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Impact of Container, Temperature and Microcarriers on Inflammation and Endothelial Activation in Human Endothelial Cells

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ABSTRACT

Human umbilical vein endothelial cells (HUVECs) were cultured on microcarrier beads to accommodate different experiment apparatus such as rotating wall vessel. In this study, fluid operating apparatus (FPA) was used. However, the effect of inflammation and endothelial activation biomarkers in HUVECs cultured on different culture surface and containers are not well established. The effects of temperature changes on these biomarkers in HUVECs grown in FPA, a spaceflight hardware, are still unclear. The objective of this study was to compare the protein and gene expression of inflammation and endothelial activation biomarkers in (i) HUVECs cultured on microcarrier beads in conventional culture flask (CCFMC) vs. conventional culture flask (CCF) (ii) HUVECs cultured on microcarrier in FPA (FPAMC) vs. CCFMC and (iii) HUVEC cultured in FPAMC with ideal temperature (37°C) (FPAMC) vs. simulated space travel temperature(25-37°C), (FPAMC-ST). sICAM-1 and sVCAM-1protein expression in HUVECs grown in CCFMC were higher than CCF. FPAMC had higher IL-6, TNF-α, ICAM-1, VCAM-1, e-selectin, NFκB and eNOS gene expression than in CCFMC. FPAMC-ST had higher ICAM-1 and e-selectin protein expression than FPAMC- in ideal temperature. HUVECs are cultured onto microcarrier in simulated space flight temperature compared with ideal temperature had higher protein expression of sICAM-1 and e-selectin but the protein and gene expression of other biomarkers of inflammation and endothelial activation are comparable. This suggests that differences in culture surface and container are have an impact on the expression of inflammation and adhesion molecule by HUVECs.

Keywords: Adhesion molecules, endothelial cells, fluid processing apparatus (FPA), inflammation, microcarrier beads

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INTRODUCTION

Vascular endothelium is an active metabolic component of tissues that has a number of important physiological functions, such as regulation of microvascular fluid and solute exchange, maintenance of anti-thrombogenic vessel surface, regulation of vascular tone and blood flow and control of leukocyte trafficking via surface expression of adhesion molecules (Kvietys & Granger, 1997). It has been postulated that endothelial cells can adapt rapidly to changes in their environment and local conditions (Infanger et al., 2006). The endothelial cells are in close contact and form a smooth layer that prevents blood cell interaction with the vessel wall. Ultra structurally, each cell can be seen to be anchored to an underlying basal lamina. Individual cells are anchored together by adhesion junctions, including prominent tight junctions, which prevent diffusion between cells (Infanger et al., 2006).

Microcarriers were specifically developed for high yield culture from a wide range of animal cells. The surface of the dextran and collagen-based beads has been optimised for efficient attachment and spreading of cells (Chen, Htay, Santos, Gillies, & Helen, 2009). The microcarriers are transparent and allow easy microscopic examination of the attached cells. Some studies have already shown that the microcarrier cell culture system produce cells that closely resemble the in vivo features of both morphological phenotype and gene-expression profile (Abbott, 2003; Chen et al., 2009). Previously, human endothelial cells were cultured on microcarriers and flown to International Space Station (ISS) in Fluid Processing Apparatus (FPA), a spaceflight hardware (Muid et al., 2010). This cell culture method has been optimised to accommodate the usage of FPA in the spaceflight experiment since FPA was initially designed for suspensions of microorganisms and not for adherence cells like HUVECs (Nawawi, Muid, Froemming, & Manaf, 2010). However, studies on inflammatory status in microcarrier cultured endothelial cells in FPA compared with conventional culture flask is scarce. Studies focusing on this would contribute to the development of FPA as a suitable culture container for growing endothelial cells in spaceflight experiment.

There is extensive evidence to indicate that heterogeneity develops in part, as a result of variation in exposure of endothelial cells (EC) to environmental stimuli and temperature changes (Cines, Pollak, Buck, & Loscalzo, 1998). During a spaceflight experiment, the cells have to undergo temperature fluctuation, especially during launch and orbiting before reaching the ISS. Upon reaching ISS, endothelial cells are transferred into a 37°C incubator, optimal temperature for the growth of cell culture. It is postulated that temperature fluctuations which occur during spaceflight travel may alter the protein and gene expression of inflammation and endothelial activation. Inflammation and endothelial activation have been involved in the pathogenesis of cardiovascular disease through atherosclerosis (Grenon, Jeanne, Aguado-Zuniga, Conte, & Hughes-Fulford, 2013). Inflammation is associated with increased cytokines such as interleukin 6 (IL-6) and tumour necrosis factor-alpha (TNF-α) leading to endothelial activation by enhanced expression of adhesion molecules [intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and e-selectin], which are critical for monocyte recruitment into the vessel wall across intact endothelium (Rajashekhar et al., 2006). Moderate hypothermia has been shown to suppress inflammatory gene transcription in human

cerebral endothelial cells (Sutcliffe, Smith, Stanimirovic, & Hutchison, 2001). However, there are limited data or studies on the effects of space travel associated temperature fluctuations on these biomarkers in endothelial cells.

Therefore, this study was designed to investigate the expression of inflammatory and endothelial activation biomarkers by: (i) endothelial cells cultured onto conventional cell culture flask with microcarrier beads (CCFMC) and conventional cell culture (CCF); (ii) endothelial cells cultured onto microcarrier beads in different culture containers (conventional culture flask vs. Fluid Processing Apparatus (FPA)- FPAMC; and (iii) endothelial cells cultured on microcarrier beads in FPA at ideal temperature (FPAMC) versus space travel temperature fluctuations (FPAMC-ST).

MATERIALS AND METHOD

Materials

Medium 200 and Low Serum Growth Supplements (LSGS) were obtained from Cascade Biologics, USA. Accutase was purchased from ICN Biomedical, USA. ELISA test kits for e-selectin, sICAM-1, sVCAM-1, IL6 and TNF-α were purchased from Bender MedSystems, Austria. Cytodex 3 microcarrier beads were purchased from GE Healthcare Biosciences AB, Uppsala, Sweden. Phosphate-buffered saline (PBS) was obtained from MP Biomedicals, France while fluid processing apparatus (FPA) was obtained from Bioserve, USA.4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline was obtained from Gibco, USA and accutase was purchased from ICN Biomedical, USA. QuantigenePlex 2 assay kit was purchased from Panomics, USA.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Cascade Biologics, USA and cultured in medium 200 (M200) supplemented with LSGS in a humidified incubator set at 37°C and 5% carbon dioxide (CO_2) until 80% confluency. Sub-cultivation ratios of HUVECs were 1:3 (culture: medium).

Preparations of Microcarrier Beads

Twenty-five mg of microcarrier beads (cytodex-3) were added into 25 ml Schott Duran bottle. Micropipette was used to transfer 5 ml of PBS (without Magnesium and Calcium) into the bottle to wash the microcarrier beads. The PBS was left to settle down at the bottom of bottle and then decanted. This washing step was repeated 3 times. This microcarrier beads were then sterilised by autoclaving at 121°C, 15 psi in 10 ml of PBS. The microcarrier beads were stored in room temperature before. Before the addition of HUVECs, the sterilised microcarrier beads were washed once with PBS. Subsequently, they were washed again twice with M200 culture medium. Finally, the microcarrier beads were soaked in M200 culture medium for 30 minutes prior to addition of HUVECs.

HUVECs Cultured in Culture Flask

HUVECs was added into T-75cm^2 cell culture flask with 100,000 cells seeding density/ml. Ten ml M200 culture medium was added into the flask and incubated at 37°C incubator for 12 days. Culture medium M200 (5 ml) was added into the flask periodically every three days.

HUVECs Cultured on Microcarrier Beads in Culture Flask (CCFMC)

M200 culture medium from the T-25 cm2 flask was aspirated and discarded. Monolayer of HUVECs in the culture flask was washed with PBS without magnesium (Mg) and calcium (Ca). Two millilitre of PBS was added into culture flask. Culture flask was agitated for 5 seconds. PBS was then discarded from the culture flask. Three millilitre of accutase was added onto the HUVECs monolayer in the culture flask and incubated in the humidified environment at 37°C in CO_2 incubator for 3 minutes. Detachment of HUVECs was observed under phase-contrast microscope. The HUVECs' suspension was transferred into 15 ml centrifuge and spun at 1000 RPM for severely minutes. Supernatant was carefully discarded. The HUVECs pellet was dissolved with 1 ml of M200 medium and suspended for several times to break the cell clumps and produced a uniformly dispersed cell suspension. The number of cells was determined by haemocytometer. A volume of suspension cells containing 100,000 cells was transferred into a sterile siliconized petri dish (60 mm × 20 mm) containing microcarrier beads. Petri dish was positioned in the 45-degree angle. The HUVECs were allowed to attach on the microcarrier beads surface for one hour with gentle agitation for 10 second for every 15 minutes. The HUVECs on the microcarrier beads were then transferred into a T-75 cm^2 cell culture flask. Then 10 ml of M200 culture medium was added into the cell culture flask and incubated at 37°C humidified in CO_2 incubator for 12 days. Culture medium M200 (5 ml) was added periodically to the flask every three days.

HUVECs in FPA Grown in Ideal (37oc) and Changes of Temperature (FPAMC and FPAMC-ST)

The FPA bio container is a syringe-like device with a cylindrical glass container housed within a Lexan™ sheath. A plunger is provided at the open end of tube (Figure 1). Three rubber septa are provided so as to divide the glass barrel into three chambers, namely culture chamber, stasis chamber and fixative chamber. When the plunger is depressed, a fluid bypass allows fluid from the stasis chamber to be injected into the culture chamber for activation of the culture. Similarly, a termination reagent can be injected from the fixative chamber into the growing culture for growth cessation at the predetermined termination point. Method of culturing HUVECs in FPA were previously described (Nawawi, Muid, Froemming, & Manaf, 2010). Briefly, 25 mg of HUVEC-coated microcarrier beads (with 5×10^5 cells) were prepared according to the above-mentioned method and added into the culture chamber of the FPA. Three millilitre of the M200 culture medium having 20 mM HEPES-buffered saline was also added into the culture chamber. A rubber septum was inserted. An amount of 1.5 ml of M200 culture medium having 20 mM HEPES-buffered saline was added into the stasis chamber. A rubber septum was then inserted. Next, 1.5 ml of M200 culture medium having 20 mM HEPES buffered saline was

added into the fixative chamber. One group of FPAMC was incubated in ideal temperature at 37°C in incubator for 12 days (FPAMC). Other sets of FPAMC were grown according to a simulated temperature changes associated with space travel (FPAMC-ST) as stated in Table 1. Three sets (triplicates) of FPAs having the above configuration were prepared.

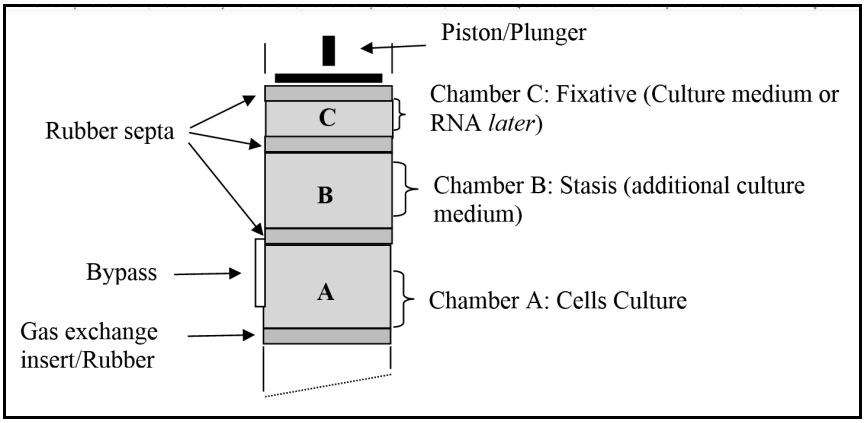

Figure 1. FPA diagram showing the three chambers separated by septa. The fluid bypass enables fluid from the stasis chamber to be injected into the culture chamber during activation and enables the termination reagent to be injected into the growing culture for growth cessation during termination

Table 1
Temperature profile from day 1-12

Time (days)	Temperature (°C)
Day 1	25
Day 2	25
Day 3	37
Day 4-9	37
Day 10	25
Day 11	25
Day 12	37

Soluble Expressions of Cytokine and Adhesion Molecules Measurement in the Supernatant

Soluble inflammatory markers of IL-6, TNF-α, ICAM-1, VCAM-1 and e-selectin in supernatant of HUVECs were measured by ELISA standard kits (Bender Med System, Vienna, Austria). Each test was performed in triplicates according to the instructions provided by the manufacturer. At the end of ELISA testing, absorbance was obtained by spectrophotometer (Micro Quant, Biotek Instruments) at 405 nm wavelength.

DNA Hybridisation Method

The RNA of HUVECs from all samples was extracted using an RNA extraction kit (Qiagen, USA). Cells lysate was obtained from HUVEC cultured in FPAMC-IT and FPAMC-ST. Samples were collected and preceded with QuantigenePlex assay according to manufacturer's protocols. The plate was immediately read using the Bio-Plex system (Bio-Rad Laboratories, Hercules, CA). Results were normalised against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine guanine phosphoribosyl transferase-1 (HPRT-1) to obtain standard norm of the ratio. Primer sequence and annealing temperature for each measured gene are shown in Table 2 (Muid et al., 2010).

Table 2
Primer sequence for mrna expression

i) IL-6
Forward primer: GCC TTC GGT CCA GTT GCC TT
reverse primer : GCA GAA TGA GAT GAG TTG TC
ii) ICAM-1 (CD54)
Forward Primer AGAGGTCTCAGAAGGGACCG
Reverse Primer GGGCCATACAGGACACGAAG
III) VCAM-1 (CD106)
Forward primer: GGTGGGACACAAATAAGGGTTTTGG
Reverse primer: CTTGCAATTCTTTTACAGCCTGCC
IV) E-selectin
Forward primer: TGAAGCTCCCACTGAGTCCAA
Reverse primer: GGTGCTAATGTCAGGAGGGAGA
V) TNF-α
Forward primer: CCGGGCGTGGTGGTGAG
Reverse primer: TCTGCCTTTTGGGTCTTGTGAATA
VI) eNOS
Forward primer: ATGGGCAACTTGAAGAGCGTGG
Reverse primer: TAGTACTGGTTGATGAAGTCCC
Reference gene:
i) GAPDH
Forward primer: CCACCCATGGCAAATTCCATGGCA
Reverse primer: TCTAGACGGCAGGTCAGGTCCACC
iii) HPRT-1:
Forward primer: GGCAAAACAATGCAAACCTT
Reverse primer: CAAGGGCATATCCTACGACAA

Statistical Analysis

Data was analysed using Statistical Package for the Social Sciences version 22.0. Significant value was set at $p<0.05$. Results were expressed as mean +standard error means. Independent T- Test was performed to compare the differences between two different groups.

RESULTS

Soluble Protein Expressions of Cytokine and Adhesion Molecules Measurement in the Supernatant

The CCFMC had higher sICAM-1 ($p<0.0001$) and sVCAM-1 ($p<0.0001$) level compared with CCF. There was no significance difference in IL-6, TNF-α and e-selectin level was found between CCFMC and CCF. FPAMC had lower IL-6 ($p<0.05$) and VCAM-1 ($p<0.05$) levels compared with CCFMC. FPAMC-ST had higher protein expression of sICAM-1 and e-selectin compared with FPAMC-. Both FPAMC-ST and FPAMC- in ideal temperature had similar protein expression of IL-6, TNF-αand VCAM-1. Data is shown in Table 3.

Table 3
Protein expression of inflammation and endothelial activation biomarkers

Biomarkers/ Samples	CCF	CCFMC	FPAMC	FPAMC-ST
IL-6 (pg./ml)	37.8 + 8.0	47.4 + 7.7	14.0 + 2.0 #	13.1 + 2.0
TNF-α (pg/ml)	78.5 + 4.1	59.4 + 1.6	58.2 + 2.7	65.4 + 1.5
sICAM-1 (pg/ml)	1055.4 + 62.1	2778.4 + 61.9 ****	2601.8 + 47.1	7738.7 + 318.2 $
sVCAM-1 (pg/ml)	450.8 + 55.9	12747.7 + 336.0 ****	5322.6 + 1590.1 #	7013.0 + 1895.4
e-selectin (pg/ml)	1506.5 + 82.5	1953.3 + 126.0	1609.2 + 41.2	2059.5 + 137.9 $

**** $p<0.0001$ compared to CCF, # $p<0.05$ compared to CCFMC, $ $p<0.05$ compared to FPAMC
CCF: Conventional culture flask; CCFMC: HUVECs cultured on microcarrier beads in conventional culture flask; FPAMC: HUVECs cultured on microcarrier in FPA; FPAMC-ST: HUVECs cultured in FPAMC with simulated space travel temperature (25-37°C). Data is expressed as Mean + SEM (n=3)

mRNA expression of IL-6, TNF-α, ICAM-1, VCAM-1 and e-selectin

There were no significant differences in the gene expression of IL-6, TNF-α, ICAM-1, VCAM-1, e-selectin, NFκB and eNOS between CCFMC and CCF. The IL-6 ($p<0.0001$) and TNF-α ($p<0.0001$) gene expression in FPAMC were higher than in CCFMC. Similarly, ICAM-1 ($p<0.0001$), VCAM-1 ($p<0.0001$), e-selectin ($p<0.0001$) levels in FPAMC were higher than in CCFMC. The involvement of NFκB pathway on the increment of cytokines and adhesion molecule was investigated and it was found that NFκB gene expression in endothelial cells

cultured in FPAMC was higher than in CCFMC ($p<0.01$). eNOS gene expression in endothelial cells cultured in FPAMC was higher than CCFMC ($p<0.05$). Both FPAMC and FPA MC-IT expressed similar gene expression of IL-6, TNF-α, ICAM-1, VCAM-1, e-selectin, NFκB and eNOS. Data is displayed in Table 4.

Table 4
Gene expression of inflammation and endothelial activation biomarkers

Biomarkers/ Samples	CCF	CCFMC	FPAMC	FPAMC-ST
IL-6	0.05+ 0.00	0.02+ 0.005	0.58+ 0.03	1.65+ 0.03
Norm ratio			####	
TNF-α	0.001+ 0.00	0.001+ 0.00	0.13+ 0.04	0.18+ 0.02
Norm ratio			####	
ICAM-1	0.22+ 0.02	0.23+ 0.03	2.81+ 0.12	9.68+ 2.14
Norm ratio			####	
VCAM-1	0.001+ 0.00	0.003+ 0.00	0.77+ 0.28	0.63+0.2
Norm ratio			####	
e-selectin	0.006+ 0.00	0.004+ 0.00	0.39+ 0.08	0.59+ 0.08
Norm ratio			####	
NFκB	0.17 + 0.01	0.006 + 0.00	0.29 + 0.00	1.10 + 0.1
Norm ratio			##	
eNOS	0.10 + 0.005	0.02 + 0.005	2.89 + 0.10	2.15 + 0.05
Norm ratio			#	

$p<0.0001$, ## $p<0.01$, # $p<0.05$ compared to CCFMC

CCF: Conventional culture flask; CCFMC: HUVECs cultured on microcarrier beads in conventional culture flask; FPAMC: HUVECs cultured on microcarrier in FPA; FPAMC-ST: HUVECs cultured in FPAMC with simulated space travel temperature (25-37°C). Data is expressed as Mean + SEM (n=3)

DISCUSSION

Microcarriers are commonly used to grow anchorage dependent cell lines in suspension culture (Hamid, McCluskey, McClenaghan, & Flatt, 2000). They are inert materials with surface characteristics suitable for promoting cell culture including charge, polarity, chemical structure and hydrophobicity (Levin, Ting-Beall, & Hochmuth, 2001). In this study, Cytodex 3 microcarriers with a surface layer of denatured collagen covalently bound to a matrix of cross linked dextran was used. It has been suggested that in comparison with conventional monolayer culture methods on stacking flat surface, microcarriers provide better surface for cell attachment and a large surface area can be contained in a given reactor volume or other culture system such as fluid operating apparatus (FPA) with a relatively uniform environment (Wong, Peshwa, & Hu, 2004). Even so, there are limited studies on the expression of inflammatory and endothelial activation biomarkers by the endothelial cells grown on microcarrier compared with those grown on flat surface of conventional (polystyrene) culture flask.

This present study has shown that endothelial cells grown on microcarrier (CCFMC) expressed 2.6 and 28.3-fold higher soluble ICAM-1 and VCAM-1 protein expression respectively, than those cells grown on flat surface of cell culture flask (CCF). It has been suggested that the process of cell attachment to any foreign surface can be viewed as a combination of two separate processes which are the initial absorption of cells to microcarrier surface followed by adhesion molecule mediated attachment and cell spreading. The cell adhesion molecule-mediated interactions are important to develop a firm grip of cells so that the cells are remained attached on the microcarrier beads (Wong, Peshwa, & Hu, 2004). The expression of adhesion molecules, either covalently bound to microcarriers or in soluble form in medium promotes subsequent development of firm grip of cells on microcarriers and facilitate cell spreading (Kan, Minamoto, Sunami, Yamam, & Umeda, 1982; Varani, Fligiel, Inman, Beals, & Hillegas, 1995). In most cases, the cell synthesises adhesion molecules on their own (Wong, Peshwa, & Hu, 2004). It can be explained the increased ICAM-1 and VCAM-1 protein expression by endothelial cells grown on microcarrier beads in this present study. However, it is interesting to note that there were no differences in the protein expression of e-selectin and other biomarkers of inflammation between the two groups. In addition, the gene expression of all the studied biomarkers was comparable between both groups. It appears that ICAM-1 and VCAM-1 are most important in providing firm endothelial cell adherence onto the microcarrier beads in facilitating cell spreading and growth in CCF.

Fluid operating apparatus (FPA) is a flight hardware that is widely used by space scientists. FPA comprises three chambers namely, a culture chamber, a stasis chamber and a fixative chamber. It is generally known that FPAs are more suitable as a culture container for non-anchorage dependent cells. To the best of our knowledge, FPA has not been previously utilised for growing anchorage cells including human endothelial cells. Hence, in the present study, the use of FPA in growing HUVECs, one example of anchorage cells was optimised for spaceflight experiment. In addition, microcarrier beads were added to culture HUVECs in FPA. Endothelial cells cultured onto microcarrier beads in FPA had lower ICAM-1 and sVCAM-1 protein expression compared with microcarrier beads in culture flask. However, the result of protein expression was in contrast with gene expression where endothelial cells cultured onto microcarrier in the FPA had higher IL-6, TNF-α, ICAM-1, VCAM-1, e-selectin, NFκB and eNOS gene expression compared with those cultured in cell culture flask. The reason for the different expression of cytokines and adhesion molecules in the protein and gene levels may be due to the post-translational modification of the protein which can stabilise or enhance degradation (Aiken, Kaake, Wang, & Huang, 2011). Therefore, the protein expression is not always parallel to mRNA abundance, and enhanced gene expression does not guarantee that this gene will result in protein synthesis (Vogel et al., 2010). Another possible reason is protein translation and increased cell surface expression in response to increase gene expression. However, there is a possibility that this cell surface protein is not being secreted into supernatant to become soluble protein expression of adhesion molecule. Therefore, it is suggested that future studies should address and investigate this finding. In this present study, gene expression analysis showed that culturing endothelial cells in the FPA may enhance the expression of cytokines and adhesion molecules. Therefore, it can be postulated that growing endothelial cells in different culture container/apparatus may give a different expression of cytokines and

adhesion molecules, at least in this study. Therefore, in the spaceflight experiments, it is of great importance to have controlled culture cells that have similar environment to avoid any confounding factors.

Since temperature changes occur during spaceflight travel, it is important to investigate whether this actual temperature profile due to space travel temperature fluctuations can give an impact on the expression of inflammation and endothelial activation by endothelial cells cultured in FPA. In this present study, we have found that ICAM-1 and e-selectin protein expression are affected in terms of being temperature sensitive. It has been shown that ICAM-1 and e-selectin expression by HUVECs cultured in FPA with space travel temperature fluctuations is higher than those in ideal temperature (37°C). In our experiment, the temperature changes range is between 25-37°C. This temperature profile was based on the temperature reading recorded by HOBO temperature recorder in the International Space Station (ISS) during a spaceflight mission. Johnson, Haddix, Pohlman and Verrier (1995) reported the down regulation of e-selectin expression in HUVECs incubated at 25°C. In contrast, our study shown e-selectin and ICAM-1 protein expression was higher in space travel temperature profile compared with that at ideal temperature of 37°C. Therefore, it can be suggested that temperature changes can lead to increment of adhesion molecules protein expression in endothelial cells. It also suggested that ICAM-1 and e-selectin are a sensitive towards temperature fluctuations in endothelial cells.

It can be concluded that HUVECs in culture flask with microcarrier beads expressed higher soluble protein expression of ICAM-1 and VCAM-1 but the protein expression of other biomarkers of inflammation and endothelial activation and gene expression of these biomarkers are comparable. Interestingly, endothelial cells cultured onto microcarrier beads in FPA compared with culture flask have increased gene expression of biomarkers of inflammation and endothelial activation. In contrast, the expression of soluble IL-6 and VCAM-1 protein expression is higher in culture flask compared with FPA while there was no difference in other protein biomarkers. This suggests that differences in culture surfaces and containers have impact on the expression of inflammation and adhesion molecule by HUVECs. The HUVECs cultured onto microcarrier beads in simulated space flight temperature compared with ideal temperature had higher protein expression of sICAM-1 and e-selectin but the protein and gene expression of other biomarkers of inflammation and endothelial activation are comparable.

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