# Increased Endothelial Cell Growth in Culture Supplemented with Outdated Human Platelet Lysate

*Lisa Rinanda Amir[1,3]*, Endang Winiati Bachtiar[1,3], Ria Puspitawati[1], Agnes Henny Puspitasari[2], Benita Kurniawan[2]*

1. Department of Oral Biology, Faculty of Dentistry, Universitas Indonesia, Jakarta 10430, Indonesia.
2. Undergraduate Program, Faculty of Dentistry, Universitas Indonesia, Jakarta 10430, Indonesia.
3. Oral Sciences Research Center Universitas Indonesia, Jakarta 10430, Indonesia.

## Abstract

The potency of Human Platelet Lysate (hPL) as an animal serum replacement in cell culture has been reported. The effect of outdated hPL derived from platelets that are stored beyond its expiry date is currently unknown. The study aimed to evaluate whether the outdated hPL retain its capacity as an animal-free based serum replacement in endothelial cell culture medium. Human Umbilical Vein Endothelial Cells (HUVECs) were cultured with basal medium of M200 supplemented with fresh hPL (5 days), outdated hPL (10 days) or FBS as control group. Cell proliferation, total protein and angiogenesis associated genes and total protein was compared. HUVECs proliferation and total HUVECs protein concentration was higher in hPL groups compared to those with FBS ($p<0.05$). The highest total protein concentration was seen in hPL fresh group ($p<0.0001$). Total protein and mRNA expression of endothelial markers tested in outdated hPL group were comparable to fresh hPL or FBS.

The present study showed outdated hPL stimulated higher HUVECs proliferation than fresh hPL and control FBS. Outdated hPL affected HUVECs protein profile while genes associated with angiogenesis remained unaffected by hPL supplement. This implies platelet that has reach its expiry date could still be used for HUVECs culture, thus reducing the blood product waste that otherwise be discarded.

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## Introduction

Angiogenesis is crucial for development, remodeling and regeneration of bone.[1,2] Cells reside in the blood vessel walls or in the close vicinity of the blood vessel wall are believed to secrete osteogenic factors and some of these cells appeared to have osteogenic potential.[3,4]

Reciprocally, osteoblasts secrete a number of angiogenic related molecules and the intimate cross-talk between osteoblasts and endothelial cells has been widely reported.[2] In bone tissue engineering, adequate vascularization in the tissue construct is essential to prevent central necrosis.[5] The vasculature role transport oxygen, nutrients, soluble factors is necessary for the viability of the transplanted cell scaffold construct.[5] As the oxygenation and nutrition by diffusion from the adjacent capillary is limited to an extend of approximately 150 μm, lack of functional microvasculature has been identified as a major challenge in tissue engineering.[5,6] Information of the cellular and molecular interactions of blood forming cells and bone cells will lead to a better understanding of the future development of vascularized bone scaffold constructs, which eventually results in optimum integration of the reconstructed bone with the surrounding host tissue.[1-6]

Studies on angiogenesis in bone tissue engineering have been demonstrated using co-culture of mesenchymal stromal/stem cells (MSCs) and endothelial cells.[7,8] Clinical application of human MSCs or endothelial cells for cellular therapy or tissue engineering

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

necessitate ex-vivo cell expansion to regenerate sufficient number of cells to be transplanted in the reconstructed tissues.[5-8] It requires adequate culture conditions and culture media to support the growth of the cells ex-vivo. Animal-derived serum such as Fetal Bovine Serum (FBS) is the most widely used growth supplement for cell culture media because of its high content of embryonic growth promoting factors.[9] It has been known to satisfy metabolic requirements of cultured cells. Yet, for tissue engineering purpose, the use of FBS as the growth supplement could potentially rising the risk of animal-pathogen transmission when cells, scaffold and signal are transplanted to the defect area.[9] The ideal situation would be to avoid the use of animal-derived serum during human stromal cells and endothelial cell manipulation.[10-19]

One of the alternative supplements which serves as the substitute for animal serum in clinical-scale expansion of cells and has gain recent increasing interest is human platelet lysate (hPL).[10-18] Human platelet lysate (hPL) is prepared by a simple freeze-thaw procedure of platelet units. Platelet unit contains abundant of growth factors and cytokines that support tissue regeneration.[15] Numerous studies have reported the application of human platelet lysate (hPL) as an alternative to animal serum for clinical-grade cell expansion.[15-18] Studies using mesenchymal stromal cells or other cell types showed hPL is superior or comparable to FBS.[10,12,14,17-19]

We recently showed that in addition to hPL role as substitute for animal-derived serum, it could also eliminate the need for exogenous growth factors in HUVECs culture system (Data not published). For blood platelet transfusion as a live-saving procedure for patients, there is a shelf-life restriction for up to 5-7 days to ensure its safety.[20] The restriction is related to the potential increased risk of pathogen contamination while platelet is stored longer at room temperature and the availability of growth factors may be reduced due to platelet storage lesions and degradation.[20] In blood bank, long storage of hPL that is not adequate for blood transfusion would be discarded as a blood product waste. Recent studies suggest the possibility to use outdated platelets to improve cell culture techniques.[11] The characteristic of MSCs is not affected by culture condition using either outdated or fresh hPL.[11] However, It is not clear whether outdated hPL support endothelial cell culture system. This study aimed to evaluate the capacity of human platelet lysate as a substitute for animal serum in HUVECs culture. It is hypothesized that there is less shelf-life restriction for platelet to be used for endothelial cells culture media supplement. Outdated hPL, prepared form platelet that has reached its expiry date of 5 days, retain its capacity on supporting endothelial cell growth. It is of interest to find out whether long storage time of platelet that is no longer suitable for blood platelet transfusion could still be used as a supplement for HUVECs culture medium.

## 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 ). Platelet-rich concentrates ($10^9$ per mL) were obtained from the Indonesian Red Cross, Jakarta, Indonesia. Platelet lysate (hPL) was prepared according to protocol developed by Schallmoser et al (2009). Fresh hPL was prepared from platelet-rich concentrates within 5 days from blood collection while outdated hPL was prepared from platelet rich concentrates within 10 days from blood collection. The pooled concentrates were centrifuged at 340 *g* for 6 minutes at 22ºC, the supernatant immediately stored in -20ºC. Frozen pooled concentrates were placed in room temperature for 10 minutes and subsequently incubated at 37°C for 2 hours in a water bath. The 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). 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. For HUVECs culture experiments, 5 thawed hPL pool was used to minimize variations between donors.

*HUVECs Isolation*

The collection of umbilical cord was performed at Budi Kemuliaan Maternity Hospital, Jakarta, Indonesia. Only breech deliveries with caesarean section were included in this study. All patients have signed informed consent. HUVECs isolation was performed according to protocol developed by Baudin et al, with some

modification.[20] Umbilical cords of approximately 15 cm were stored in cord buffer transport medium containing 2% glucose (sigma, USA) in PBS with 1% penicillin-streptomycin (Gibco, USA) and processed within 2 hours. Cannulae was inserted in the vein, fixed with surgical clamp, and sterile PBS was flushed through the vein to remove the remaining blood clot. Another cannulae was inserted at the other cord extremity, fixed with surgical clamps, 0.2% collagenase I (Gibco, USA) was then injected into the umbilical vein and incubated briefly for 7 minutes in 37°C incubator to avoid contamination of fibroblast or smooth muscle cells. The cords were gently massaged to facilitate cells detachment and were washed with M200 medium (Gibco, USA). HUVECs were cultured in 6 well-plate (Nunc, Denmark) in M200 medium supplemented with Low Serum Growth Supplement (LSGS kit, Gibco, USA) containing 2% v/v fetal bovine serum, 1 mg/mL hydrocortisone, 10 ng/mL human epidermal growth factor, 3 ng/ml basic fibroblast growth factor, and 10 mg/mL heparin, for approximately 6 days until it reached 80% confluency. HUVECs was identified microscopically by their polygonal phenotype and by flow cytometry using CD34, VCAM-1 antibodies for positive markers and CD45 antibody for negative marker (BD Biosciences, USA).

*HUVECs proliferation*

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.[21] Briefly, $3x10^3$ HUVECs were plated in 96-well plates (Nunc, Roskilde, Denmark) and cultured with 2% fresh or outdated hPL or 2% FBS as control group for up to 14 days. MTT solution was added to the medium and incubated for 3 h 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 microplate reader (Benchmark, Biorad, USA) at 655 nm after normalized with blank. Samples were examined in quadruplicate and the experiments were repeated three times. Data were presented as the percentage of viable cells in the treated over untreated groups.

*Protein Profile Assay*

For protein and gene expression 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) were 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 gel was analyzed semi-quantitatively through its band intensity and thickness using ImageJ 1.45r (National Institute of Health, Bethesda, Md, USA).

*Gene Expression Analysis*

Real-Time PCR for the analysis of angiogenic markers of VCAM-1, Integrin αv and Integrin β3 was performed after 7 and 14 days of HUVECs culture. RNA isolation, cDNA synthesis and Real-Time PCR procedures have been previously described in detail.[21]Briefly, total RNA from HUVECs culture was isolated using TRIzol reagent (Gibco, USA). 5 µg of glycogen (Invitrogen, USA) was added to isopropanol to increase RNA yield. Reverse transcriptase was performed with M-MuLV reverse transcriptase (First Strand cDNA synthesis kit, Thermo Fisher Scientific, Lithuania) in iCycler PCR (Biorad, USA) with 2 µg total RNA in 20 µL final reaction volume. RT-PCR analysis was performed in duplicate with SYBR Green PCR Master Mix (Applied Biosystems, UK) on a StepOne Real-Time PCR System (Applied Biosystems, USA). Housekeeping gene of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous reference to normalize VCAM-1, Integrin αv and Integrin β3 expressions (Table 1). VCAM-1 and GAPDH primers were purchased from Invitrogen, USA while Integrin αv, Integrin β3 primers were obtained form 1st Base, Singapore.

| Gene | Primers | Sequence (5' to 3') |
|---|---|---|
| VCAM | Forward | ACAAAGTTGGCTCACAATTAAGAAGTT |
| | Reverse | TGCAAAATAGAGCACGAGAAGCT |
| Integrin αv | Forward | ATGGCAAACTCCAAGAGGTG |
| | Reverse | GAGATGGGACTGCGTTCAAG |
| Integrin $b_3$ | Forward | GCGGCAAGTGTGAATGTG |
| | Reverse | ACTGAGAGCAGGACCACCAG |
| GAPDH | Forward | ATGGGGAAGGTGAAGGTCG |
| | Reverse | TAAAAGCAGCCCTGGTGACC |

**Table 1.** Primers used for Real-Time PCR.

*Statistical analysis*

Data were calculated using Graphpad

Prism 6 for Mac OS X and presented as mean and standard deviation. 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

*Effect of Outdated hPL on HUVECSs Cell Proliferation.*

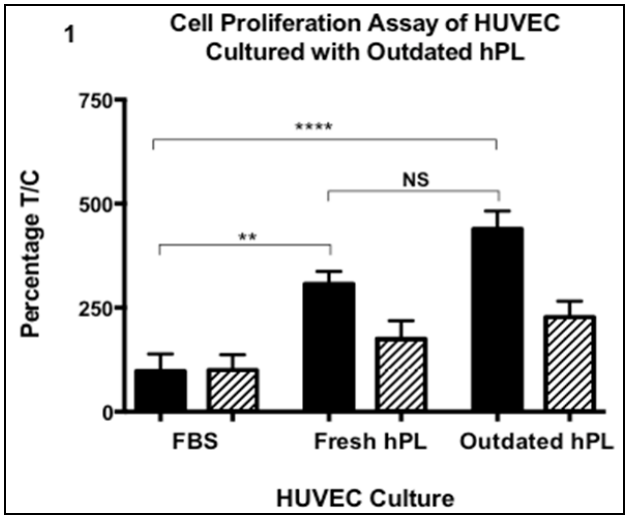


**Figure 1.** Cell Proliferation Assay of HUVECs Cultured with Outdated hPL. Note the consistently high HUVECs cell proliferation in outdated hPL group. Black bar: Median with range from day 3. Slant line bar: Median with range from day 14. ** P=0.0014, **** $p<0.0001$

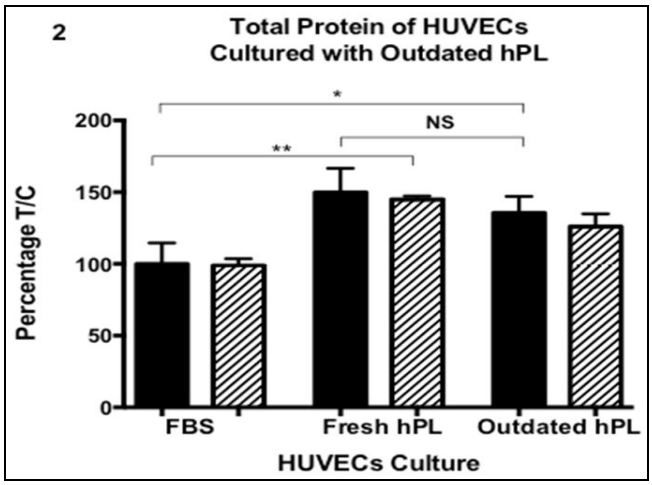


**Figure 2.** HUVECs Total Protein Cultured with Outdated hPL. No difference was found in HUVEC total protein between fresh and outdated hPL. Black bar: Median with range from day 3. Slant line bar: Median with range from day 14. *P=0.012, **P=0.007

First, we analyzed HUVECs metabolism as an indirect measure of HUVECs proliferation. Our data showed a significant increased in cell proliferation of HUVECs treated with both hPL groups compared to the control group of FBS (Fig.1). The increase was found in the early culture time of day 3 while in longer culture time of day 14, HUVECs cell proliferation was comparable in all groups ($p>0.05$). No significant different in HUVECs cell proliferation was observed in outdated hPL group compared to fresh hPL group.

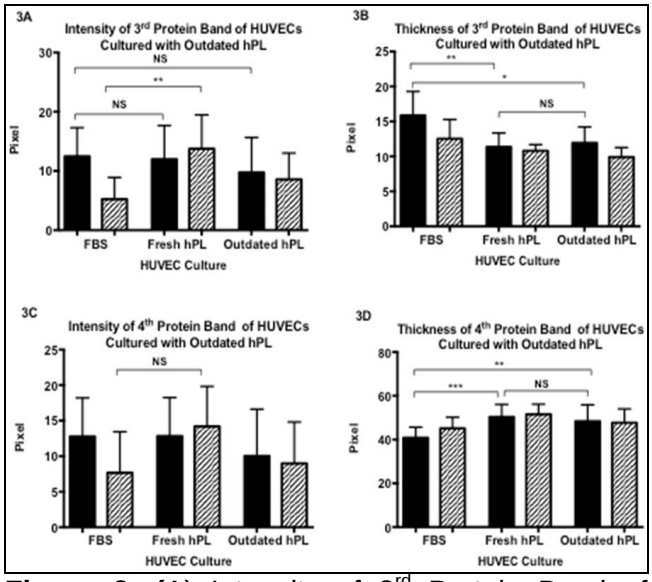


**Figure 3. (**A) Intensity of 3rd Protein Band of HUVECs Cultured with Outdated hPL. **P=0.009; (B) Thickness of 3rd Protein Band of HUVECs Cultured with Outdated hPL. *P=0.02, **P=0.002; (C) Intensity of 4th Protein Band of HUVECs Cultured with Outdated hPL; (D) Thickness of 4th Protein Band of HUVECs Cultured with Outdated hPL. Note that higher band intensity was found between fresh hPL group and control FBS. **P=0.003, *** P=0.0002 Black bar: Median with range from day 7. Slant line bar: Median with range from day 14.

*Effect of Outdated hPL on HUVECs Protein Profile*

HUVECs secreted proteins represented by the total proteins in HUVECs supernatant were measured. Total protein concentrations of HUVECs cultured with both fresh and outdated hPL were significantly higher compared to the control group of FBS (Fig 2, $p<0.01$).

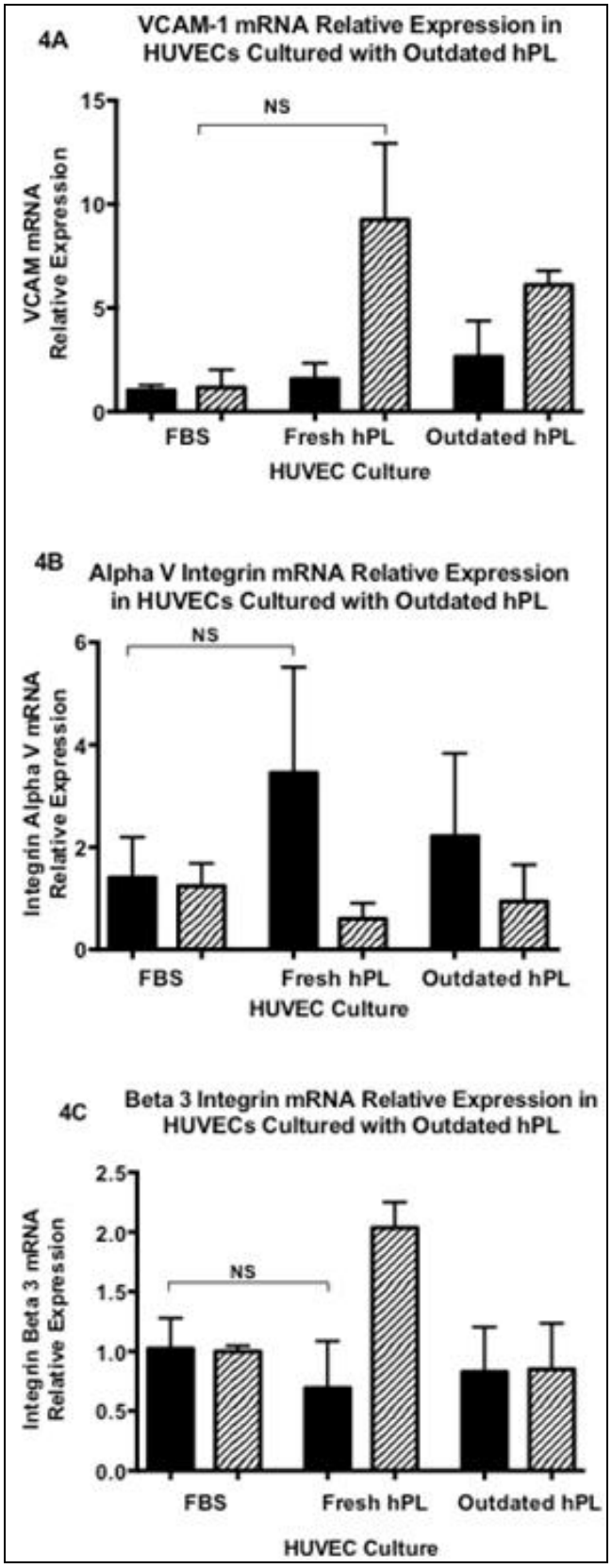


**Figure 4.** (A) VCAM-1 mRNA Relative Expression in HUVECs Cultured with Outdated hPL; (B) $\alpha_v$ Integrin mRNA Relative Expression in HUVECs Cultured with Outdated hPL; (C).$\beta_3$ Integrin mRNA Relative Expression in HUVECs Cultured with Outdated hPL. Comparable angiogenic genes expressions in all group tested. Black bar: Median with range from day 7. Slant line bar: Median with range from day 14. NS: not significant.

In line with HUVECs cell proliferation data, comparable total protein value of HUVECs cultured with fresh and outdated hPL was observed ($p>0.05$). HUVECs total proteins in each sample were standardized up to 0.5 mg/mL to evaluate the HUVECs protein profile by means of SDS PAGE. Protein molecular weight was determined by band analysis quick guide program on gel documentation system.

Out of total 18 SDS-PAGE gel analyzed, the 3rd and 4th protein bands gave consistent strong expressions, allowing us to determine the protein consentration in gel bands using Image J 1.45r (National Institute of Health, USA). The analysis was carried out to measure the intensity and thickness of protein bands. Higher protein bands intensity was found in the fresh hPL group compared to control group at day 14 of culture period ($p<0.01$). The finding was detected in only in 3rd protein band tested that represent protein molecular weight range 89.7–104.6 kD. A tendency to increase in protein bands intensity was seen in the outdated hPL group compared to the control group at day 14, although it was not statistically significant ($p>0.05$). Higher intensity could indicate higher protein concentration in the 3rd and 4th protein bands, range from 53.3-114.2 kDa. The thickness of protein bands was found the highest in the control group range form 78.6-100.8 kDa and 74.2-99.3 kDa for the 3rd and 4th protein band, respectively. Significant lower values of 3rd band thickness was observed in hPL groups compared to control FBS group ($p<0.001$, Fig. 3B), while higher value of 4th protein band thickness was detected in hPL groups compared to control group ($p<0.001$, Fig. 3D).

*Effect of Outdate hPL on angiogenic genes expressions*

Analysis of angiogenic genes expressions revealed comparable VCAM-1, Integrin $\alpha$V and Integrin $\beta$3 mRNA expression in hPL groups and FBS group in 7 and 14 days of HUVEC culture. A tendency of higher expression in the hPL groups was detected in all genes tested, although it was not statistically significant ($p>0.05$, Fig. 4A-C).

The expression genes related to angiogenesis were unchanged in both hPL group.

## Discussion

The rapidly expanding field of tissue engineering and cell therapy demands a well-defined and safe in vitro cell culture method involving animal component-free culture condition. The present study analyzed outdated hPL, prepared from platelet concentrate beyond its expiry date, as the potential of HUVECs growth supplement compared to the standard growth supplement of FBS and fresh hPL. We found faster HUVECs proliferation in hPL groups than in control FBS group, while angiogenic markers expressions in HUVEC cultured with hPL groups were unchanged.

A number of research groups have reported similar results on the effect of fresh hPL on cell proliferation. The use of fresh hPL as animal-derived serum replacement in the cell media has been tested for the isolation of numerous cell types including MSCs from various tissue origin, human fibroblast, epithelial cell line and endothelial cells.[10-14,16,22] Total population doubling of bone marrow-derived MSCs cultured with hPL was increased 1.3-fold compared to control FBS.[11] Other MSCs isolated from adipose tissue cultured with fresh hPL revealed almost 2-fold increased of total population doubling compared to FCS.[12] The less pronounce growth stimulation of hPL in bone marrow MSCs might indicate difference nutritional requirements among these different origin MSCs. Furthermore, MSCs expanded in the presence of platelet-derived growth factors retain their immunosuppressive properties.[11-13,16,18,22]

Our study demonstrated that hPL proliferative stimulation appeared to be higher in HUVECs culture, despite low concentration of 2% hPL added to media. We showed approximately 4.5-fold increase of HUVECs proliferation at early culture period culture and reduce to 2-fold increase at late culture period. On the contrary, several studies suggested that hPL is not superior to FCS/FBS in endothelial cell culture.[15-17,22] A study performed by Hofbauer and colleagues showed a decreased in metabolic activity of lymphatic ECs (LECs) and outgrowth ECs (OECs) while HUVECs showed comparable MTT values than FCS group.[22] Other study showed increase ECs proliferation and migration were only seen in higher concentrations of hPL (10% and 20% v/v). At low hPL concentrations (1% and 5% v/v), decreased ECs proliferation and the number of migrated ECs were observed.[15] Recent study by Chou and colleagues reported higher Corneal Endothelial Cells Growth was observed on complete medium supplemented 5% FBS compared to hPL groups. Significantly better cell viability was exhibited at higher hPL concentrations up to 10%.[16] The discrepancies on the effect of hPL in endothelial cell culture presented by other research groups might be related to (1) difference in combination media used, and (2) difference in HUVECs passage. We used earlier passages 1-3 while their study used HUVECs passage 7-13.[22] It has been known that the cell proliferation capacity is reduced at late passages.[22] Futhermore, one could argue that exogenous growth factors used in this study might influence the HUVECs proliferative stimulation by hPL. We have recently tested the use of fresh hPL in HUVEC culture with no addition of EGF and bFGF. No differences were detected between medium supplemented with FBS and exogenous growth factors and medium supplemented with hPL with no exougenous growth factor groups (Amir et al. Manuscript in preparation). The result indicates the hPL proliferative stimulation is independent to the exogenous growth factors added to the media. This highlight the additional role of hPL as animal-derived serum replacement in culture media, it can also be used in replacement of exogenous growth factors in HUVECs culture.

The data presented in this study showed HUVECs cell proliferation in the outdated hPL group was comparable to the fresh hPL group. The result is in agreement with other study on outdated hPL using different cell types.[11,18] No differences were demonstrated in the population doubling time of bone marrow MSCs cultured with outdated hPL.[17] Supplementation of neither fresh nor outdated hPL affects the expression of surface antigen, osteogenic differentiation and immunomodulatory of MSCs.[16] The expression of Runx2 mRNA was transiently higher in outdated hPL group compared to fresh hPL, and became comparable at later incubation time.[17] Analysis of hPL composition showed that it contains higher levels of $\alpha$-granule factors PDGF-AB, EGF, bFGF, HGF, TGF-$\beta$1 and VEGF than human serum, whereas the level of liver-derived serum marker IGF-1 was higher in the serum

sample.[16,23] These growth factors are known for their potent mitogenic properties. The stability of growth factor in hPL stored in 22°C remains up to 5 months. This study demonstrates that outdated hPL still retain its capacity as serum replacement for HUVECs cell culture.

The characteristic of HUVECs protein profiles was changed by hPL supplements. As the 3rd and 4th protein bands gave consistent strong expressions, we further analyzed these protein bands quantitatively by measuring the intensity and thickness of the band, as well as the range of protein molecular weight. The intensity of the protein bands tested was comparable between all groups in early culture time. Significant increased band intensity was detected in the protein band at molecular weight range from 53.3 to 114.2 kDa at late culture period between fresh hPL and control FBS, indicating higher concentration of polypeptides. A number of proteins within this molecular weight range includes CD146, CD34 and CD105/Endoglin that are believed to be involved in HUVECSs proliferation. Other proteins with molecular weight within the range of the 4th protein band include CD102 or ICAM-2 (60 kDa). ICAM-2 (60 kDa) is known to play a role in the process of angiogenesis, as it activate the guanosine triphosphatase (GTPase) Rac, which is needed in the formation of new blood vessels.[24]

Vascular cell-adhesion molecule-1 (VCAM-1), VEGF, integrin $\alpha_v$ and integrin $\beta_3$ have been demonstrated to function as angiogenic mediators. VCAM-1 expression is activated in endothelial cell migration, a critical process in angiogenesis, involving a series of integrin-mediated adhesion and de-adhesion events.[24] Integrin, including $\alpha_v\beta_3$ is linked to TNF-$\alpha$-induced angiogenesis and considered as one of the central molecules in capillary formation.[25]

It is the adhesion reception on endothelial cells for several extracellular matric proteins containing Arg – Gly – Asp tripeptide motive such as vitronection, fibronectin and collagen.[25]

Besides VEGF critical role in angiogenesis, numerous studies have reported the role of VEGF in the communication between endothelial cells and bone forming cells of osteoblasts.[3,7] VEGF is released by osteblasts and its primary action is by endothelial cells. The comparable expression of these angiogenic genes following HUVECs culture with outdated hPL underscore the effectiveness of outdated to be used for HUVECs culture media supplements. Growth factors content in the hPL appears to be related to its preparation method.[26] Platelet activation by means of repeated freezing/thawing method produced maximum yield of growth factors. High concentrations of the $\alpha$-granule derived growth factors in the hPL have been shown to be potent mitogenic factors. Moreover, due to its growth factors-rich content, the use of hPL in HUVECs culture can eliminate the need for exogenous growth factors. The proper choice for serum replacement in culture system is therefore distinct for each cell types.

## Conclusions

This study present evidence that the outdated hPL processed from platelets that had passed the shelf life could still stimulate HUVECs growth equivalent to fresh HPL or FBS. The use of outdated supplement in HUVECs media could be an alternative in the condition where there is a shortage of platelet and fresh platelet would be more favorable for transfusion purpose. The outdated platelet could be produced according to Good Manufacturing Practice procedures and the accepted standards for the platelet production of transfusion materials for human patients, and use for cells expansion for tissue engineering.

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## Declaration of Interest

The authors report no conflict of interest.

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