortenbach, ICM, Martens ACM. Bone-forming capacity of mesenchymal stromal cells when cultured in the presence of human platelet lysate as substitute for fetal bovine serum. Tissue Eng Part A. 2009;15: 3741-51.
5. Lohmann M, Walenda G, Hemeda H, Joussen S, Drescher W, Jockenhoevel S, Wagner W. Donor age of human platelet lysate affects proliferation and differentiation of mesenchymal stem cells. PLoS ONE. 2012; 7: 14-6.
6. Naaijkens BA, Niessen HWM, Prins HJ, Krijnen PAJ, Kokhuis TJA, De Jong N, Juffermans LJM. Human platelet lysate as a fetal bovine serum substitute improves human adipose-derived stromal cell culture for future cardiac repair applications. Cell Tissue Res. 2012; 348: 119-30.
7. Barsotti MC, Losi P, Briganti E, Sanguinetti E, Magera A, Al Kayal T, Soldani G. Effect of platelet lysate on human cells involved in different phases of wound healing. PLoS ONE. 2013; 8: 84753.
8. Chou ML, Burnouf T, Wang TJ. Ex vivo expansion of bovine corneal endothelial cells in xeno-free medium supplemented with platelet releasate. PloS ONE. 2014; 9: 99145.
9. Hofbauer P, Riedl S, Witzeneder K, Hildner F, Wolbank S, Groeger M, Holnthoner W. Human platelet lysate is a feasible candidate to replace fetal calf serum as medium supplement for blood vascular and lymphatic endothelial cells. Cytother. 2014; 16: 1238-44.
10. Amir LR, Bachtiar EW, Puspitawati R, Puspitasari AH, Kurniawan B. Increased Endothelial Cell Growth in Culture Supplemented with Outdated Human Platelet Lysate. J Int Dent Med Res. 2016; 9(Special Issue): 368-375.
11. Labitzke R, Friedl P. A serum-free medium formulation supporting growth of human umbilical cord vein endothelial cells in long term cultivation. Cytotechnol. 2001; 35: 87-92.
12. Maguire, P.B. and Fitzgerald, D.J. Platelet proteomics. J Thromb Haemost. 2003; 1: 1593-1601.
13. Eppley BL, Woodell JE, Higgins J. Platelet quantification and growth factor analysis from platelet-rich plasma: implications for wound healing. Plast Reconstr Surg. 2004; 114: 1502-8.
14. Rauch C, Feifel E, Amann E, Spötl H, Schennach H, Pfaller W, Gstraunthaler G. Alternatives to the use of fetal bovine serum: human platelet lysate as a serum substitute in cell culture media. Altex. 2011; 28: 305-16.
15. Schallmoser K and Strunk D. Preparation of pooled human platelet lysate (pHPL) as an efficient supplement for animal serum-free human stem cell cultures. J Visual Exp. 2009; 20-3.
16. Baudin B, Bruneel A, Bosselut N, Vaubourdolle M. A protocol for isolation and culture of human umbilical vein endothelial cells. Nature Prot. 2007; 2: 481-5.
17. Amir LR, Suniarti DF, Utami S, Abbas B. Chitosan as a potential osteogenic factor compared with dexamethasone in cultured macaque dental pulp stromal cells. Cell Tissue Res. 2014; 358: 407-15.
18. Marin V, Kaplanski G, Gres S, Farnarier C, Bongrand P. Endothelial cell culture: protocol to obtain and cultivate human umbilical endothelial cells. J Immunol Methods. 2001; 254: 183-90.
19. Pusztaszeri MP, Seelentag W, Bosman FT. Immunohistochemical expression of endothelial markers CD31, CD34, von Willebrand Factor, and Fli-1 in normal human tissues. J Histochem Cytochem. 2006; 54: 385-95.
20. Siemering MJ, Klaassen I, Vogels IM, Griffioen AW, Van Noorden CJ, Schlingemann RO. CD34 marks angiongenic tip cells in human vascular endothelial cell cultures. Angiogenesis. 2012; 15: 151-163.
21. Shaw J. Hematopoietic stem cells and endothelial cell precursors express Tie-2, CD31 and CD45. Blood Cells Mol Dis. 2004; 32:168-175.
22. Santos MI, Reis RL. Vascularization in bone tissue engineering; physiology, current strategies, major hurdles and future challenges. Macromol Biosci. 2010; 10: 12-27.
23. Novosel EC, Kleinhans C, Kluger PJ. Vascularization is the key challenge in tissue engineering. Adv Drug Deliv Rev. 2011; 63: 300-311.
24. Grellier M, Bordenave, L, Amédée J. Cell-to-cell communication between osteogenic and endothelial lineages: implications for tissue engineering. Trends Biotechnol. 2009; 27: 562-571.
25. Herzog DPE, Dohle E, Bischoff I, Kirkpatrick CJ. Cell communication in a co-culture system consisting of outgrowth endothelial cells and primary osteoblasts. Bio Med Res Int. 2014; Article ID 320123: 1-15. doi:10.1155/2014/320123.