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Research Article

EXPLANT CULTURE DERIVED MOUSE PRIMARY FIBROBLASTS TOLERATE CO_2 FLUCTUATIONS IN HIKARYOXL™ MEDIUM

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ABSTRACT
During cell culture practices, when a carbon dioxide incubator is shared among many researchers frequent opening of the door of the incubator is inevitable. This results in loss of CO_2 concentration. In addition, long term microscopic observation of the culture without on stage CO_2 supply also causes loss of CO_2 from the culture medium. The depletion in the CO_2 concentration affects pH of the culture medium. Recently, I have demonstrated that HikaryoXL™ gives significant protection to mouse dermal fibroblasts cultured by cell dissociation technique. In the present study, the primary culture of mouse dermal fibroblasts was carried out by explant culture technique. The explant culture was fed with three different cell culture media; viz. DMEM supplemented with 10% serum, RPMI supplemented with 10% serum and HikaryoXL™. After initiation of cell migration from the periphery of the explants, the cultures were subjected to hypocapnic stress of 3.5% CO_2 for 24 hours, followed by exposure to 2.5% CO_2 for next 24 hours. Thereafter, the CO_2 level was maintained to 5% as the recovery phase. After 24 hours, cells were observed under inverted phase contrast microscope. DMEM and RPMI 1640 fed hypocapnic stressed groups revealed cell debris, whereas, HikaryoXL™ fed group exhibited change in the cell morphology from typical fibroblastic nature to rounded form without appearance of cell debris, indicating that HikaryoXL™ gives cytoprotection during hypocapnic stress.

Keywords: Hypocapnia, Primary cell culture, Explant culture, fluctuations in CO_2 level in animal cell culture

INTRODUCTION
Maintenance of the pH of the culture medium is vital important for the metabolic activities of the cells (Ceccarini and Eagle 1971). *In vitro* condition depends upon the exogenous buffering system unlike the *in vivo* system. All buffers have an optimum pH range to resist changes in hydrogen ion concentration. This range is a factor of the dissociation constant (Ka) of a weak acid used in preparation of a buffer. pKa can be determined by Henderson-Hasselbalch equation, which is a logarithmic transformation of the expression of the dissociation constant (Ishizaki *et al.*, 1971). At 5% CO_2, the pH of the culture medium is maintained in a range of 7.2 to 7.4 by carbonic acid-bicarbonate ion buffering system. The amount of dissolved CO_2 in the culture medium depends upon the concentration of CO_2 in the carbon dioxide incubator. The depletion in the CO_2 concentration adversely affects the pH of the culture medium (Ham and McKeehan 1979)

Taking into account the CO_2 depletion during cell culture procedures such as frequent opening the door of the incubator, long term observation of cells under microscope without onstage CO_2 supply etc. there is a need to select an appropriate culture medium, which facilitates the cells to tolerate the CO_2 fluctuations during unavoidable circumstances. Of course the choice of the culture medium depends upon the cells to be cultured (Ham and McKeehan 1979); however, for cells like fibroblasts traditional cell culture media like DMEM, M199, RPMI 1640 also suffice. Recently, I have demonstrated that HikaryoXL™ confers significant cytoprotection during hypocapnic stress in the primary culture of mouse dermal fibroblasts cultured by cell dissociation technique (Deshmukh 2016).

In the present investigations the effect of hypocapnic stress was studied on the explant culture derived primary mouse dermal fibroblasts using different cell culture media such as DMEM, RPMI 1640 and HikaryoXL™.

MATERIALS AND METHODS

Sterile PBS, DMEM, RPMI 1640, HikaryoXL™, FBS, Gentamycin, T25 flasks, etc.

Collection of the dermal tissue: Dermal tissue of female albino mouse (*Mus musculus*) of age two months was used with ethical clearance of the Institutional Animal Ethics Committee. (1825/PO/EReBi/S/15/CPCSEA). All the procedures were carried out in the aseptic condition in laminar air flow. The dermis was minced in a sterile beaker containing chilled Phosphate Buffered Saline (PBS) supplemented with gentamycin (50µg/ml) and washed repeatedly until the PBS was clear.

Explant Culture: Minced pieces of dermis of the size1.00 mm^2 were placed in T25 flasks and allowed to adhere to the surface of the flasks for 30 min in laminar air flow. The explants were fed with three different culture media each supplemented with 10% FBS (i.e. DMEM, RPMI1640 and HikaryoXL™).

Study groups: Initially the explant culture flasks were divided into three groups of different culture media i.e. DMEM + 10% FBS fed culture, RPMI 1640 + 10% FBS fed culture and HikaryoXL™ fed culture. Once the migration of cells was initiated these cultures were divided into two groups as the control group and the hypocapnic stressed group.

Control group: In this group, the cells were cultured at 5%CO_2 and 37^oC in the humidified CO_2 incubator without any modification in the CO_2 level.

Hypocapnic stressed group: Once the migration of cells was initiated from the periphery of the explant, the hypocapnic stress was induced by setting the CO_2 level to 3.5% for first 24 hours, followed by 2.5% CO_2 for next 24 hours. After this, the CO_2 level was maintained at 5% as the recovery phase.

Microscopic studies: After recovery phase, the cells from both the groups were observed for cytological alterations.

Statistical analysis: The cytological alterations were quantified and expressed as the average $\pm$ standard deviation. The level of significance was computed by unpaired t test using MS-Excel.

RESULTS

The cells from the control group and experimental group were observed under inverted phase contrast microscope and live cell imaging was carried out. The images are displayed in the Plate I. Figure No.1, 2 and 5 (figures at the left) show control group explant culture maintained at 5%CO_2 and 37^oC in the humidified CO_2 incubator. Figure No. 2, 4 and 6 (figures at the right) show hypocapnic stressed explant culture. The cells fed with DMEM and RPMI 1640 could not tolerate the hypocapnia. It was evidenced by cell debris resulted due to death of the cells. In the HikaryoXL™ fed explant, the cells exhibited alteration in their morphology and appeared spherical in shape. The size of the cell was also reduced and cell debris was not seen in this group. The pH of the culture medium was 7.4 at 5% CO_2 level, at 3.5% CO_2, the pH was increased to 7.8 and at 2.5% CO_2 level, it was further increased to 8.2.

DISCUSSION

The cells from DMEM and RPMI 1640 fed group could not tolerate hypocapnic stress, whereas, the cells from the HikaryoXL™ fed hypocapnic stressed group although were surviving but showed spherical morphology. This might be due to conformational changes in the cell adhesion proteins due to change in the pH resulted during hypocapnic stress. Lengheden (1994) demonstrated that at pH levels above 7.8, both attachment and growth of human lung fibroblasts and peritoneal ligament fibroblasts decreased significantly. Lengheden and Jansson (1995) studied the effect of pH on experimental wound healing of human fibroblasts *in vitro* and found that the surviving cells in the wounds showed cytoplasmic vacuoles and blebbing when the pH was above 7.8. Mackenzie *et al.* (1960) demonstrated that cells exposed to a pH of 8.0 to 8.2 grew at the maximal rate for the first 12 to 24 hours, then ceased abruptly and the cells

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Plate I

Phase contrast images (200X) showing Cellular alterations after hypocapnic stress in explant culture derived mouse dermal fibroblast grown in different cell culture media

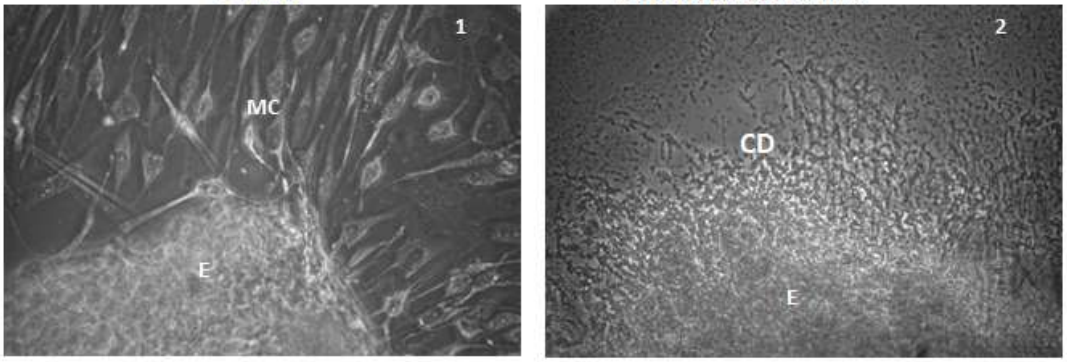

Figure 1 and 2 explants fed with Dulbecco's Modified Eagle Medium (DMEM)

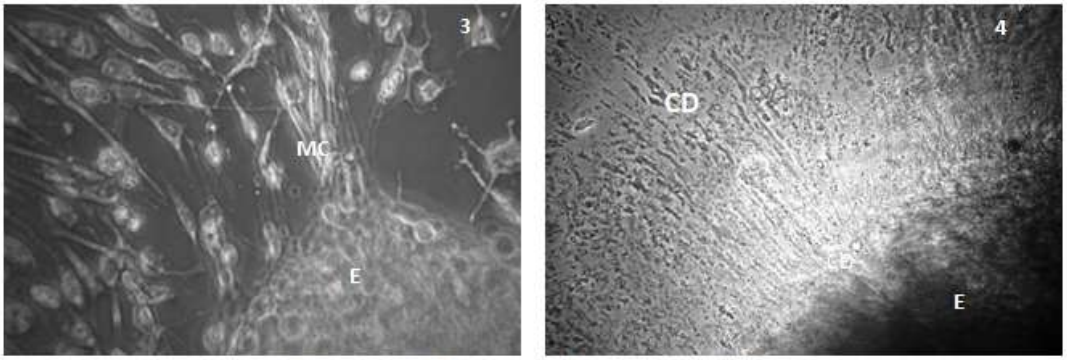

Figure 3 and 4 explants fed with RPMI1640

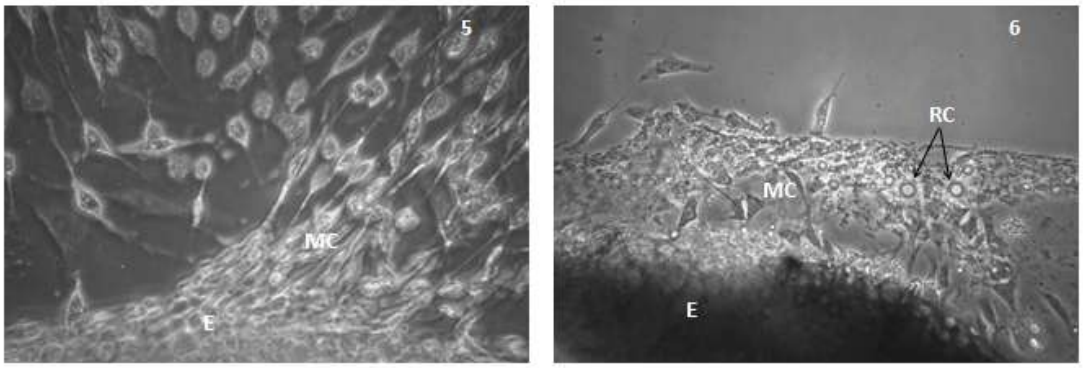

Figure 5 and 6 explant fed with HikaryoXL™

E: Explant CD: Cell debris MC: Migrating cells RC: Rounded cells

Table 1: Effect of hypocapnic stress on number of fibroblasts migrating from dermal explants fed with different cell culture media (Mean ± SD)

Group	DMEM	RPMI1640	HikaryoXL™
Control group	45.16 ± 3.06/mm^2	46.67 ± 4.46/mm^2	49.33 ± 4.59/mm^2
Hypocapnic stressed group	0 ***	0***	17 ± 0.89/mm^2***

**** indicates p<0.001 i.e. highly significant (When experimental group compared with control group and HikaryoXL™ compared with DMEM and RPMI 1640 fed group)*

entered a steady state which was followed by cytoplasmic retraction and cell death. In the present study, the cells from the DMEM and RPMI1640 fed group exhibited cell death after hypocapnic stress. On the contrary, the cells from HikaryoXL™ fed group demonstrated significant cytoprotection. HikaryoXL™ is the licensed product of Himedia promoted for the short term *in vitro* culture of peripheral blood lymphocytes for cytogenetic studies. It consists of basal medium RPMI 1640 supplemented with L-Glutamine, FBS, Phytohaemagglutinin (PHA-M), Penicillin, Streptomycin and Sodium bicarbonate. The major difference between the other two media and Hikaryo XL™ is the presence of a plant lectin Phytohaemagglutinin (PHA), it acts as a mitogen for lymphocytes and stimulates cell division. Sell and Costa (2003) studied the effect of Phytohaemagglutinin on human gingival fibroblasts proliferation and found that PHA at a concentration of 0.1 to 10 µg/ml does not influence cell proliferation. Moreover, when these fibroblasts were treated with PHA at a concentration of 20 µg/ml resulted in loss of fibroblastic morphology and appearance of rounded cells. In the present investigations, HikaryoXL™ fed dermal fibroblasts exhibited rounded morphology. At a concentration of 150 and 300 µg/ml there was 100% death of the fibroblasts, concluding that PHA does not act as mitogen for fibroblasts. Asaga and Yoshizato (1992) found that PHA recognizes the glycochain of collagen receptor at the cell surface. Kulkarni and McCulloch (1995) examined the role of cell surface carbohydrates in apoptosis. Binding of these cell surface receptors by PHA may inhibit the apoptotic cascade. Being the licensed and proprietary product of Himedia, the concentration of Phytohaemagglutinin is not disclosed by Himedia. However, the results from the control group indicate that PHA in the HikaryoXL™ neither promotes cell division nor induces cell death. Similar results were observed in enzymatically dissociated mouse primary dermal fibroblasts fed with HikaryoXL™ (Deshmukh 2016). Cunha *et al.* (2013) found that Phytohaemagglutinin improves the development of *in vitro* cultured goat preantral follicles. Supplementation of culture medium with 10µg/ml PHA maintains the follicular viability and ultrastructure and promotes formation of antral cavity after six days of culture *in vitro*.

Cells need the basic nutritional conditions to grow *in vitro*, that includes twelve essential amino acids (arginine, cysteine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, histidine, tyrosine and valine), sugar (glucose), vitamins, inorganic ions and trace elements (Eagle 1955). These nutritional requirements were fulfilled by the DMEM, RPMI1640 and HikaryoXL™. Although there are minor variations in the concentration of salts, amino acids and vitamins in these three culture media, these variations do not account for the results observed; since there was no any significant difference seen in the morphology of fibroblasts from the control group fed with these media. The unique

feature of HikaryoXL™ that makes it different from the other two culture media is Phytohaemagglutinin. Thus, Phytohaemagglutinin may confer protective role in combating the hypocapnic stress.

CONCLUSION

HikaryoXL™ confers protection to explant culture derived mouse primary fibroblasts to tolerate CO_2 fluctuations.

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