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ORIGINAL ARTICLE

Effects of Secretome from Dynamic 3D Cell Culture System onto Growth and Cytoprotection of Nasal Fibroblast

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ABSTRAK

Model tiga dimensi (3D) menyerupai ciri-ciri persekitaran tisu asli, justeru morfologi dan isyarat-isyarat sel daripada kultur 3D selalunya lebih menyerupai fisiologi asal berbanding sel kultur dua dimensi (2D). Diketahui juga, rembesan sel mempunyai kesan parakrin kepada pertumbuhan sel-sel lain. Dalam kajian ini, pengkulturan fibroblast hidung menggunakan system kultur sel 3D telah dioptimumkan dan kesan bahan rembesan (BR) daripada kultur 3D terhadap kadar pertumbuhan dan perlindungan sel telah dikaji. Fibroblas hidung dipencilkan daripada turbinate hidung manusia. Mikrosfera yang sesuai telah dipilih melalui pengkulturan fibroblast pemindahan ke-3 pada pelbagai jenis mikro sferapolisterin PolyGEM™. Kemudian, sel-sel telah dikulturkan pada mikrosfera yang terpilih menggunakan system kultur 3D dan media terkondisi (MT) telah dikumpulkan. Media terkondisi tiga dimensi (MT3D) telah ditambah kepada fibroblast untuk mengkaji kadar perlekatan sel, kadar proliferasi, dan perlindungan sel terhadap kesitotoksikan Centella asiatica. Asai protein asid bicinchonic dijalankan untuk mengetahui kuantiti protein di dalam BR. Elektroforesis gel poliakrilamida-Sodium Dodesil Sulfat (SDS-PAGE) telah dilakukan untuk memperoleh profil awal protein dan membandingkan profil MT3D dengan protein media terkondisi dua dimensi (MT2D). Kajian ini menunujukkan MT3D tidak menggalakkan perlekatan dan proliferasi sel secara signifikan. BR didapati memberikan perlindungan sel yang signifikan pada fibroblast hidung terhadap kesitotoksikan Centella asiatica. MT3D mempunyai kepekatan protein yang lebih tinggi berbanding MT2D. SDS-PAGE menunjukkan MT3D mempunyai 3 jalur ekslusif manakala MT2D mempunyai

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4 jalur eksklusif. Kajian masa depan harus dijalankan keatas penggunaan BR fibroblast hidung untuk perlindungan sel terhadap agen-agen yang memudaratkan di alam sekitar dan produk herba yang sitotoksik.

Kata kunci: epitelium salur pernafasan, Centella asiatica, perlindungan sel, microsfera, respiratori

ABSTRACT

Three dimensional (3D) models mimic the features of native tissue environment. Thus, morphology and signalling of cells from 3D culture are often more physiological than routine two dimensional (2D) cell culture. It is also known that the cell-secreted products have paracrine effect on other cells growth. In this experimental study, we optimised the nasal fibroblast culture on a 3D cell culture system and studied the effects of secretome from the 3D culture (3DCM) onto fibroblast growth and cytoprotection. Nasal fibroblast was isolated from human nasal turbinates. The suitable microcarrier was selected by culturing the fibroblasts in passage 3 on various types PolyGEM™ polystyrene microcarriers. Then, the cells were cultured on selected microcarrier using a 3D culture system and the conditioned medium (CM) was collected. 3DCM were supplemented to fibroblasts to study for attachment, proliferation, and cytoprotective effects against cytotoxicity of *Centella asiatica*. Bicinchonic Acid Assay (BCA) was performed to quantify protein amount in CMs. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for preliminary profiling and comparison of 2DCM and 3DCM protein profile. Our study showed that the 3DCM did not significantly enhance cell attachment and proliferation. The secretome of both 2DCM and 3DCM found to have significant cytoprotective effect onto nasal fibroblast against cytotoxicity of *C.asiatica* extract. 3DCM had higher protein concentration than 2DCM. SDS-PAGE showed three exclusive proteins in 3DCM and four exclusive proteins in 2DCM. Future study should be conducted on utility of nasal fibroblast secretome on cytoprotection against harmful agents in environment and cytotoxicity of natural products.

Keyword: airway epithelium, *Centella asiatica,* cytoprotection, microsphere, respiratory

INTRODUCTION

Secretomics can be described as the global study of secreted proteins of cell, tissue or organism at any particular time or condition. The constituents of secretome control and regulate various biological and physiological processes, thus making it valuable biomarker targets and also therapeutic candidates

(Hathout 2007). Cultured cells release a number of extracellular matrix proteins (ECM), cytokines, chemokines, angiogenic factors, and growth factors into the surrounding growth medium. These factors synergistically found to enhance attachment of the cell to the substratum and the cell growth. In addition, these secreted factors are also known to promote rapid migration and proliferation of epithelial cells to replace the injured cells (Walter et al. 2010). The fibroblast secreted ECM plays an important role in tissue remodelling during disease state or following tissue injury (Brandsma et al. 2013). Among the molecules identified as released into the medium by fibroblasts are plasminogen activator (Mira-y-Lopez et al. 1983), collagenase (Werb & Aggeler 1978) and fibronectin (Burridge et al. 1988). Chowdhury et al. (2012) reported that the supplementation of human skin fibroblasts' secretome enhanced in vitro expansion of autologous skin keratinocytes by increasing efficiency of cell attachment and providing better condition for proliferation of keratinocytes compared to control condition. It was suggested that the dermal fibroblasts supplemented with conditioned medium could induce production of extracellular matrix components that promote cell attachment. Previous study by Walker (2013) suggested that the use of mesenchymal stem cells (MSC) derived conditioned medium enhanced skin wound closure by affecting both dermal fibroblast and keratinocyte migration besides facilitating the formation of extracellular matrix. It was successfully proven that MSC conditioned medium (MSC-CM) obtained from serum free conditions considerably improve the wound closure rate of both skin cells separately and in co-culture. In the other study, conditioned medium derived from Activin-directed human embryonic stem cells (hESC) differentiated cell population showed a differential reparative effect on alveolar epithelial cells (AEC) wound repair model. This report suggested a paracrine mediated epithelial injury healing mechanism by hESC secreted products, which have potential to be developed in to acellular therapy. Akram et al. (2014) also demonstrated that human MSC-CM stimulate migration of AEC and small alveolar epithelial cells (SAEC) during wound repair in a serum-dependent and -independent manners, respectively. Besides, conditioned medium from chronic obstructive pulmonary disease (COPD) fibroblasts also found to promote epithelial-to-mesenchymal transition (EMT) in normal human bronchial epithelial cells (NHBE), which propose that interactions between fibroblast and epithelial cells are is essential in the EMT process in chronic obstructive pulmonary disease (COPD) (Nishioka et al. 2015). On other hand, the release of cytokine and other signalling molecule by airway epithelial cells during epithelial injury trigger proinflammatory responses in fibroblasts, where the activated fibroblast, in turn, produce ECM for tissue remodelling and also produce other signalling molecules to regulate immune response (Wynn & Barron 2010).

Cells are commonly cultured in vitro on two-dimensional (2D) surfaces due to familiarity, ease, convenience, and also the cost but 2D surfaces has its limitation such as cell flattening, which can affect nuclear shape and lead to differences in gene expression and protein synthesis. It is important for the in vitro culture microenvironment to mimic the native niche of the cell and three-dimensional (3D) models are better than 2D models in this sense. 3D models mimic the features of *in vivo* environment and cell morphology and signalling are often more physiological than routine 2D cell culture (Green & Yamada 2007). 3D models were better candidates for replication of physiological conditions of *in vivo* cellular responses to external stimuli compared to the 2D monolayer (Edmondson et al. 2014). Along this line, 3D culture surface can play a major role in enhancing the cell growth and function, thus increasing the quality of the produced secretome. Besides that, the dynamic mixing of the culture enables uniform temperature, pH, dissolved oxygen level and nutrients/metabolites concentration across the culture. Furthermore, the dynamic culture also increases the gas exchange rate and prevent the sedimentation microspheres (Meng et al. 2013). Secretome from 2D culture has some disadvantages such as low concentration of secreted proteins and contaminations with cellular debris due to cell lysis, while the concentration of protein obtained from 3D culture was considerably higher (>5-fold, $p<0.05$) compared to 2Dculture (Tit-Oon et al. 2014). The 3D surface can be categorized into to scaffold-free such as spheroid that consists of multi-cellular aggregates and scaffold-based culture systems, which are broadly divided into two forms - hydrogels and solid scaffold.

Secretome also was proven to have cytoprotective effects on cells, where the secretome or the paracrine mechanism of the mononuclear cells found to protect the cells from various injury caused by disease conditions (Hoetzenecker et al. 2012; Tögel et al. 2007). Furthermore, MSC-CM was found to protect the cells from cell death caused by serum deprivation or hypoxia in endothelial cells and cardiomyocytes in in vitro culture (Yamahara et al. 2014). In our study, we used *Centella asiatica* as cytotoxic agent. *C.asiatica* is well-known to have neuroprotective and neuroregenerative properties besides acting as antioxidant. Many researchers have embarked on study on indication of *C.asiatica* for, Parkinson's disease, Alzheimer's disease, neurotoxicity, learning and memory enhancement, epilepsy, and other mental illnesses such as depression and anxiety (Lokanathan et al. 2016). Asiatic acid (AA), which is one of the major components of *C.asiatica* has been shown to possess antioxidant and anti-inflammatory activities which prevents the infiltration of inflammatory cells in bronchoalveolar lavage fluid (BALF) of cigarette smoke exposure mice (Lee et al. 2016). However, the extract of *C.asiatica* has shown significant cytotoxicity on various human cells including airway epithelial cells above certain concentration (Mohd Heikal et

al. 2014; Sampson et al. 2001).

Therefore, in this study *C.asiatica* was used to create cytotoxicity model by supplementing *C.asiatica* to fibroblasts and testing the nasal fibroblast secretome on cells against *C.asiatica* cytotoxicity. Cytoprotection conferred by the conditioned medium may enable use of higher dose C.asiatica for therapeutic purposes.

This study was conducted to optimise the culture of nasal fibroblasts on a dynamic 3D culture system and then to compare the effect of secretome from cells cultured on 2D and 3D surface onto airway fibroblasts in term of cell attachment and proliferation, and the cytoprotection conferred by the secretome to cells against *C.asiatica* cytotoxicity.

MATERIALS & METHODS

This study was approved by Universiti Kebangsaan Malaysia Research Ethics Committee (UKM PPI/111/8/JEP-2016-233).

SAMPLE COLLECTION AND CELL ISOLATION

Redundant nasal turbinate was collected from consented patients undergoing turbinectomy and stored in normal saline normal saline at 4˚C before being processed within 24 hrs from surgery. The turbinate was cleaned by washing it several times with Dulbecco's Phosphate-Buffered Saline (DPBS; Sigma-Aldrich, USA). Then, the cartilaginous tissue part was removed and the remaining tissue was minced into small pieces before being digested using 0.6% collagenase type I (Worthington, USA) for 1-2 hrs at 37˚C. Digested tissue containing fibroblast and respiratory epithelial cells (RECs) was centrifuged and the pellet was resuspended in co-culture medium [(Airway Epithelial Cell Growth Medium (AECGM) (Promocell, Germany) and Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham; FD (Sigma-Aldrich, USA) with the 1:1 ratio)] supplemented with 5% Fetal Bovine Serum (FBS) (Biowest, France) and seeded into a six-well plate. The cells were kept at 37˚C in 5% CO_2 incubator with culture medium being replaced every 2–3 days. When the cells reached 80%-90% of confluency, fibroblasts were differentially trypsinized using 0.05% Trypsin-EDTA (Sigma, USA) and the cells were counted using heamocytometer. The fibroblasts were maintained in FD supplemented with 10% FBS.

CULTURE OF FIBROBLASTS ON MICROBEADS

To select the best type of PolyGEM™ polystyrene microcarriers (SoloHill Engineering, Inc., USA) for fibroblast culture, five types of microcarriers with varying coatings [plastic (no coating), Fact III (cationic type I porcine collagen), collagen (type I porcine collagen), Hillex® II (cationic trimethyl ammonium) and ProNectin® F (recombinant fibronectin)] were prepared by mixing 1g of PolyGEM™ with 5 ml of miliQ water (25 mg/ml). Then the stocks were autoclaved for 20 mins at 121˚C. After that, 2ml FD + 10%

FBS was used to wash the microbeads. Then, the solution was removed and replaced with fresh 2ml FD+ 10% FBS. Prior to adding the microbeads into 24-well plate, the plate was coated with 1% Poly (vinyl alcohol) (PVA) (Aldrich, USA) in DPBS at 37°C for 15 mins to prevent the attachment of fibroblast to the plate surface. Then, the plate was washed with 250 µL of DPBS twice and dried. Each well of 24-well plate were added with 200 µL of microcarrier stock and 2.0 x 10^5 of fibroblasts and the final volume was made into 1 ml. Culture medium was changed every 2-3 days. At seventh day, the microcarriers were stained with 4′, 6-diamidino-2-phenylindole (DAPI; Molecular Probes, USA) for viewing under the confocal microscope (Nikon A1R, Japan). Three images were taken for each type of microcarrier used and the number of cells attached on each microcarrier was counted to determine the best microcarrier for fibroblast attachment.

COLLECTION OF 3D CONDITIONED MEDIUM

Approximately, 70%-80% confluent fibroblast P_3 was trypsinized and was seeded at density of 4.0x10^6 per 2 mL of Hillex II® microcarrier stock solution in a Levitube™ (Hamilton, Switzerland) and the final volume was brought to 10 ml. The culture medium inside the Levitube™ was maintained as mentioned in Table 1. On day 4 onwards, 20 ml of culture medium was removed and was replaced with another 20 ml fresh FD+10% FBS on alternate days.

Table 1: Amount of FD added with days

Days	Amount of FD added (ml)	Total volume (ml)
0	10	10
2	10	20
3	20	40

The cells were cultured in BioLevitator™ 3D Cell Culture System (Hamilton, Switzerland) following the manufacturer recommended parameters of inoculation and culture. For 24 hrs inoculation period, the parameters were set as: rotation speed - 50 rpm, rotation period -1 second, agitation period - 4 mins, and agitation pause -30 mins. The Levitube™ in BioLevitator™ was maintained in above culture condition until the fibroblasts reached 100% confluency. To check for confluency of fibroblast on microbeads, 100 µL of microbeads were placed onto glass slide and viewed under inverted microscope. Once the cells reached 100% confluency, the medium was replaced with AEBM and continued the culture in BioLevitator™ for another 72 hrs. Then, conditioned medium of 3D culture was collected and stored at -80°C until used. Conditioned medium was collected from fibroblasts of two human nasal turbinates, which hereafter referred as 3D1 and 3D2. They were individually tested on allologous fibroblast cultures for their effect on cell attachment, proliferation and cytoprotection.

COLLECTION OF 2D CONDITIONED MEDIUM

Fibroblast at P_3 was cultured in 175 cm^2 culture flasks and once it reached 100% confluency, the culture medium was changed to Airway Epithelial Cell Basal Medium (AEBM) without growth factors. Then, the conditioned mediums were collected after further 72 hrs of culture and stored at -80˚C. Conditioned medium was collected from fibroblasts of 3 human nasal turbinates, which hereafter referred as 2D1, 2D2 and 2D3.

CELL COUNT USING HOECHST DYE STAINING

The fibroblasts used for attachment and proliferation assays were stained with Hoechst dye prior to seeding. The 0.1% Hoechst dye in FD pure was added into the 50 ml tube containing the cell pellet. The pellet was dissolved and incubated for 30 mins. Then the cells were pelleted again and was washed with 10mL of DPBS and finally resuspended in FD + 10% FBS before they were seeded.

ATTACHMENT AND PROLIFERATION ASSAY

Fibroblast at P_1 was seeded into two 24 well plates with density of 5,000 cells/cm^2. The plates were divided into two sets whereby the CMs were supplemented during cell seeding for the first set, while for the second set the CMs were added once the cells reached 40% confluency. All experiments were carried out in triplicate. Each well of the 24-well plate was added with 150 µL of CM and 350 µL of FD + 10% FBS. Cells cultured in FD + 10% FBS served as the controls. The cells were observed under fluorescent microscope after 24 hrs of CM supplementation to estimate the number of attached cells by Hoechst dye stained nuclei counting. The medium was changed on every alternate day up to 9 days and the cells were observed under fluorescent microscope for 9 days to estimate the proliferation rate. Images were taken at three random points for quantitative analysis and the proliferation rate was calculated (n=2).

CYTOTOXIC DOSE DETERMINATION

Fibroblast at P_1 was seeded into 24 well plates with cell density of 5,000 cells/cm^2. Raw extract of *C.asiatica* (L.) Urban (RECA) with concentrations of 0, 200, 400, 600, 800, 1000 µg/ml were added to the cells once the cells reached 40% confluency. The cells were observed and cell number was calculated as mentioned earlier. *C.asiatica* concentration that inhibit proliferation of more than 50% of cells (inhibitory concentration 50%, IC_{50}) was calculated (n=2).

CYTOPROTECTION

Fibroblast at P_1 (n=2) was seeded into 48 well plates with density of 5,000 cells/cm^2. The plates were divided into 2 sets whereby for the first set, 30% of CM and 70% of fresh medium containing 800 µg/ml of *C.asiatica*

were added during cell seeding. For the second set, 30% of CM and 70% of fresh containing 800 µg/ml of *C.asiatica* were added once fibroblast reached 40% confluency. All experiments were carried out in triplicate. Approximately, 24 hrs after the *C.asiatica* was added to the cells, PrestoBlue® Cell Viability Assay was performed to investigate cell viability and proliferation. The cells were washed with DPBS before added with 20 µL of PrestoBlue® reagent (Molecular Probes, USA) and 180 µL FD per well. Three wells without cells in each plate served as a blank. The plates were incubated for 2 hrs in dark to increase the sensitivity of detection. Then, 100 µL of medium from each well was transferred into 96 well plate and the absorbance was read at 570 nm and 600nm using spectrophotometer (BioTekPowerWave XS, Biotek, USA).

PROTEIN PRECIPITATION AND QUANTIFICATION

One volume of 100% (w/v) Tricholoroacetic acid (TCA) stock was added to 4 volumes of protein sample and it was incubated for 1 hr at -20˚C. The tube was spun at 14,000 rpm for 15 mins and the supernatant was removed. The whitish and fluffy precipitate was washed with 500 µL cold acetone and was spun down at 14,000 rpm for 10 mins. The acetone wash was repeated once before the pellet was dried at room temperature to dry off the acetone. Then, 100 µL of ultrapure water was added in each protein sample to dissolve the pellet.

Bicinchoninic acid assay (BCA assay; Sigma-Aldrich, USA) was conducted according to manufacturer's protocol to quantify the amount of protein in 2D- and 3D-culture conditioned medium. Protein standard was prepared by serial dilution of bovine serum albumin (BSA; Sigma Aldrich, USA). Approximately, 25 µl sample and 200 µl of BCA working reagent was added into 96 well plate and incubated at 37˚C for 30 mins. Then, the absorbance was read at 562nm. The standard curve was plotted using BSA protein standards absorbance and the amounts of proteins in the condition medium were calculated.

SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE according Laemmli (1970) protocol was done with 8% separating gel and 4% stacking gel. Protein sample were prepared by mixing protein loading buffer (Transgen Biotech, China) and CMs at 1:5 ratios and the wells were loaded with 6.25 µL of protein sample. The electrophoresis was run at voltage of 80V for 20 mins. Then the voltage was changed to 180V and electrophoresis was continued for another 45 mins. Then, silver staining was performed on the gel according to Heukeshoven and Dernick (1988). Several optimisations were done including usage miliQ water instead of distilled water, sensitizing duration was increased from 30 mins to 40 mins, washing time in between of silver reaction and developing was changed to twice for one min each. Besides, developing duration was increased from 4 mins to 12 mins and there was

no preserving step was done.

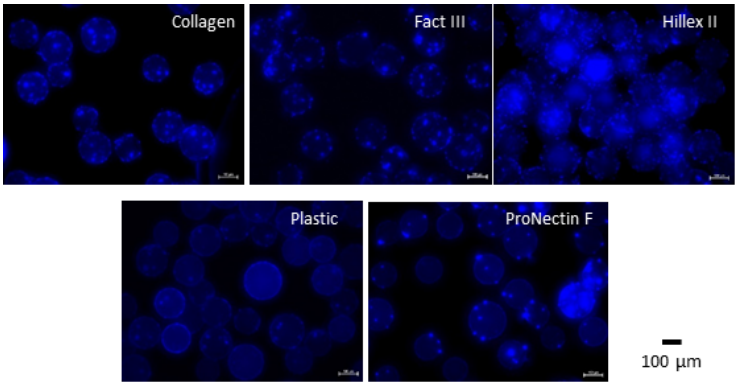
RESULTS

CULTURE OF FIBROBLASTS ON MICROBEADS

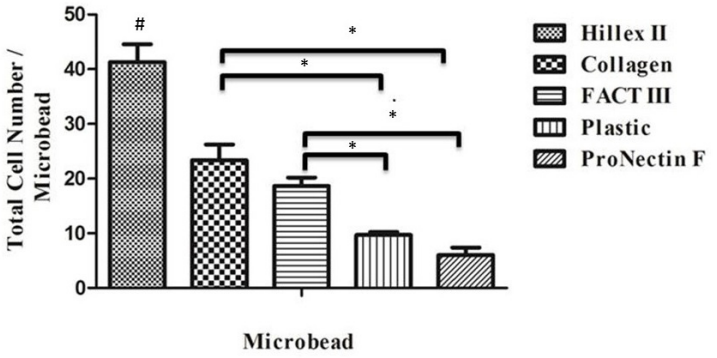
Culture of nasal fibroblast on various types of PolyGEM™ microcarriers showed that Hillex™ II had significantly higher number of cells (41 cells per bead) attached onto it after 7 days of culture, although all types of PolyGEM™ microcarriers showed presence of attached cells (Figure 1). So, Hillex™ microcarrier II was chosen for culture of fibroblast thereafter.

ATTACHMENT AND PROLIFERATION ASSAY

For total cell attachment, 3DCM supplemented culture (3DCMSC) showed the higher number of attached cells compared to 2DCM supplemented

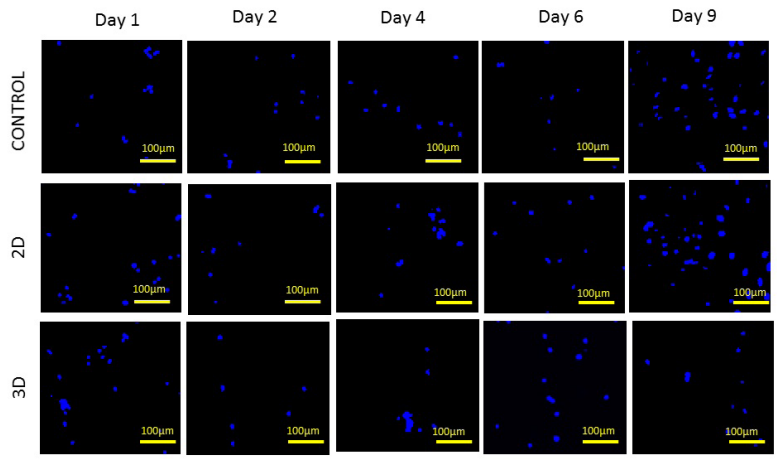


(a)



(b)

Figure 1: Nasal fibroblast on various PolyGEM™ microcarriers. a) The cell distribution on various PolyGEM™ microcarriers with their nucleus stained with DAPI; b) Selection of microbeads for maximum fibroblast attachment. Hillex II showed the highest cell number per beads, followed by Collagen, FACT III, Plastic and lastly ProNectin F. # indicates significant difference between Hillex II and all other tested group ($p<0.05$), * indicates significant difference ($p<0.05$).



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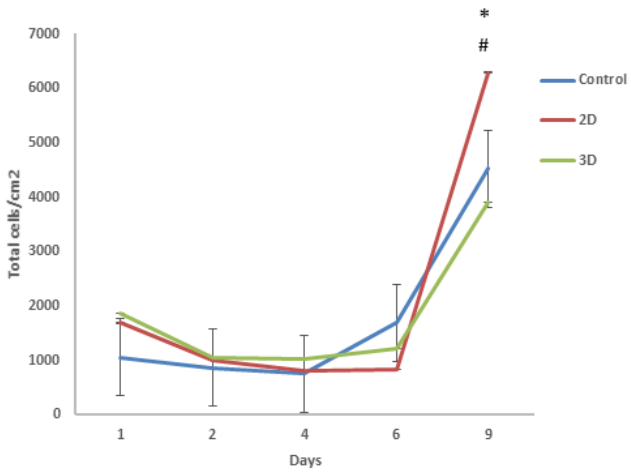

(b)

Figure 2: a) Hoechst stained cells on conditioned medium supplemented culture and control culture; b) Total amount of cells over 9 days of culture period. * indicates significant difference between 3DCMSC and Control group ($p<0.05$). # indicates significant difference between 3DCMSC and 2DCMSC ($p<0.05$).

culture (2DCMSC) but the differences were not statically significant (Figure 2). Attached fibroblast was 1854, 1684 and 1052 cells/cm^2 in 3DCMSC, 2DCMSC and control, respectively, at day 1 (Figure 2). The cell number for all three groups decreased from day 1 until day 4 while increased from day 4 till day 9. At day 9, 2DCMSC had significantly higher number of cells (6269 cells per cm^2) compared to both control and 3DCMSC. 3DCMSC showed the highest attached cell number at day 1 (Figure 2). The proliferation was determined based on the cell proliferation from day 6 to day 9. The 2DCMSC had the highest proliferation rate of fibroblast (0.021121 h^{-1}), followed by 3DCMSC and control, respectively 0.012165 h^{-1} and 0.010275

h^{-1}. However, the differences between all three groups were not statistically significant (Figure 3).

CYTOTOXICITY MODEL

C.asiatica supplementation once fibroblast reached 40% confluency showed that the total cell number of control group and 200 µg/mL continued to increase until day 6 while that of 1000 µg/mL decreased from day 2 to day 6. The other groups (400-800 µg/mL) showed a decrease from day 2 to day 4 and then an increasing trend from day 4 to day 6 although the increase was lower than that of control group (Figure 4). The total cell number in control group was significantly higher than all *C.asiatica* supplemented groups on day 6. The IC_{50} of *C.asiatica* on fibroblast, calculated from the total cell number on day 2 of *C.asiatica* supplementation, was determined as 820 g/mL (Figure 5).

CYTOPROTECTION

Supplementation of 800 µg/ml *C.asiatica* and 30% CM (v/v) since cell seeding onwards showed that 2DCM and 3DCM have cytoprotective effect as the number of attached cells in 2DCMSC and 3DCMSC group was significantly higher than that of control group. However, there was no significant difference between 2DCM and 3DCM cytoprotection effect during the cell attachment phase (Figure 6). Supplementation of 800 µg/ml *C.asiatica* and 30% CM (v/v) once the cells reached 40% confluency showed that 2DCM has slightly higher

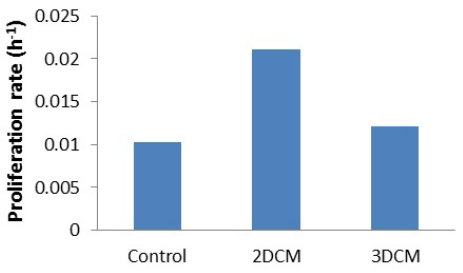

Figure 3: Proliferation rate of fibroblast from day-2 to day-9.

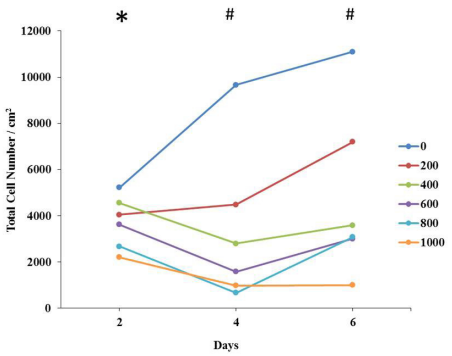

Figure 4: Cytotoxicity of C.asiatica supplemented once fibroblast reach 40% confluency. 5000 cells/cm2 was seeded at Day 0. # indicates significant difference between 0 g/mL and all tested group ($p<0.05$). * indicates 0 g/mL had significant difference from 800 g/mL and 1000 g/mL ($p<0.05$).

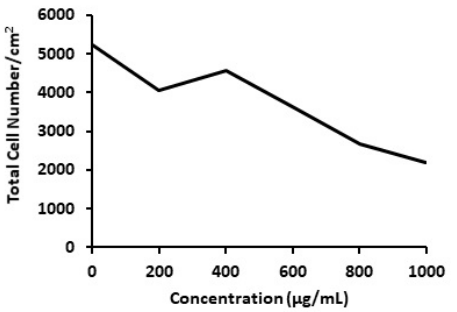

Figure 5: Inhibitory concentration 50% of C.asiatica supplemented once fibroblast reached 40% confluency.

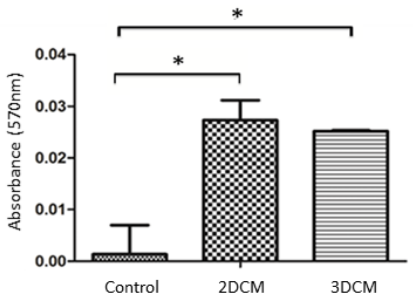

Figure 6: Fibroblast viability at 800 µg/ml of C.asiatica supplemented since cell seeding. * indicates significant difference ($p<0.05$)

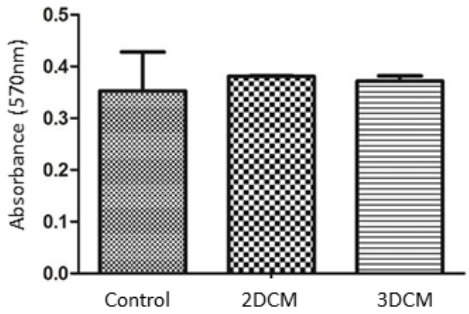

Figure 7: Fibroblast viability at 800 µg/ml of C.asiatica supplemented once cells were at 40% confluency

cytoprotective effect than control and 3DCM at however there was no statistically significant difference (Figure 7).

PROTEIN QUANTIFICATION AND SDS-PAGE ANALYSIS

Protein quantification by BCA Assay showed that protein concentration in 3DCM (225.7 µg/mL) was significantly higher than 2DCM (50.4 µg/mL) (Figure 8). The SDS-PAGE analysis showed presence of 4 exclusive bands in 2DCM and 3 exclusive bands in 3DCM (Figure 9). The exclusive proteins were seen approximately at 20,30,35 and 40kDa in 2DCM while approximately at 10,

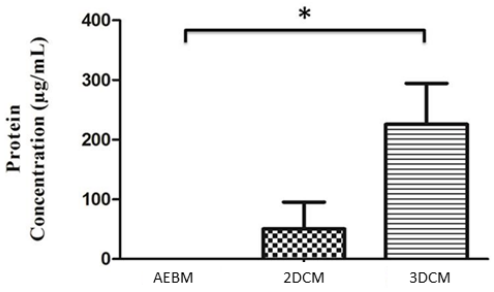

Figure 8: 2D and 3D cell culture conditioned medium protein quantification. * indicates significant differences ($p<0.05$).

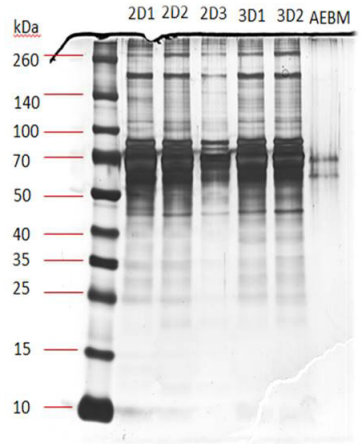

Figure 9: SDS-PAGE analysis for 2DCM and 3DCM

20 and 35kDa for 3DCM. Besides that, sample to sample variation was observed among the samples of 2DCM and 3DCM especially the 2DCM3 was found to have lower concentration of proteins and some protein bands was not clearly distinguishable compared to 2DCM1 and 2DCM2.

DISCUSSION

The identification of optimal coating of PolyGEM™ microcarriers showed Hillex® II had the highest nasal fibroblasts growth. Hillex® II is microcarrier with modified polystyrene

core and coated with tri-methylamine (TMA). The other microcarriers tested in this study were of crosslinked polystyrene core instead of modified polystyrene core and coated with different proteins or not coated instead of coated with TMA. Previous study has shown that Hillex® II can be used for efficient propagation of human skin fibroblast (Phillips et al. 2008). Microcarriers together with agitated suspension culture systems have been used for culture of anchorage dependent cells for production of virus (as vaccines), growth factors and antibodies (Merten 2015). The same basis was used in this study to obtain higher quality and quantity of nasal fibroblast secretome compared to the conventional monolayer cultured cell secretome. The dynamic culture parameters of Biolevitator™ was optimised to ensure the maximum number of nasal fibroblast attached to the microcarriers before they start proliferating. Since the microbeads have 3D surfaces the confocal microscope was used to capture z-stack images of microbeads and the maximum intensity projection image was reconstructed before DAPI-stained cells counting.

It was found that 2DCM and 3DCM obtained in this study did not have any significant effect on cell attachment compared to the control group. On other hand, 2DCM significantly increased proliferation of nasal fibroblasts in 2DCMSC compared to control group and 3DCMSC. Although it was expected that the CM generally will increase the cell attachment and 3DCM will be better than 2DCM in promoting cell growth as reported in previous studies (Chowdhury et al. 2012; Frith et al. 2009), our result did not indicate this. This may be being caused by high sample to sample variation during the secretome collection, and also the sample to sample variation, variation in cell seeding and variation in growth kinetics during the attachment and proliferation assay, which caused higher variation in the outcome, thus making the differences not significant. Other probable cause could be the basal medium that was used for secretome collection, AEBM, which is an airway epithelial cells culture medium without any growth factors, is not suitable for fibroblast growth. Besides the, the secretome collection time and CM supplementation percentage need to be optimised as the higher amount waste products in the secretome might mask the positive effects of the secretome. The prolonged lag phase, up to at least 4 days in all the groups as seen in figure 2B, is an observation that could not be explained. Even the fibroblasts that was not supplemented with conditioned medium was found to have the prolonged lag phase in this study.

The effect of the waste products also might be the cause of 2DCMSC having higher proliferation rate and 3DCM having lower proliferation rate compared to that of control group. As 3DCM had higher protein concentration as compared to 2DCM, there was also high concentration of waste product such as ammonia and lactate in 3DCM that may have a cytotoxic and inhibitory effects towards

the attachment and proliferation of fibroblasts (Schneider et al. 1996). The 3D condition medium contained significantly higher protein compared to 2D and control (AEBM) because the seeding density in 3D surface (4×10^6 per Levitube) was higher compared to 2D surface (3.75×10^5 in 75mL flask). Thus when the supplementation is done based on volume, the high concentration of waste products in 3DCM translates into higher final concentration of waste product in 3DCM supplemented culture compared to 2DCM supplemented culture.

In this study, a cytotoxic model of fibroblast was created using *C.asiatica* extract. Concentration of *C.asiatica* ranging from 200-1000 µg/mL was used to determine the inhibitory dose on fibroblast proliferation. Supplementation of *C.asiatica* to fibroblast at 40% confluency showed inhibitory effects as the fibroblasts proliferation rate decreased in all the *C.asiatica* supplemented groups except for 400 µg/mL group. The inhibitory effects was due to the cytotoxicity of *C.asiatica* that caused the cells to detach and lyse. The survived cells continued to proliferate thus showing an increase in cell number after day 4 till day 6 except in 1000 µg/mL *C.asiatica* supplemented group. Our results also showed that the effect of *C.asiatica* against fibroblast proliferation was dosage dependent and this was in agreement with study by Mohd Heikal et al. (2014).

The result showed that CM provide cytoprotection against *C.asiatica* cytotoxicity during fibroblast attachment as shown by CM supplementation at cell seeding that significantly enhance fibroblast attachment. On other hand, supplementation of CM to 40% confluent monolayer of fibroblast containing 800 µg/mL *C.asiatica* showed no significant cytoprotective effect. Thus, it can be safely assumed that the CM collected in this study provide cytoprotection in our cytotoxicity model at attachment phase but not in proliferation phase.

The SDS-PAGE analysis showed presence of differentially expressed proteins in 2DCM and 3DCM. Previous studies have shown that 3D cultured cells produce more unique proteins than 2D cultured cells and also the common secreted proteins in 2DCM and 3DCM was found to be enriched in 3DCM (Mishra et al. 2012; Tit-Oon et al. 2014). Thus, an in-depth proteomic analysis of the secretome can reveal the identity and function of the unique proteins found in this study. The future study on comparison of 2DCM and 3DCM should normalise the cell number per culture area across the surfaces to enable quantification of secreted proteins relative to the cell number. This will enable determination of CM production efficiency and its usage in a normalised manner in the downstream comparisons. The CM also should be filtered or dialysed to remove the waste products such as lactate and ammonia that negatively affect the cell growth. Furthermore, as it is known that nasal fibroblast secretome enhance attachment, proliferation and wound healing properties of respiratory epithelial

cells (RECs), the way forward will be testing the 2DCM and 3DCM on RECs for a greater utility of the conditioned medium from nasal fibroblasts. As the conditioned medium also can alleviate the cytotoxicity of natural products when taken together, it will enable intake of higher dose of natural product for a more preferred effects of the natural product. However, a more systematic and extensive study using more standardized conditioned medium need to be carried out to ascertain the extent of cytoprotection provided by conditioned medium to human cells.

CONCLUSION

We have successfully optimised the culture of nasal fibroblast cells in Biolevitator™ using Hillex™ II microcarriers for an efficient production of conditioned medium from cells in 3D Culture. The cytoprotective effect against cytotoxicity of *C.asiatica* on proliferating nasal fibroblast cells by 3DCM was comparable to that of 2DCM. Future optimisation in aspect of cell number normalisation and conditioned media clean-up need to be done before further study on utility of nasal fibroblast secretome in cell growth and cytoprotection against harmful agents in environment and drugs, especially on respiratory airway cells, can be performed.

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