Sains Malaysiana 46(3)(2017): 463–468
http://dx.doi.org/10.17576/jsm-2017-4603-14

Isolation Methods and Culture Conditions of Human Umbilical Vein Endothelial Cells from Malaysian Women

(Kaedah Pengasingan dan Keadaan Kultur Sel Endotelium Vena Umbilikus daripada Wanita Malaysia)

RAJA NOR SUHAILA & SABREENA SAFUAN*

ABSTRACT

*Human umbilical vein endothelial cell (HUVEC) isolated from umbilical cord is widely used as endothelial cell model. However, HUVEC has been characteristically hard to maintain and showed molecular heterogeneity depending on the umbilical cord donors. Commercial HUVEC is commonly derived from European and Caucasian population which have different molecular characteristics from Asian women. This study aimed to optimize the isolation and culture condition of HUVEC using combinations of growth factors and extracellular matrix components so that the isolated HUVEC will purely represent the population under study. Umbilical cords were obtained from women post-labour. Different incubation times and digestive enzymes were used during endothelial cells isolation process. The culture conditions were optimized based on the coating materials and the media supplements. The results showed that 0.1% collagenase for 40 min incubation was the optimal isolation condition of HUVEC. HUVEC grown in 0.2% gelatin coated plate with 10% heat-inactivated fetal calf serum showed higher proliferative capacity and reduced cell death compared to other conditions (*p*<0.05). The results generated from this study provide a basic protocol of HUVEC isolation and culture conditions in order to generate working endothelial cell populations purely represent the Malaysian population.*

Keywords: Endothelial cells; HUVEC; isolation; umbilical cord

ABSTRAK

*Sel endotelium vena umbilikus manusia (HUVEC) yang dipencilkan daripada tali pusat digunakan secara meluas sebagai model sel endotelium. Walau bagaimanapun, HUVEC sukar untuk diselenggara dan menunjukkan keheterogenan molekul bergantung kepada penderma tali pusat. HUVEC komersial biasanya diperoleh daripada populasi Eropah dan Caucasian yang mempunyai ciri molekul yang berbeza daripada wanita Asia. Objektif kajian ini adalah untuk mengoptimumkan kaedah pengasingan dan keadaan kultur HUVEC menggunakan kombinasi faktor pertumbuhan dan komponen matriks ekstrasel supaya HUVEC yang dipencilkan ini akan mewakili populasi yang dikaji sahaja. Tali pusat diperoleh daripada wanita selepas bersalin. Masa pengeraman dan enzim pencernaan berbeza digunakan semasa proses pengasingan sel endotelium. Keadaan kultur dioptimumkan berdasarkan bahan salutan dan media tambahan. Keputusan menunjukkan bahawa 0.1% enzim kolagenase selama 40 minit tempoh pengeraman adalah keadaan pengasingan optimum HUVEC. HUVEC yang dikultur di atas 0.2% pinggan disalut gelatin dengan menggunakan 10% serum anak lembu yang diaktifkan haba menunjukkan kapasiti proliferatif yang lebih tinggi dan kurang kematian sel berbanding keadaan lain (*p*<0.05). Keputusan yang diperoleh daripada kajian ini menyediakan protokol asas mengenai pengasingan and keadaan kultur HUVEC untuk menjana populasi sel endotelium yang mewakili penduduk Malaysia secara khusus.*

Kata kunci: HUVEC; pengasingan; sel endotelium; tali pusat

INTRODUCTION

Endothelial cell is crucial in the development of blood and lymphatic vessels. This cell produces inflammatory regulatory molecules and regulate vascular homeostasis. *In vitro* endothelial study is beneficial in order to understand the biology and physiology of blood and lymphatic vessels. Depending on the origin, endothelial cells play significant role in angiogenesis and lymphangiogenesis, hence, this cell model is valuable in research involving drugs development and disease diagnosis.

Endothelial cells can be isolated from the capillaries, arterioles, venules and lymphatics. They are many types of endothelial cell models including human umbilical vein endothelial cells (HUVEC), human aortic endothelial cells (HAEC), human coronary artery endothelial cells (HCAEC), human dermal microvascular endothelial cells (HDMEC) and human pulmonary artery endothelial cells (HPAEC). Of these, HUVEC is widely used as endothelial cell model because of their robustness and data reproducibility. However, despite its usefulness as a model system, there are few issues of commercial HUVEC. The different origin of vascular bed used during isolation of HUVEC would contribute to molecular heterogeneity especially if the umbilical cords are from multidonor origin (Hughes 1996). The heterogeneous population of HUVEC would expressed varied markers and exhibited differences in term

of the proliferative capacity and the angiogenic process (Zhenga et al. 2014). Besides that, HUVEC derived from different origins may display inter-batch variability in their physiology and function. Variation in experimental results may also be observed even from the same donor. Different passage numbers often shows varying proliferation rates (Heng et al. 2011).

In addition, commercial HUVEC, commonly derived from the white American, European and Caucasion women have different molecular characteristic from Asian women. It was shown that HUVEC isolated from the African American is distinct in term of oxidative stress level molecules and the expression of biomarkers compared to HUVEC from Caucasion women (Feairheller et al. 2011). Nitric oxide and oxygen free radicals between Black Americans and White Americans also showed significant differences (Ugusman et al. 2014). Therefore, commercial HUVEC is not a good model to be used if the population under study is from other origins such as the Asia, in particular Malaysia.

The main objective of this study was to isolate and optimize the culture condition of HUVEC obtained from general Malaysian population after childbirth. This is important in light of HUVEC application in research settings in Malaysia and Asia. HUVEC from the same population as the sampel population is required to produce results reflective of the population under studied.

Materials and Methods

UMBILICAL CORD SAMPLES

About 10 to 15 inches of umbilical cords was taken from seven women post-labor at the Obstetric and Gynaecological Department, Hospital Universiti Sains Malaysia. Ethical approval was granted by the Human Research Ethics Committee, Universiti Sains Malaysia (USM/JEPeM/1404151). The patients involved were free from chronic diseases including gestational diabetes, HIV and hepatitis. The age of these patients range from 24 to 43 years from different ethnic populations.

ENDOTHELIAL CELLS ISOLATION FROM UMBILICAL CORDS

Endothelial cells were isolated from human umbilical cord using enzyme digestion technique. The sample processing was carried out within 24 h after collection. Antiseptic solution was used to wipe the outer surface of the cord to remove the excess blood and debris. Then, the cord was squeezed slightly to remove any clots forming inside the vein. In order to ensure maximum sterility, 2 cm of the cord from both ends was cut-off. The umbilical vein was washed with RPMI medium containing 1% penicillin streptomycin to remove excess blood and debris using a 20 mL syringe inserted into the cord. Next, 0.1% of type I collagenase was added into the vein where it was incubated at 37°C. The incubation time was optimized in three conditions including 30 min, 40 min and overnight incubation for different cords. After that, the vein was washed with 20 mL of HUVEC media into a universal to stop the proteolytic activity of the collagenase solution and centrifuged at 1500 rpm for 5 min. The resultant pellet was resuspended in 5 mL of HUVEC medium and plated in a T25 tissue culture flask. Cells were incubated overnight and the media was changed the next day to remove cells debris. HUVEC was maintained in complete M199 media. The procedure of isolation was repeated by using 0.25% trypsin as digestion reagent.

THE INFLUENCE OF GELATIN ON THE GROWTH OF HUVEC

It was reported that HUVEC needs a coating medium for optimal adhesion and growth. Therefore, this steps were carried out to determine the influence of non-coated and gelatin-coated surfaces on the rate of cell growth. 0.5×10^4 cells per mL of HUVEC was cultured in a 12 well plate under 4 conditions: non-coated plate; 0.2% gelatin-coated; 2% gelatin coated; and 20% gelatin coated. After 24 h incubation, the HUVEC cell was trypsinized and counted with a haemocytometer using trypan blue exclusion assay. This assay is based on the principle that viable cells possess an intact cell membrane which does not take up the blue dye. Dead cells will take up the dye as their membrane can no longer control the passage of macromolecules. Procedures as above were repeated at 48, 72 and 96 h time point.

THE INFLUENCE OF SERUM ON THE GROWTH OF HUVEC

Serum is usually needed as it provides growth factor that help in cell growth. This steps were carried out to determine the optimal serum concentration on the growth of HUVEC. 0.5×10^4 HUVEC per mL was cultured in a 12 well plate under 4 different conditions based on the percentages of heat-inactivated fetal calf serum (HI-FCS) in the media which are: basal media; 2% HI-FCS; 5% HI-FCS; and 10% HI-FCS. After 24 h incubation, the HUVEC cell was trypsinized and counted with a haemocytometer using trypan blue exclusion assay. Procedures as above were repeated at 48, 72 and 96 h time point.

THE INFLUENCE OF DIFFERENT MEDIA CONSTITUENTS ON THE GROWTH OF HUVEC

HUVEC, being the primary cell needs additional constituents in the complete media for optimum growth and function. Therefore, 0.5×10^4 cells per mL of HUVEC were cultured in a 12 well plate under the following conditions: basal media; complete media (include ascorbic acid, hydrocortisone, EnGS - commercial endothelial cells growth supplement, EGF, heparin and L-glutamine); complete media without ascorbic acid; complete media without hydrocortisone; complete media without EnGS (commercial endothelial growth supplement); complete media without EGF; complete media without heparin; and complete media without L-glutamine. After 24 h incubation, the HUVEC cell was trypsinized and counted with a haemocytometer

using trypan blue exclusion assay. Procedures as above were repeated at 48 and 72 h time point.

STATISTICAL ANALYSIS

For the cell viability and the cell proliferation assay, results were reported as the number of cells ± standard error of mean. Student t-test was used to calculate the significance value between control and isolated HUVEC. A *p value* of <0.05 defined as statistical significant. All statistical analysis was carried out using GraphPad Prism and Microsoft Excel 2010.

RESULTS

ENDOTHELIAL CELLS ISOLATION FROM UMBILICAL CORDS

0.25% trypsin-EDTA was too harsh to the umbilical cord where all cells including the wall of the cord were damaged and died within 30 min incubation. Hence, 0.25% trypsin-EDTA was not a recommended to be used to isolate endothelial cells from umbilical cord. In contrast, 0.1% type I collagenase was a lot gentler than trypsin-EDTA. Based on the three incubation times used, it was observed that the umbilical cord remained intact after 30 and 40 min incubation with 0.1% collagenase. It was also observed that the yield of HUVEC was higher with 40 min incubation compared to 30 min incubation of the cord. Figure 1 shows the pictomicrograph of HUVEC after 24 h and 48 h incubation.

THE INFLUENCE OF GELATIN-COATING ON THE GROWTH OF HUVEC

Figure 2 shows HUVEC proliferation at 24, 48, 72 and 96 h post plating on non-coated and different concentrations of gelatin coated flasks. The proliferation of cells increased from Day 1 until Day 4 for all gelatin coated plate. Based on the different gelatin concentrations, 0.2% gelatin shows the highest increased compared to 2% and 20% gelatin. Interestingly, uncoated flask shows a decline in HUVEC proliferation at 72 h post-plating and continue to decrease at 96 h.

THE INFLUENCE OF SERUM ON THE GROWTH OF HUVEC

Figure 3 shows the cell viability of HUVEC in different percentages HI-FCS. 10% HIFCS shows the optimal condition of HUVEC growth compared to other HI-FCS concentrations at 24 to 96 h post plating.

THE INFLUENCE OF DIFFERENT MEDIA CONSTITUENTS ON THE GROWTH OF HUVEC

The influence of media constituents on the growth of HUVEC was investigated under 8 different conditions.

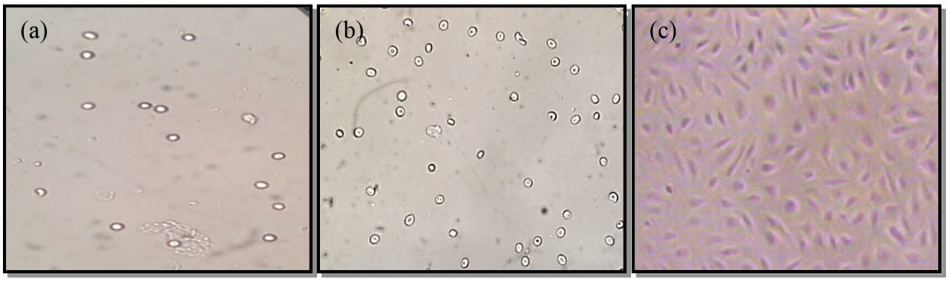

FIGURE 1. Human umbilical vein endothelial cells after 24 h (a and b) and 48 h (c) of incubation (a) shows the yield of endothelial cells with 30 min incubation with 0.1% type I collagenase and (b) shows the yield of cells with 40 min incubation with 0.1% type I collagenase

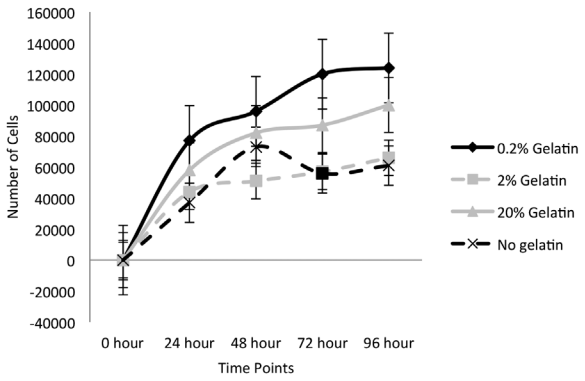

FIGURE 2. The influence of gelatin coating on HUVEC cell proliferation at 24, 48, 72 and 96 h time point. 0.2% gelatin shows the highest proliferation rate compared to the other coating conditions. Uncoated flask shows decreased cell viability at 72 and 96 h time point

HUVEC grown in complete media and without EnGS shows increase proliferaton rate from 24 to 96 h time points. The proliferation of HUVEC in complete media was 2-fold higher than cells grown in basal media. HUVEC grown in media without ascorbic acid plateau after 24 h post plating. Media without hydrocortisone shows a decrease in cell number at 72 h time point. At 24 h time point, HUVEC grown in media without heparin, L-glutamine and EGF shows a reduction in cell count. Figure 4 shows HUVEC proliferation rate under different media requirements.

Discussion

Endothelial cells have been widely used to study the vasculature and diseases associated with haemodynamic disturbances. However, endothelial cells exhibited large degrees of heterogeneity between donor and handling protocols (Gifford et al. 2004). These contribute to the variation of results and decrease the robustness of data. In addition, commercial endothelial cells derived from the European and American population differs in term of molecular characteristics and non-reflective of the population under study (Karasarides & Chi 2007). Therefore, the aim of this study was to isolate human endothelial cells isolated from the umbilical cord (HUVEC) collected among Malaysian population and to optimized the culture conditions of HUVEC.

The enzymatic digestion technique used in this study was modified from Siow (2012), but the protocol did not specifically stated the incubation period for collagenase to digest endothelial cells from the umbilical vein. Trypsin-EDTA used in this study is too harsh for the cells. This is in accordance with other study showing that trypsin incubation for 10 min in 37°C reduced cell viability and caused cellular death (Brown et al. 2007). The result from this study shows that 40 min incubation time with collagenase is the optimal digestion time to isolate endothelial cells compared to other time point.

In this study, 0.2% gelatin coated flask showed the highest proliferative capability compared to non-coated, 2% and 20% gelatin-coated flask. According to this result, coating matrix are able to help in maintaining

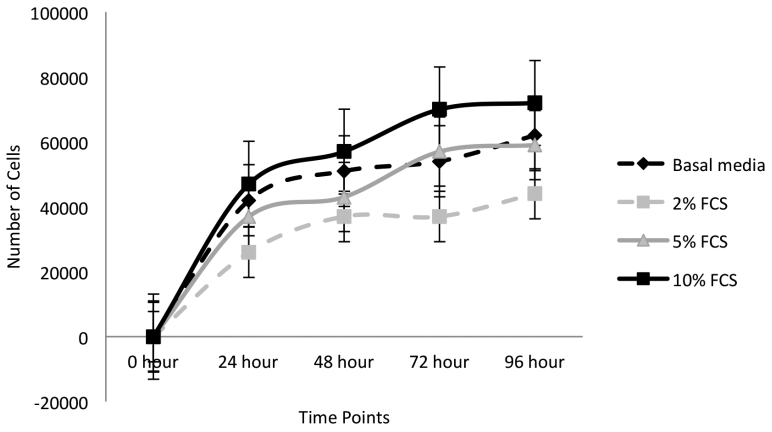

FIGURE 3. The influence of serum on HUVEC cell proliferation at 24, 48, 72 and 96 h time point. 10% HI-FCS shows the optimum cell number at all time point assessed

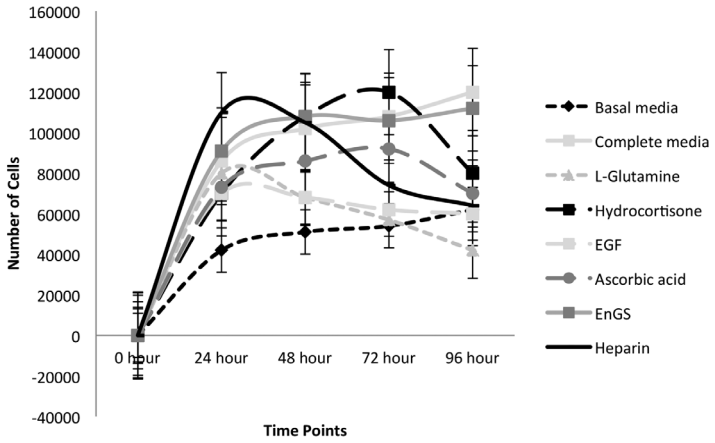

FIGURE 4. The influence of media constituents on HUVEC cell proliferation at 24, 48, 72 and 96 h time point

primary cell growth. The interaction of growth factor and regulators with extracellular matrix could improve cell behavior (Chennazhy & Krishnan 2005). However, in high serum condition, HUVEC are not dependent on an ECM for attachment and proliferation. The high-serum media provide sufficient adhesive factors, such as fibronectin and vitronectin to aid in cell attachment (Young et al. 2005). This is in line with previous study showing that the present of gelatin cause cellular propagation but the cell still also grow on raw plastic albeit slower (Feugier et al. 2005; Sipehia et al. 1996).

With regards to the percentage of HI-FCS in the media, HUVEC cell proliferation shows significant increase when cultured in 10% HI-FCS compared to other concentrations. Previous study using macrovascular cells also showed that cell growth is dependent on factors present in serum (Terramani et al. 2000) and that optimal serum concentration is needed to ensure healthy cells (Siow 2012). In addition, this study also evaluated the influence of different media supplements on the viability and proliferation of HUVEC. The results show that 0.75 U/mL heparin, 10 mM L-glutamine and 5 ng/mL EGF is a crucial factors to be added to HUVEC media in order to maintain optimal cell proliferation. Heparin acts as stabilizers of other growth factors with the release of heparin-binding factor. L-glutamine was also important in primary cell culture as it replaced the non-essential amino acids as energy source. In addition, EGF is a crucial factors to be added to HUVEC media without which, the endothelial cells will cease growing and this result is supported by other studies (Terramani et al. 2000).

The function of hydrocortisone in HUVEC media is not as crucial as heparin, L-glutamine and EGF as HUVEC shows normal proliferation rate compared to control up to 72 h time point. Hydrocortisone is involved in regulating the proliferation and differentiation of cells. Mouse epidermal cells show normal growth rate when culture in serum free media supplemented with hydrocortisone (Hirobe et al. 2004). Clearly in this this study, serum in the HUVEC media has replaced the function of hydrocortisone up to 72 h post plating. Interestingly, HUVEC grown in media lacking EnGS, which is the commercially available growth suplement shows steady proliferative rate combarable to HUVEC grown in complete media. Clearly, with this observation, the addition of EnGS has no significant advantage in term of cell proliferative capacity of HUVEC. This is very important in light of the cost required to purchase commercially made EnGS factors. We hypothesized that the addition of serum in HUVEC media coupled with EGF is able to sustain optimal growth rate of HUVEC (Siow 2012).

Conclusion

This study suggested that the optimal conditions for endothelial cells proliferation require a balance of necessary components for cellular proliferation. It is essential to optimize cell culture condition for each type of experiment and to use as long as possible identical batch reagents and media. In addition, further study is required to investigate the yield of cells based on length and types of cord. Molecular studies need to be carried out to identify the similarities and differences among cords from different individuals and populations.

Acknowledgements

This study was supported by Universiti Sains Malaysia (Short Term Grant: 304/PPSK/61312137). Special thanks to Dr Amir Ismail for the umbilical cord samples and Dr Wan Amir Wan Nizam for technical supports.

References

Brown, M.A., Wallace, C.S., Anamelechi, C.C., Clermont, E., Reichert, W.M. & Truskey, G.A. 2007. The use of mild trypsinization conditions in the detachment of endothelial cells to promote subsequent endothelialization on synthetic surfaces. *Biomaterials* 28(27): 3928-3935. doi: 10.1016/j.biomaterials.2007.05.009.

Chennazhy, K.P. & Krishnan, L.K. 2005. Effect of passage number and matrix characteristics on differentiation of endothelial cells cultured for tissue engineering. *Biomaterials* 26(28): 5658-5667. doi: DOI 10.1016/j.biomaterials.2005.02.024.

Feairheller, D.L., Park, J., Rizzo, V., Kim, B. & Brown, M.D. 2011. Racial differences in the responses to shear stress in human umbilical vein endothelial cells. *Vascular Health and Risk Management* 7: 425-431.

Feugier, P., Black, R.A., Hunt, J.A. & How, T.V. 2005. Attachment, morphology and adherence of human endothelial cells to vascular prosthesis materials under the action of shear stress. *Biomaterials* 26(13): 1457-1466. doi: http://dx.doi.org/10.1016/j.biomaterials.2004.04.050.

Gifford, S., Grummer, M., Pierre, S., Austin, J., Zheng, J. & Bird, I. 2004. Functional characterization of HUVEC-CS: Ca2+ signaling, ERK 1/2 activation, mitogenesis and vasodilator production. *Journal of Endocrinology* 182(3): 485-499.

Heng, B.C., Xia, Y., Shang, X., Preiser, P.R., Law, S.K.A., Boey, F.Y.C. & Venkatraman, S.S. 2011. Comparison of the adhesion and proliferation characteristics of HUVEC and two endothelial cell lines (CRL 2922 and CRL 2873) on various substrata. *Biotechnology and Bioprocess Engineering* 16: 127. doi:10.1007/s12257-010-0141-9.

Hirobe, T., Furuya, R., Ifuku, O., Osawa, M. & Nishikawa, S.I. 2004. Granulocyte-macrophage colony-stimulating factor is a keratinocyte-derived factor involved in regulating the proliferation and differentiation of neonatal mouse epidermal melanocytes in culture. *Experimental Cell Research* 297(2): 593-606. doi: http://dx.doi.org/10.1016/j.yexcr.2004.03.042.

Hughes, S.E. 1996. Functional characterization of the spontaneously transformed human umbilical vein endothelial cell line ECV304: Use in an *in vitro* model of angiogenesis. *Experimental Cell Research* 225: 171-185.

Karasarides, M. & Alfred L.C. 2007. Chapter 1. Standardization of primary cell culture process. In *Handbook of Primary Cell Culture: Standardization of Primary Cell Culture*. Retrieved from www.mc.vanderbilt.edu/root/pdfs/mclaughlin_lab/primary_cell_culture_handbook.pdfpp. 8-17.

Siow, R.C. 2012. Culture of human endothelial cells from umbilical veins. *Human Cell Culture Protocols*. New York: Springer. pp. 265-274.

Sipehia, R., Martucci, G. & Lipscombe, J. 1996. Transplantation of human endothelial cell monolayer on artificial vascular prosthesis: The effect of growth-support surface chemistry, cell seeding density, Ecm protein coating, and growth factors. *Artificial Cells, Blood Substitutes, and Biotechnology* 24(1): 51-63. doi: 10.3109/10731199609117431.

Terramani, T., Eton, D., Bui, P., Wang, Y., Weaver, F. & Yu, H. 2000. Human macrovascular endothelial cells: Optimization of culture conditions. *In Vitro Cellular & Developmental Biology - Animal* 36(2): 125-132. doi: 10.1290/1071-2690(2000)036<0125:HMECOO>2.0.CO;2.

Ugusman, A., Zakaria, Z., Chua, K., Mohd Nordin, N.A.M. & Mahdy, Z.A. 2014. Role of rutin on nitric oxide synthesis in human umbilical vein endothelial cells. *The Sci. World J.* 2014: Article ID. 169370. http://dx.doi.org/10.1155/2014/169370.

Young, S., Wong, M., Tabata, Y. & Mikos, A.G. 2005. Gelatin as a delivery vehicle for the controlled release of bioactive molecules. *Journal of Controlled Release* 109(1): 256-274.

Zheng, Y.W., Nie, Y.Z., Tsuchida, T., Zhang, R.R., Aoki, K., Sekine, K., Ogawa, M., Takebe, T., Ueno, Y., Sakakibara, H., Hirahara, F. & Taniguchi, H. 2014. Evidence of a sophisticatedly heterogeneous population of human umbilical vein endothelial cells. *Transplantation Proceedings* 46(4): 1251-1253.

School of Health Sciences, Universiti Sains Malaysia
1610 Kubang Kerian, Kelantan Darul Naim
Malaysia

*Corresponding author; email: sabreena@usm.my

Received: 22 February 2016
Accepted: 28 June 2016