VOL. 11, NO. 16, AUGUST 2016 ISSN 1819-6608
ARPN Journal of Engineering and Applied Sciences


www.arpnjournals.com

THE EFFECTS OF CULTURE SUBSTRATES AND MEDIA TO THE BEHAVIOR OF MICROTISSUES

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ABSTRACT

Three dimensional cell cultures are receiving increasing scientific attention recently because of the biological similarity and relevancy to the in-vivo system. Further understanding on the responses of the microtissues to the change of culture environment is lacking. In this paper, microtissues of keratinocytes or keratinospheroids were cultured using the liquid crystal based 3D culture technique. The study was undertaken to investigate the behavior of microtissues on both the liquid crystal surface and culture dish. Trypan blue exclusion assay and live/dead cell assay kit were applied to study the viability of the cells. The production of the microtissues on RPMI-1640 and DMEM culture media were also studied. The microtissues transferred to a tissue culture treated dish were found spreading into monolayer while the microtissues on the liquid crystal substrate merged into larger piece of microtissues. The trypan blue exclusion assay showed > 80 % of cell viability which agreed with the microtissue transfer results and cell stainings with Calcein Am and Ethidium Homodimer-1. The 3D cell culture in DMEM media having higher concentration of nutrients yielded higher ($p < 0.05$, t-test) number of microtissues compared with RPMI-1640 culture from Day 2 of culture. Nonetheless, microtissues migrated synchronously and merged into larger mass on the liquid crystal substrate. The physical properties of the microtissues produced are highly influenced by the culture environment.

Keywords: 3D cell culture, liquid crystal, microtissue, culture media, DMEM, RPMI-1640, keratinocytes.

INTRODUCTION

Culturing cells into three dimensions (3D) is under intensive research due to the demand from the medical sciences industry. Regenerated tissues can be applied as transplantable tissues, toxicity assessment assay and primary drug screening assay (Hsieh *et al.,* 2011, Ferro *et al*., 2014, Lee *et al.,* 2009). Various methods such as patterned hydrogel (Tibbitt and Anseth, 2009), droplet suspension technique (Amann *et al.,* 2014), micromolds (Napolitano *et al.,* 2007) and microencapsulation (Sugiura *et al.,* 2005) have been developed in order to reconstruct cells into microtissues. Most of these techniques introduced interfacial tension of curve containment which confines the cells in tight spaces leading to the aggregation of cells. Hence, our research group had developed an attractive technique to culture 3D microtissues of keratinocytes on the liquid crystal substrate. Cholesteryl ester liquid crystal (CELC) is non-toxic, stable at incubation temperature, provides suitable biochemical and physical properties for the adhesion of cells (Soon *et al.,* 2014). As demonstrated in (Hsieh *et al.,* 2011), the compliance of the planarly coated liquid crystal stimulates the cells to self-migrate and self-organise into microtissue constructs. The advantage of liquid crystal based 3D culture technique is that this technique allows the observation of the cells migratory activities and self organisation process into microtissues on the surface of the liquid crystal substrate (Soon *et al*., 2014). However, the fundamental physical behaviour of these microtissues or keratinospheroids towards different culture microenvironment requires further investigations. Previous literature reported that cells are sensitive to the stiffness of the surrounding microenvironment and they remodel according the stiffness of the extracellular matrix (ECM). Cells took on round morphology on the soft gel but broadly coupled to a stiff substrate (Engler *et al*., 2004). The question is raised if the reconstructed 3D microtissue would behave similarly or would retain the cohesiveness of the soft microtissue when cultured on a dish. Hence, the objective of this work is to study the physical responses of the cells transferred to culture dishes in comparison to those remaining on the liquid crystal surface. The viability of the cells in the microtissues would be determined using the trypan blue exclusion assay and live/dead cell staining kit. In addition, the effects of using different culture media to the growth of the keratinospheroids were also investigated.

MATERIALS AND METHODS

Preparation of cholesteryl ester liquid crystal substrate

CELC gels were prepared as reported previously (Soon *et al.,* 2014). Briefly, the LC gel was heated in a vial on a heating stage at a temperature of 100 °C until it is fully converted to clear liquid form. 40 µl of CELC were deposited to a petri dish and coated as a liquid crystal substrate using a squeegee coater developed in-house. The thickness of the coating was controlled at approximately 200 µm .

Culture of keratinospheroids

Human keratinocyte cell lines (HaCaTs) were purchased from Cell Line Services (CLS, Germany). When the cells in a 25 cm^2 culture flask reached 80%

confluency, the old culture media was discarded and the cells were washed three times with Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich, UK). There after the cells were wash in HBSS, 1 ml of crude 0.25% EDTA-trypsin (Biowest, L0931, France) was added into the culture flask and the cells were incubated at a temperature of 37 °C for 5 minutes. Subsequently, 5 ml of Dulbecco's modified eagle's medium (DMEM, Sigma-Aldrich, UK) was deposited into the culture to halt the trypsinization. The DMEM media used was supplemented with L-Glutamine (2 mM, Sigma-Aldrich, UK), Penicillin (100 units/ml, Sigma-Aldrich, UK), Streptomycin (100 mg/ml, Sigma-Aldrich, UK), Fungizone (2.5 mg/l, Sigma-Aldrich, UK) and 10 % fetal bovine serum (Biowest, France). The cells were then transferred to a 15 ml tube and centrifuged at 1500 rpm for 5 minutes. After centrifugation, the supernatants were discarded and the cell pellets were re-suspended in 6 ml of media. Cell suspensions at a density of 4.5 x 105 cells/ml were deposited to the petri dish containing the liquid crystal substrate with an addition of DMEM media. Then, the culture flask was maintained in a 5 % CO_2 incubator at 37 °C. The formation of keratinospheroids were monitored in a Nikon TS-100 phase contrast microscope and the micrographs of the keratinospheroids were recorded using a Qo-5 digital camera linked to the QCapture software. Similar experiments were repeated three times.

Cell viability study

For the trypan blue exclusion assay, keratinospheroids or microtissue of HaCaTs were harvested at day 5 of culture (dormant phase) and washed with HBSS three times. Meanwhile, 500 µl of 0.25% EDTA-trypsin was warmed on a peltier hot plate and the temperature was maintained at approximately 37°C. The keratinospheroids were transferred into warmed EDTA-trypsin and mechanically dissociated for 10 minutes. After 10 minutes of dissociation, 500 µl of DMEM was deposited to the cells to stop the trypsinization. Subsequently, 100 µl of 0.4% trypan blue (T8145, Sigma-Aldrich, USA) was added to the cells and the cells were incubated at room temperature for 5 minutes. Both the viable and dead cells were counted via Nikon Eclipse TS-100 inverted phase contrast microscope. Cell viability were calculated based on stained cells/(stained + unstained cells) x 100%. Additionally, a live/dead cell viability kit (Invitrogen, Life Technologies USA) was used to stain the live and dead cells contained in the microtissues. A mixed solution of Calcein-Am (0.5 µM) and Ethidium Homodimer-1 (0.5 µM) in HBSS was prepared and thoroughly mixed. The solution was added to a glass slide containing the microtissues for 30 minutes at room temperature (25 ºC). The fluorescence stainings were observed in an Olympus BX53 fluorescence microscope and the images were captured using DP73 digital camera. This experiment was repeated to triplicate of similar keratinospheroids.

The orthogonal diameter of the keratinospheroids was measured using the ImageJ software (National Institute of Health, US). Based on the diameter of keratinospheroid, the volume of the keratinospheroids was calculated by using the volumetric equation:

$$V=4/3\ \pi r^3 \quad (1)$$

where, $r = 1/2\ \sqrt{(d_1 \times d_2)}$, is the geometric mean radius with two orthogonal diameters (d_1 and d_2) of the keratinospheroids (Napolitano *et al*., 2007, Khaitan *et al*., 2006).

Viable microtissue transfer experiment

Upon reaching the dormant phase, the keratinospheroids were removed from the surface of the LC using a pair of tweezers in a phase contrast microscope and washed three times with HBSS. The cleaned keratinospheroids were transferred to a tissue culture (TC) treated polystyrene petri dish with a diameter of 58 cm (Greiner Bio-one, Belgium). Subsequently, 6 ml of DMEM were deposited to the petri dish and the cells were incubated in a 5% CO_2 incubator at 37 ºC. The keratinospheroids were monitored until the cell spread beyond the camera observation region.

3D cell culture in RPMI-1640 and DMEM

HaCaTs were maintained in RPMI-1640 (R5886, Sigma Aldrich, UK) and DMEM (D6171, Sigma Aldrich, UK). The cells were sub-cultured and harvested in cell suspensions. Both media were added with equal concentration of supplements and two petri dishes with LC coatings were prepared. The cells at a density of 4.5 x 10^5 cells/ml were grown on the liquid crystal substrates containing in 6 ml of RPMI-1640 and DMEM, respectively. The cells were incubated in a 5% CO_2 incubator at 37 ºC to form 3D cells. After 24 hours of culture, the keratinospheroids were observed in a phase contrast microscope and the micrographs were captured using a Qo-5 digital camera.

Statistical analysis

The quantity of the microtissues (mean ± SE) grown on the LC in DMEM and RPMI-1640 media were analyzed for significant differences ($N = 200$, $P < 0.05$) using independent t-test in a statistical package for social sciences (SPSS) software.

RESULTS AND DISCUSSION

On the surface of the LC substrate, the microtissues were observed with interesting migratory behaviour that allowed them to migrate and united with the adjacent colony of microtissue as indicated in Figure-1. The merged microtissues appeared as dark spheroids indicated the constituents of higher density of cells in comparison to individual microtissues of lower cell density. Based on the previous report (Soon *et al.,* 2014), individual cells migrate to form cell microspheroids. The current study revealed that the microspheroids could migrate synchronously and merged to other microspheroids to produce larger mass of

microtissues. The 3D cells culture using other techniques may not be able to provide these valuable observations (Tibbitt and Anseth, 2009, Amann *et al.*, 2014, Svoronos *et al.*, 2014).

Cholesteryl ester liquid crystal would be changed to the lyotropic phase when it is immersed in the culture media. The color of the polarizing images indicated the superimposed lyotropic birefringence surface (Figure-2). The optical line path and annulus ring around the keratinospheroids were found possibly due to the disturbance of the birefringence property of the LC as a result of cell migration and the attachment of microtissues on the substrate. Besides, the structure of lyotropic surface was analogous to the bilayer biological phospholipid membranes that comprise a pair of hydrophobic tail and hydrophilic head (Blinov *et al*., 1988). Thus, cholesteryl ester lyotropic liquid crystal substrate could provide keratinocyte cell a more mimic environment to proliferate into 3D microspheroid.

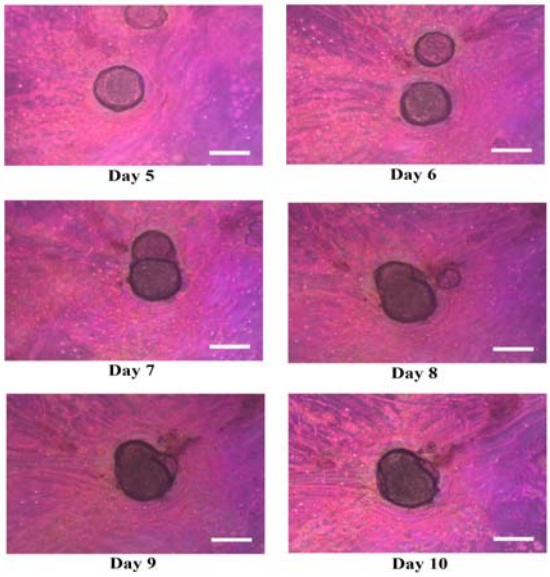


Figure-1. Photomicrographs of inverted phase contrast microscopy of the migration and integration of keratinospheroids (scale bar: 100 µm).

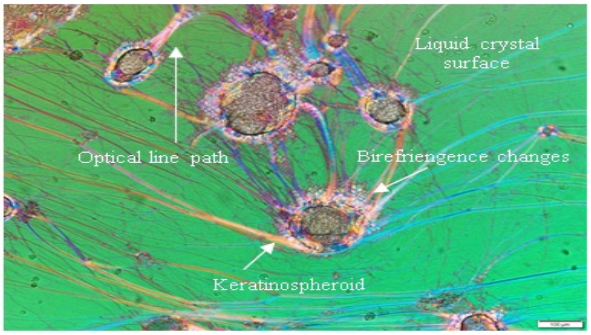


Figure-2. Crossed-polarizing images of liquid crystal substrate (scale bar: 100 µm).

The average cell viability from the microtissues of similar sizes (99 - 132 µm) cultured for 5 days on the LC substrate is approximately 80% (Table-1). The microtissues contained approximately 593 – 1283 of cells. The size of the microtissue is inversely proportional to the viability of cells. Larger microtissue yielded higher cell density but a lower percentage of cell viability. This can be explained by diffusion limit of thicker microtissues in restricting the mass transport, catabolic exchange, and nutrients in-reach leading to the starvation and death of cells at the central region of the microtissue. Microtissues of smaller volume could manage to reduce catabolism and hence, results in lower cell death. Keratinospheroids of approximately 100 µm are more efficient for mass transport and gas exchange (Ferro *et al.*, 2014). After prolonged culture, some of the cells experienced necrosis during the dormant phase leading to the shrinkage of microtissue (Soon *et al.*, 2014). A further experiment with live and dead cell staining kit indicated the locations of the non-viable cells were identified at the outer region of keratinospheroids (Figure-3). The majority of the cells survived for microtissues were approximately 200 µm diameter. Similar stainings were found in all the replicated experiments.

Table-1. Physical characteristics of the keratinospheroids and percentage of cell viability.

Keratinospheroid	Radius (µm)	Volume (µm³)	Viable cells	Dead cells	Cell viability (%)
1	99.49	$4.13x10^6$	495	98	83.47
2	110.14	$5.59x10^6$	667	140	82.65
3	132.47	$9.73x10^6$	1023	260	80.73

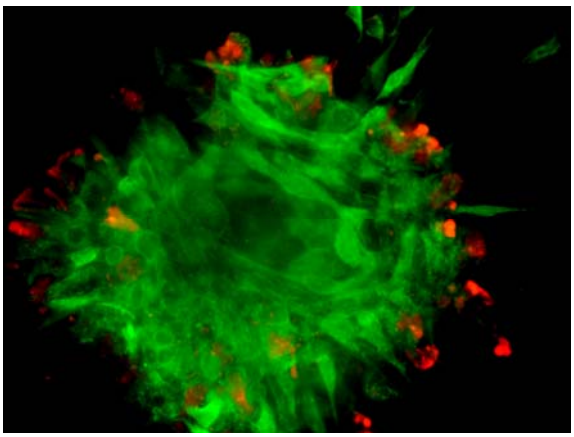


Figure-3. A photomicrograph of fluorescence stainings of live and dead cells in a microtissue. The cells labeled in green and red indicate the live and dead cells stained using Calcein-Am and Ethidium Homodimer-1, respectively. (Scale bar: 100 µm).

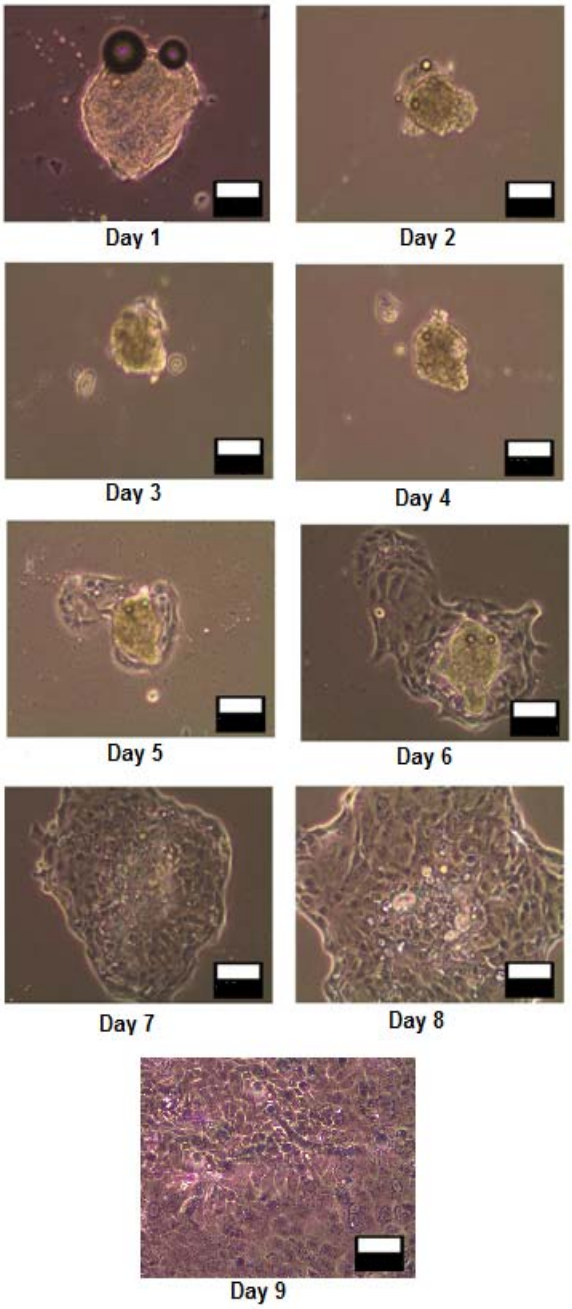
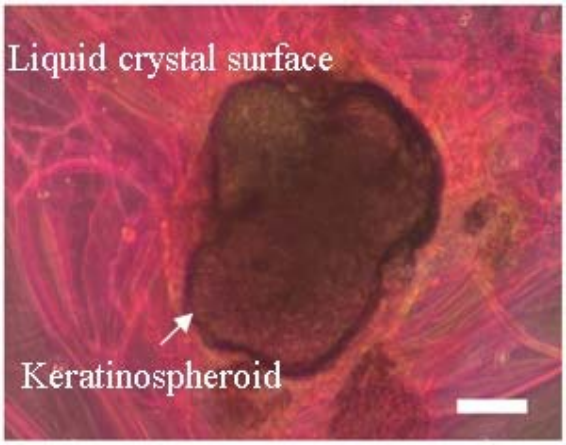


Figure-4. Photomicrographs of phase contrast microscopy of keratinospheroid transferred to the surface of a TC treated culture dish. (Scale bar: 100 μm).

This work raised the question if the viable microtissues stay integrated if they were to be transferred to a plain culture dish. Hence, we extracted and seeded some similar keratinospheroids in a Petri dish and found that the keratinospheroids did not migrate or merge to other colonies of microtissues but presented a change of physical behaviour in the tissue cultured (TC) treated dish. After a few minutes of transfer, the microtissues were found adhering to the surface of the culture dish (Figure-4). The adhesion was confirmed by gently shaking the Petri dish to see if the microtissue detached and float in the culture media. On Day 2, individual leading cells at the periphery of the microtissue migrated out of the microtissues (Figure-4). As more cells migrated out of the microtissues after 1 days of transfer, monolayer of cells formed around the microtissue. The 2D cells continued to proliferate with an enlarged cover area in the dish and the microtissues gradually disintegrated. The results show that cells preferably attached to a culture dish which may be due to the stiffness of the culture dish (Yeung *et al*., 2005) or the negative charged surface of the TC-treated culture dish, despite that the origin microtissue body is more compliant. Negatively charged polystyrene surface can attract the deposition of positively charged amino acids that created a surface in which the cells shown an affinity (Amstein and Hartman, 1975, Ryan, 2008). As a result, the cells of the microtissue spread and adapted to the treated culture dish.

(a)



(b)

Figure-5. 3D keratinospheroids cultured on the LC substrate in (a) RPMI-1640 and (b) DMEM media. (Scale bar: 100 μm).

In addition to the culture substrate which has an influence to the growth of microtissues, the type of the culture media also plays a role in regulating the growth of microtissues cultured on the LC substrate (Figure-5). There is no significant differences in the quantity of keratinospheroids formed during first day of culture ($p = 0.38$, $p > 0.05$, t-test) in both DMEM (12 ± 1) and RPMI-1640 (13 ± 1) media (Figure-6). The DMEM yielded a significantly higher quantity of keratinospheroids as compared to RPMI media from day 2 onwards ($p < 0.05$, t-test). Overall, the quantity keratinospheroids grown in DMEM was three fold higher than those grown in RPMI from day 3 to day 7 of culture. For DMEM, the number of keratinospheroids dramatically increased from 12 ± 1 to 138 ± 18 for day 1 and day 5, respectively, and subsequently decreased to 121 ± 20 on day 7. In contrast, the keratinospheroids culture in RPMI media increased moderately from 13 ± 1 on day 1 to 46 ± 7 on day 5, and a slight decline to 44 ± 7 on day 7 of culture. The decrease in the number of keratinospheroids for both cultures may be attributed to the merging of the keratinospheroids as indicated in the result of Figure-6.

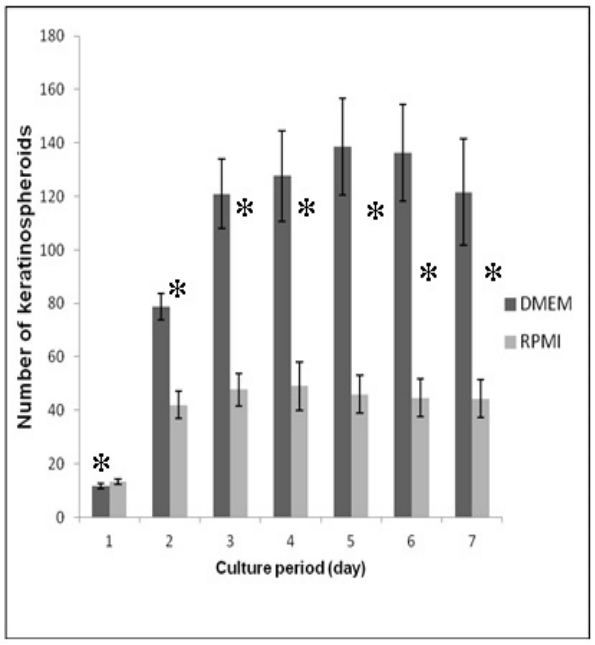


Figure-6. The effects of DMEM and RPMI media to the growth of keratinospheroids cultured on the LC substrate.

The asterisks indicate that the quantity of keratinospheroids cultured in the presence of DMEM and RPMI-1640 media is significantly different at $p = 0.009$, 0.008, 0.016, 0.01, 0.01 and 0.02 for Day 2 to Day 7 of culture, respectively ($p < 0.05$, t-test, $N = 200$).

Table-2. List of amino acids and vitamins in DMEM and RPMI-1640 (obtained from the Sigma Datasheet).

	DMEM	RPMI -1640
Component	g/L	g/L
Amino Acids		
L-Arginine.HCI	0.084	0.2
Glycine	0.03	0.01
L-Histidine.HCI.H_2O	0.042	0.015
L-Isoleucine	0.105	0.05
L-Leucine	0.105	0.05
L-Lysine.HCI	0.146	0.04
L-Methionine	0.03	0.015
L-Phenylalanine	0.066	0.015
L-Serine	0.042	0.03
L-Threonine	0.095	0.02
L-Tryptophan	0.016	0.005
L-Tyrosine.2Na.2H_2O	0.10379	0.02883
L-Valine	0.094	0.02
Vitamins		
Choline Chloride	0.004	0.003
Folic Acid	0.004	0.001
myo-Inositol	0.0072	0.035
Niacinamide	0.004	0.001
Pyridoxine.HCI	0.00404	0.001
Riboflavin	0.0004	0.0002
Thiamine.HCI	0.004	0.001
D-Pantothenic Acid.1/2Ca	0.004	0.00025
Other		
D-Glucose	4.5	2
Add		
L-Glutamine	0.584	0.3

The significant differences in the number of keratinospheroids generated in the DMEM and RPMI media were further analyzed by comparing the nutrients composition in both media. The DMEM contents were higher than the quantity of each type of amino acids and vitamins contained in RPMI media (Table 2). Cell culture media is a synthetic chemical medium to provide growth factors to support cell proliferation, survival and metabolic process for in-vitro culture (Vander Heiden *et al.,* 2001). The amino acid is categorized as essential and non-essential amino acid. A Cell cannot synthesize sufficient essential amino acid for cell metabolism by itself and require supplement from culture media whereas, the non-essential amino acid can be synthesized by itself through the chemical reaction between amino acid. The amino acid is used to control the protein synthesis (Yang *et al.,* 2010) and cell metabolism (Cellarier *et al.,* 2003) whereas vitamin is important for cells morphogenesis, lipid synthesis, cell growth and metabolism (Soltani *et al.*, 2012, Liu *et al.,* 2010, Zeisel and Blusztajn, 1994). The provided information may suggests that higher contents of amino acids, vitamins and glucose in DMEM had

promoted the growth, metabolism and formation of keratinospheroids grown on the LC substrates.

CONCLUSIONS

The transfer of microtissue to a new culture environment changed the physical behavior of the keratinospheroids. Microtissues of keratinospheroids turned into a monolayer of cells when transferred to a Petri dish despite that the percentage of cell viability was approximately 80%. In contrast, microtissues merged to form a larger colony of microtissues on the LC substrate. Nonetheless, the viability determined by trypan blue exclusion assay is consistent with the observation revealed in dead/live stainings. In comparison to RPMI-1640 media, higher content of amino acids and vitamins in the DMEM may enable cells to secrete more adherent protein and energy, that perhaps supported the 3D reconstruction of keratinocytes. Our results clearly revealed the change of culture environments strongly influence the physical characteristics of the microtissues.

ACKNOWLEDGEMENTS

The authors would like to thank the Malaysia Ministry of Higher Education for providing research funding support under the Fundamental Research Grant Scheme (FRGS Vot No. 1482) and Science Fund (Vot. No. S024 or Project No. 02-01-13-SF0104).

REFERENCES

[1] Amann, A., Zwierzina, M., Gamerith, G., Bitsche, M., Huber, J. M., Vogel, G. F., Blumer, M., Koeck, S., Pechriggl, E. J. & Kelm, J. M. (2014). Development of an Innovative 3D Cell Culture System to Study Tumour-Stroma Interactions in Non-Small Cell Lung Cancer Cells. PloS One, 9(3), p. e92511.

[2] Amstein, C. F. & Hartman, P. A. (1975). Adaptation of plastic surfaces for tissue culture by glow discharge. Journal of Clinical Microbiology, 2(1), pp. 46-54.

[3] Blinov, L. M., Davidyan, S. A., Petrov, A. G., Todorov, A. T. & Yablonskiĭ, S. V. (1988). Manifestation of ferroelectricity in a lyotropic liquid crystal with a chiral impurity: a structural analog of a biological membrane. JETP Lett, 48(5), pp. 259-262.

[4] Cellarier, E., Durando, X., Vasson, M., Farges, M., Demiden, A., Maurizis, J., Madelmont, J. & Chollet, P. (2003). Methionine dependency and cancer treatment. Cancer Treatment Reviews, 29(6), pp. 489-499.

[5] Engler, A., Bacakova, L., Newman, C., Hategan, A., Griffin, M. & Discher, D. (2004). Substrate compliance versus ligand density in cell on gel responses. Biophysical journal, 86(1), pp. 617-628.

[6] Ferro, F., Baheney, C. S. & Spelat, R. (2014). Three-dimensional (3D) cell culture conditions, present and future improvements. Razavi International Journal of Medicine 2, pp. e17803 (1-9).

[7] Hsieh, C.-H., Wang, J.-L. & Huang, Y.-Y. (2011). Large-scale cultivation of transplantable dermal papilla cellular aggregates using microfabricated PDMS arrays. Acta Biomaterialia, 7(1), pp. 315-324.

[8] Khaitan, D., Chandna, S., Arya, M. & Dwarakanath, B. (2006). Establishment and characterization of multicellular spheroids from a human glioma cell line; Implications for tumor therapy. Journal of Ttranslational Medicine, 4(1), pp. 12.

[9] Lee, J., Lillly, D., Doty, R. C., Podsiadlo, P. & Kotov, N. A. (2009). In vitro toxicity testing of nanoparticles in 3D cell culture. Small, 5(10), pp. 1213-1221.

[10] Liu, H., Huang, G.-w., Zhang, X.-m., Ren, D.-l. & Wilson, J. X. (2010). Folic Acid supplementation stimulates notch signaling and cell proliferation in embryonic neural stem cells. Journal of Clinical Biochemistry and Nutrition, 47(2), pp. 174-180.

[11] Napolitano, A. P., Dean, D. M., Man, A. J., Youssef, J., Ho, D. H., Rago, A. P., Lech, M. P. & Morgan, J. R. (2007). Scaffold-free three-dimensional cell culture utilizing micromolded nonadhesive hydrogels. BioTechniques, 43(4), pp. 494-500.

[12] Ryan, J. A. (2008). Evolution of cell culture surfaces. BioFiles, 3(8), pp. 21.

[13] Soltani, L., Kafilzadeh, F. & Karami Shabankareh, H. (2012). The effect of various concentrations of myo-inositol in culture medium on development of bovine embryos. Iranian Journal of Reproductive Medicine, 10(5), pp. 409-412.

[14] Soon, C. F., Goh, Z. P., Ku, L. C., Lee, T. T. & Tee, K. S. (2014). A Squeegee Coating Apparatus for Producing a Liquid Crystal Based Bio-Transducer. Applied Mechanics and Materials, 465, pp. 759-763.

[15] Soon, C. F., Omar, W. I. W., Berends, R. F., Nayan, N., Basri, H., Tee, K. S., Youseffi, M., Blagden, N. & Denyer, M. C. T. (2014). Biophysical characteristics of cells cultured on cholesteryl ester liquid crystals. Micron, 56, pp. 73-79.

[16] Sugiura, S., Oda, T., Izumida, Y., Aoyagi, Y., Satake, M., Ochiai, A., Ohkohchi, N. & Nakajima, M. (2005). Size control of calcium alginate beads containing living cells using micro-nozzle array. Biomaterials, 26(16), pp. 3327-3331.

[17] Svoronos, A. A., Tejavibulya, N., Schell, J. Y., Shenoy, V. B. a. & Morgan, J. R. (2014). Micro-mold design controls the 3D morphological evolution of self-assembling multi-cellular microtissues. Tissue Engineering, 20, pp. 1134-1144.

[18] Tibbitt, M. W. & Anseth, K. S. (2009). Hydrogels as extracellular matrix mimics for 3D cell culture. Biotechnology and Bioengineering, 103(4), pp. 655-663.

[19] Vander Heiden, M. G., Plas, D. R., Rathmell, J. C., Fox, C. J., Harris, M. H. & Thompson, C. B. (2001). Growth factors can influence cell growth and survival through effects on glucose metabolism. Molecular and Cellular Biology, 21(17), pp. 5899-5912.

[20] Yang, J., Chi, Y., Burkhardt, B. R., Guan, Y. & Wolf, B. A. (2010). Leucine metabolism in regulation of insulin secretion from pancreatic beta cells. Nutrition Reviews, 68(5), pp. 270-279.

[21] Yeung, T., Georges, P. C., Flanagan, L. A., Marg, B., Ortiz, M., Funaki, M., Zahir, N., Ming, W., Weaver, V. & Janmey, P. A. (2005). Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. Cell Motility and the Cytoskeleton, 60(1), pp. 24-34.

[22] Zeisel, S. H. & Blusztajn, J. K. (1994). Choline and human nutrition. Annual Review of Nutrition, 14(1), pp. 269-296.