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Research Article

HIKARYO XL™ CONFERS PROTECTION DURING HYPOCAPNIC STRESS IN THE PRIMARY CULTURE OF MOUSE DERMAL FIBROBLASTS

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ABSTRACT
Maintaining the pH of the culture medium is of crucial importance for survival and growth of cells in culture. For mammalian cells, pH 7.4 is required; the pH of body fluids, extracellular fluid and the cytoplasm. This pH is maintained by the carbonic acid-bicarbonate ion buffering system in carbon dioxide incubator at 5% level of CO_2. However, there are certain unavoidable circumstances, like leakage of the regulator/valve of the CO_2 cylinder, long term observation of cells under microscope with lack of stage top CO_2 incubation system, etc., causing remarkable decrease in the concentration of carbonic acid from the culture medium causing increase in the pH (alkalosis). This stress is termed as hypocapnic stress. In the present investigations, mouse dermal fibroblasts were cultured in different cell culture media like DMEM, RPMI1640 and HikaryoXL™ supplemented with 10% fetal bovine serum. After attaining the Confluency, the fibroblasts were subjected to hypocapnic stress of 3.5% CO_2 for initial 24 hours, followed by 2.5% CO_2 for the next 24 hours. Thereafter, CO_2 concentration was maintained at 5%. After 24 hours, cellular morphology and apoptotic changes were studied. The results show that fibroblasts grown in MEM and RPMI1640 could not tolerate hypocapnic stress, whereas, Hikaryo XL™ gives significant protection in terms of decreased incidences of apoptotic alterations, healthier cell morphology and improved cell survival.

Key words: *Primary cell culture, hypocapnia, CO_2 tension and pH, apoptotic alterations*

INTRODUCTION
Cell culture refers to growing the cells in the laboratory in the artificial environment simulating the *in vivo* condition. It is one of the major tools used in cell and molecular biology, toxicology, developmental biology etc. It provides an excellent model system for studying the cytology, physiology and biochemistry of the cells e.g. metabolic studies, cellular alterations, cell senescence etc. (Swim *et al.* 1957, Ehrlich *et al.*1978 Deshmukh 2013). It is used in testing the mutagenicity and carcinogenicity of the drugs (Alley *et al*. 1988) and large scale manufacturing of compounds such as monoclonal antibodies, vaccines and therapeutic proteins (Selisteanu *et al*. 2015, Houghten *et al.*, 1991, Milian and Kamen 2015). The major advantage of cell culture for any of these applications is the consistency and reproducibility of the results.

CO_2 incubator is the most essential component of the cell culture laboratory. It helps maintain the pH of the culture medium which is critical for cell survival. The carbonic acid-bicarbonate ion buffering system (H_2CO_3 & HCO_3^-) is commonly used to maintain the pH of the culture medium in a physiological range of pH 7.2- 7.4. The carbonic acid (H_2CO_3) is formed by the dissolution of carbon dioxide (CO_2) in water (H_2O). However, the carbon dioxide generated during cellular respiration is not enough to produce desired quantity of H_2CO_3 required to maintain the equilibrium with HCO_3^-. Therefore, the concentration of carbonic acid is maintained by supplying the CO_2 at a concentration of 5%. Most of the cells in culture grow well at pH 7.4 and their growth is inhibited at pH 6.8 (Ham and McKeehan 1979).

Fluctuation in the CO_2 level is deleterious to the cells growing in culture. There are unavoidable circumstances during which the CO_2 level gets adversely affected, such as long term observation of culture under microscope lacking stage top CO_2 incubation system, leakage of CO_2 regulator/valve of

CO_2 cylinder, frequent power failure and lack of power backup (particularly in developing countries) etc. This leads to depletion in the CO_2 level. The decline in the CO_2 concentration below threshold or desired level is termed as hypocapnia (Laffey and Kavanagh 2002). Hypocapnic stress results in the fluctuations in the pH affecting the metabolism.

In the present investigations, effect of fluctuations in the CO_2 level were studied on the primary culture of mouse dermal fibroblasts grown in different cell culture media like DMEM, RPMI1640 and KikaryoXL™ to know if culture media provide any protection against damage caused due to depletion in CO_2 level.

MATERIALS AND METHODS

***Chemicals and Media*:** Phosphate Buffer Saline (PBS), Antibiotics – Penicillin 50U/ml and Streptomycin 50µg/ml, Trypsin (0.25%), Collagenase 50µg/ml, L-Glutamine 200mM, Fetal Bovine Serum, DMEM, RPMI1640, HikaryoXL™, T flasks (T25 area 25cm^2)

***Collection of dermal tissue*:** All procedures were carried out in aseptic condition in laminar air flow. Female albino mouse (*Mus musculus*) of age two months was used to collect the dermis with due permission of the Institutional Animal Ethics Committee. (1825/PO/EReBi/S/15/CPCSEA). The mouse was sacrificed by cervical dislocation. The abdominal skin was wiped with 70% alcohol and shaved with sterile surgical blade. The skin was cut open and dermis was excised without any interference of fur and transferred to a sterile beaker containing chilled and sterile Phosphate Buffer Saline (PBS) having gentamycin (50µg/mL). Dermis was finely minced into pieces and washed four times with PBS until the PBS was clear.

Enzymatic Disaggregation of cells: The minced pieces of dermis were transferred in 15ml sterile falcon tube and treated with 0.25% Trypsin and Collagenase 50 µg/ ml at 37°C for 10 min. The protease action was critically monitored by observing the fuzzy appearance of the minced pieces of the dermis. The suspension was aspirated using sterile Pasteur pipette. 100µl of serum and 50µl of 1mM EDTA were added to inhibit the residual trypsin and collagenase activity respectively. 2ml of PBS was added in it. The protease digested tissue was triturated using sterile fire polished glass pipette to dissociate the cells. The content was filtered through sterile cell strainer in laminar air flow. The filtrate was used for the cell viability assay.

Cell Viability By Trypan Blue Dye Exclusion Method: 100µl cell suspension was transferred to sterile Eppendorf tube and 100 µl 0.2% trypan blue was added in it. The sample was mixed and loaded on haemocytometer. The final cell concentration was set to 1×10^5 viable cells per ml.

Seeding of the cells: Cells were seeded in T-25 flasks and allowed to adhere for 30 min. The DMEM and RPMI1640 were supplemented with 10% FBS, 200mM L-glutamine 34µl/10ml and 40µg/ml gentamycin. HikaryoXL™ is a complete medium containing serum, L-glutamine and antibiotic, therefore nothing was added in it exogenously. After cell adhesion to T flasks, cells were fed with different culture media viz. DMEM, RPMI1640 and HikaryoXL™. The culture flasks were transferred to humidified CO_2 incubator set at 5%CO_2 and 37°C.

Study groups: There were six study groups, two study groups of each culture medium: one as the control group and the other as the hypocapnic stressed (experimental) group. Each group was run in triplicate and five trials were conducted.

Control group: In this group, the cells were cultured continuously at 5%CO_2 and 37°C in the humidified CO_2 incubator.

Hypocapnic stressed group (experimental): In this group, after attaining the confluency, the cells were exposed to hypocapnic stress of 3.5% CO_2 for initial 24 hours, followed by 2.5% CO_2 for next 24 hours. Thereafter, CO_2 concentration was maintained to 5% for 24 hours as a recovery phase.

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

Statistical analyses: The data for cellular morphology and alterations were quantified per hundred cells. The results are calculated as average and standard deviation. To evaluate the statistical significance unpaired t test was employed. All the statistical calculations were carried out using MS-Excel.

Plate I: Phase contrast microscopic images (200X) showing Cellular alterations after hypocapnic stress in Primary culture of mouse dermal fibroblast grown in different cell culture media

Control group **Hypocapnic stressed group**

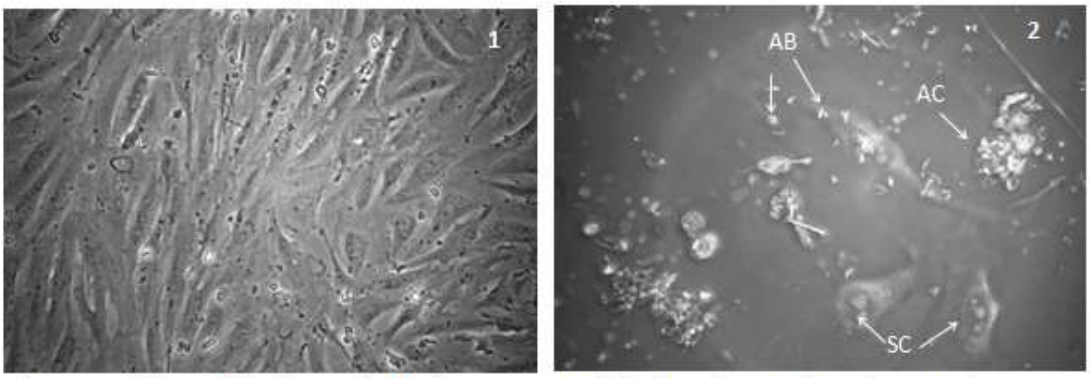


Figure 1 and 2 Cells grown in Dulbecco's Modified Eagle Medium (DMEM)

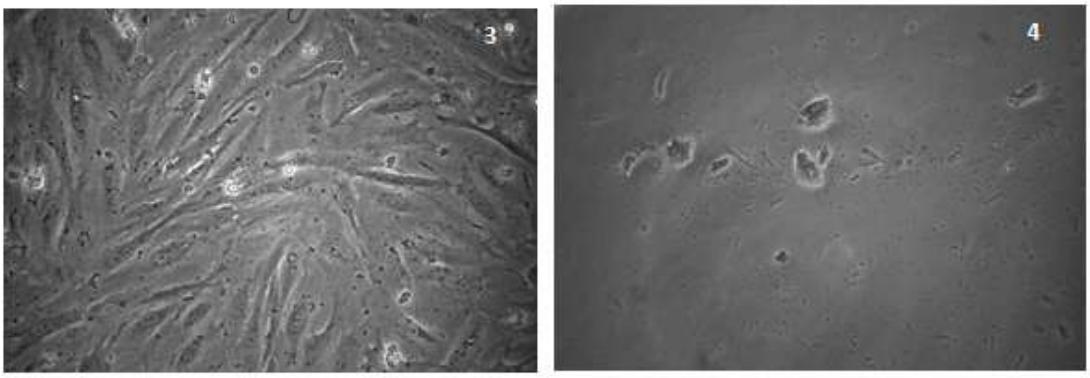


Figure 3 and 4 Cells grown in Roswell Park Memorial Institute -1640 Medium (RPMI 1640)

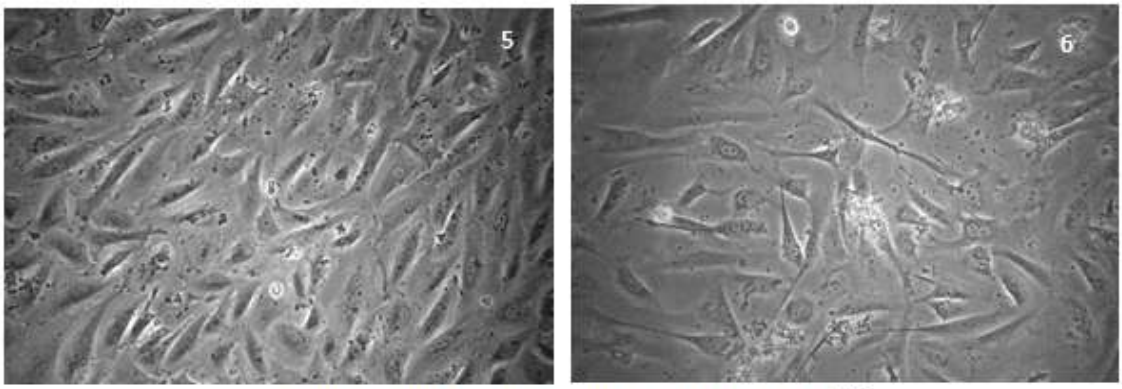
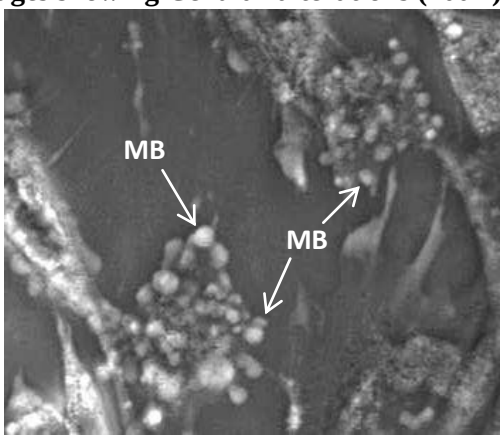


Figure 5 and 6 Cells grown in Hikaryo XL™

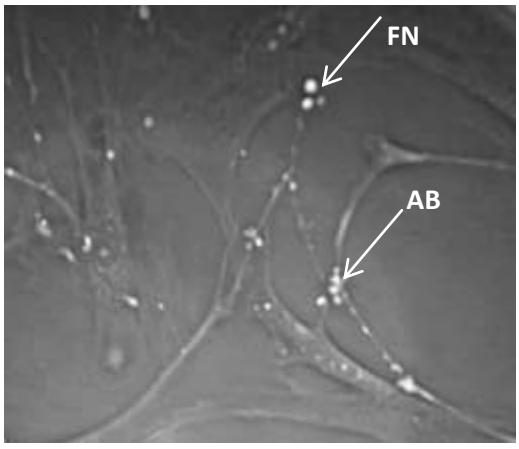
AB: Apoptotic body, AC: Apoptotic Cell, SC: Swollen cell

Plate II

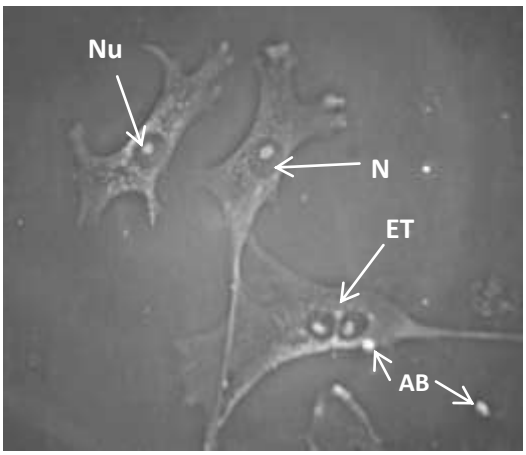
Phase contrast microscopic images showing Cellular alterations (400X) after Hypocapnic Stress



Cells grown in DMEM

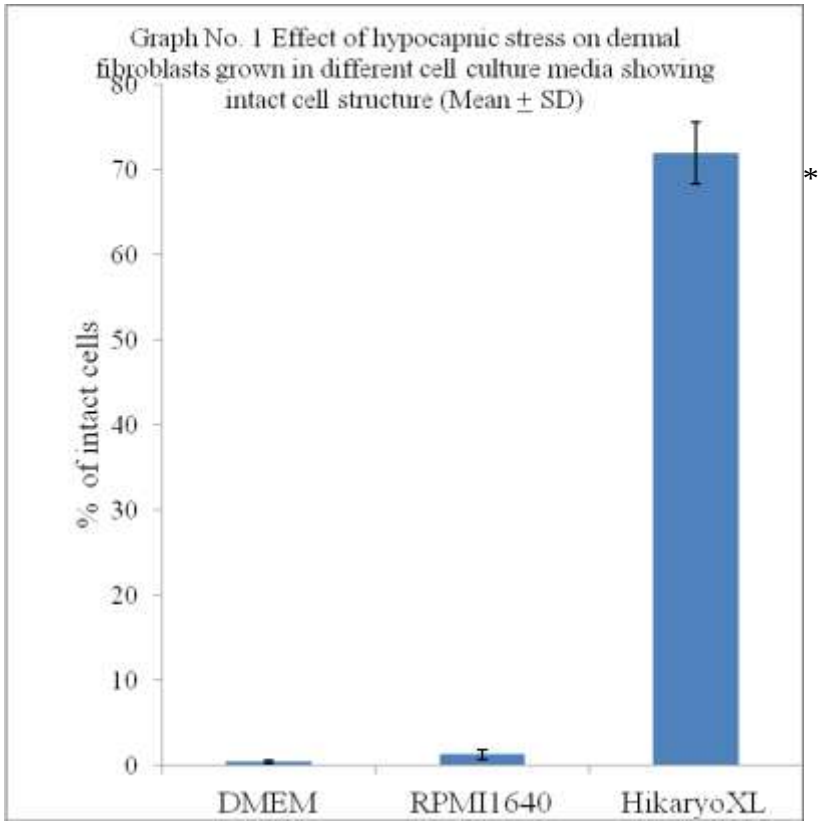


Cells grown in RPMI1640

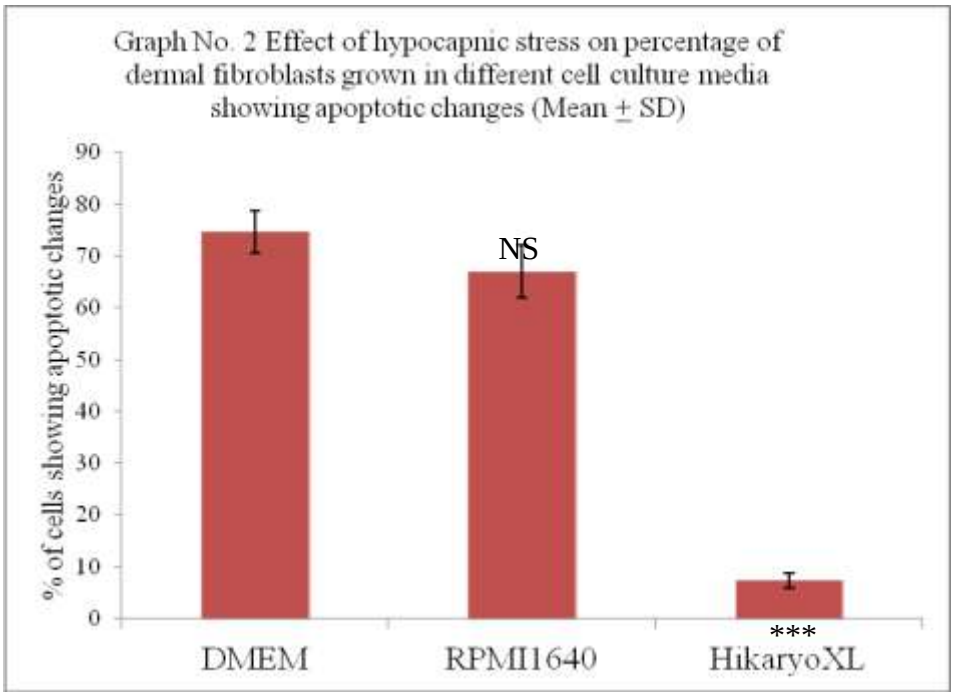


Cells grown in HikaryoXL™

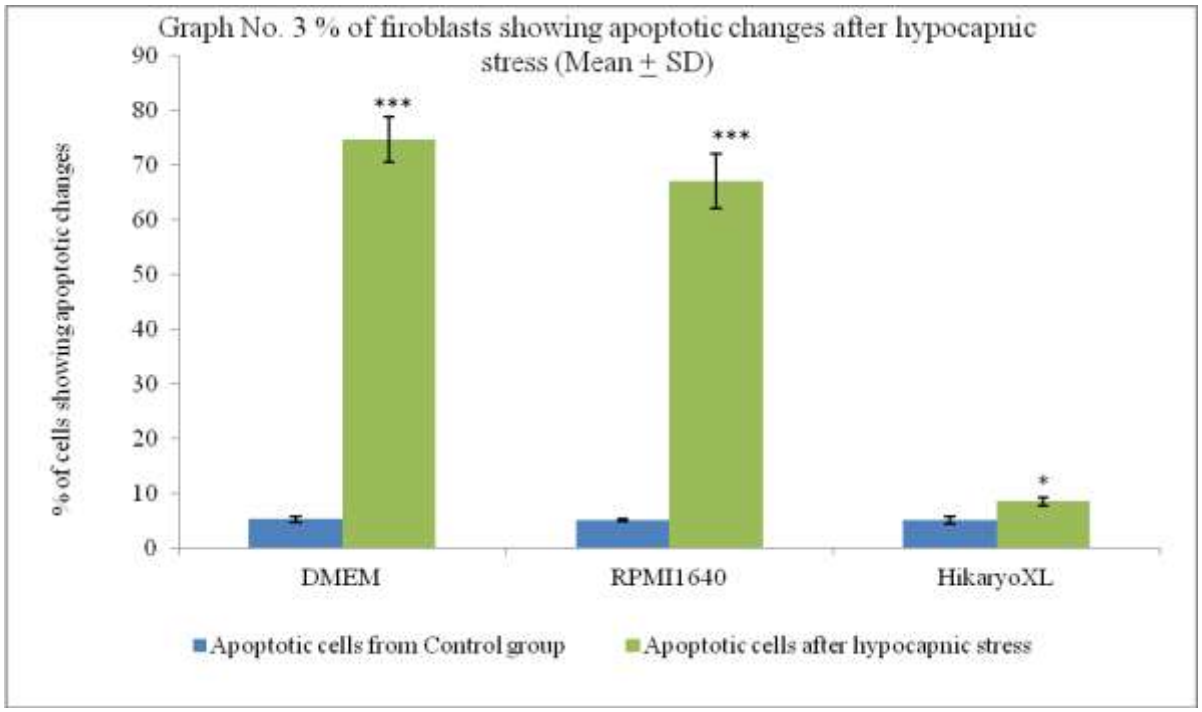
MB: Membrane blebbings, AB: Apoptotic bodies, FN: fragmented nucleus, N: Nucleus, Nu: Nucleolus, ET: Early telophase



NS



**** indicates p<0.001 i.e. highly significant Hikaryo XL compared with DMEM and RPMI1640, NS indicates nonsignificant RPMI 1640 compared with DMEM*



**** indicates p<0.001 i.e. highly significant, * indicates p<0.05 i.e. statistically significant Hypocapnic group compared with control group*

Table No.1: Number of intact cells grown in different cell culture media

Group	DMEM	RPMI1640	HikaryoXL
Control group	94.67 ± 4.23	94.84 ± 3.94	94.87 ± 3.27
Experimental group i.e. after Hypocapnic stress	0.44 ± 0.19***	1.33 ± 0.58***	72 ± 3.6***
	*		

**** indicates p<0.001 i.e. highly significant (When experimental group compared with control group and HikaryoXL compared with DMEM and RPMI 1640) * indicates p<0.05 i.e. statistically significant (when RPMI1640 compared with DMEM)*

Table No. 2: Number of cells grown in different cell culture media showing apoptotic changes

Group	DMEM	RPMI 1640	HikaryoXL
Control group	5.33 ± 0.57	5.16 ± 0.28	5.13 ± 0.7
Experimental group i.e. after Hypocapnic stress	74.66 ± 4.16***	67.0 ± 5.0***	8.55 ± 0.69*
	NS		

**** indicates p<0.001 i.e. highly significant and * indicates p<0.05 i.e. statistically significant (when experimental group compared with control group and HikaryoXL compared with DMEM and RPMI 1640) NS indicates nonsignificant, when RPMI1640 compared with DMEM*

RESULTS AND DISUCUSSION

Results: At 5% CO_2, the pH of the culture medium was 7.4. At 3.5% CO_2, it was increased to 7.8 and at 2.5% it was further increased to 8.2. The images of the culture, obtained by live cell imaging are displayed in Plate No. I and II.

Plate I shows the images of primary culture of the dermal fibroblasts. Cells from the control group are displayed in figures 1, 3 and 5; while, cells from the hypocapnic stressed group are displayed in figures 2, 4 and 6. In the DMEM fed culture, (PLATE I Figure 1) maintained at 5% CO_2 (i.e. DMEM Control group), there were 94.67 ± 2.23% cells showing intact and typical fibroblastic morphology. Whereas, DMEM fed hypocapnic stressed culture exhibited 0.44 ± 0.19% intact cells. There was complete loss of cellular architecture in this group. Floating apoptotic bodies were revealed in the culture medium (PLATE I Figure 2). 74.66 ± 4.16% cells from this group exhibited apoptotic alterations such as membrane blebbings (PLATE II Figure 1). In control group such alterations were seen in 5.33 ± 0.57% cells. There is highly significant difference ($p< 0.001$) between the control group and hypocapnic stressed group.

The RPMI1640 fed culture maintained at 5%CO_2 (RPMI1640 Control group), exhibited 94.84 ± 3.94% cells with typical fibroblastic morphology (PLATE I Figure 3), the cells appeared healthy. However, RPMI1640 fed hypocapnic stressed group exhibited 1.33 ± 0.38% intact cells (PLATE I Figure 4). There were 5.16 ± 0.28% cells from the RPMI1640 fed control group showing apoptotic alterations, whereas, 67.0 ± 5.0% cells from the hypocapnic stressed group underwent apoptotic changes (PLATE II Figure 2). The difference observed between the RPMI1640 fed control group and the hypocapnic stressed group is highly significant ($p<0.001$).

The HikaryoXL™ fed culture maintained at 5% CO_2 exhibited 94.87 ± 1.27% cells with intact and typical fibroblastic morphology (PLATE I Figure 5); whereas, in the hypocapnic stressed group 72 ± 3.6% cells exhibited typical fibroblastic morphology (PLATE I Figure 6). The observed difference is highly significant ($p<0.001$). 5.13 ± 0.7% of the cells from HikaryoXL™ fed control group exhibited apoptotic changes, whereas, 8.55 ± 0.69% cells from HikaryoXL™ fed hypocapnic stressed group exhibited apoptotic changes.

As compared to DMEM and PRMI1640 fed culture, the cells grown in HikaryoXL™ exhibited highly significant ($p<0.001$) preservation of the cell morphology and survival (PLATE II figure 3) even after hypocapnic stress (Table No.1, Graph 1). Moreover, the fibroblasts from HikaryoXL fed hypocapnic stressed group exhibited highly significant reduction in the apoptotic features as compared to DMEM and RPMI1640 fed group, exposed to hypocapnic stress (Table No.2 Graph No.2)

Discussion: In the present study, the observed alkalosis was due to the depletion of CO_2 tension from the incubator and hence from the culture medium. According to Henry's law the partial pressure of CO_2 in the culture medium depends upon the partial pressure of CO_2 in the humidified air of the CO_2 incubator.

PCO_2 (Liquid) = PCO_2 (Gas) ----------I

According to Henderson Hasselbalch equation, 5% CO_2 is such a concentration which equilibrates the culture medium to the physiological pH of 7.4. Oberving the culture for a long time under microscope lacking stage top CO_2 incubation system, frequent opening the door of CO_2 incubator, leakage of regulator/ Cylinder/ Power failure lead to depletion of CO_2 from the incubator. This can be represented as

PCO_2 (Liquid) > PCO_2 (Gas) ----------II

This leads to dissociation of carbonic acid from the culture medium into CO_2 and H_2O, to maintain the equilibrium shown in equation I

$$H_2CO_3 \rightleftharpoons CO_2 + H_2O \text{ ----------III}$$

As a result, there is a decrease in the concentration of carbonic acid from the carbonic acid–bicarbonate conjugate acid-base pair that affects the pH. Carbonic acid being a weak acid undergoes reversible dissociation into H+ and HCO_3^-.

$$H^+ + HCO_3^- \rightleftharpoons H_2CO_3 \text{ ---------IV}$$

As the concentration of the carbonic acid is depleted, the bicarbonate ions consume H^+ ions to regenerate H_2CO_3. Therefore, the concentration of H^+ ions from the culture medium gets decreased resulting into increase in the pH of the culture.

In the present investigations, the decrease in the number of healthy cells from DMEM fed and RPMI1640 fed hypocapnic stressed group was due to intolerance of pH fluctuations. The cells exhibited morphological features of apoptotic cell death, such as membrane blebbings and presence of floating apoptotic bodies in the culture medium. Formation of membrane blebbings and finally fragmentation of cell into apoptotic bodies are the characteristic features of aototic cell death. (O'Brien, 1998). In the RPMI 1640 fed fibroblast culture, there were few swollen cells in hypocapnic stressed group which demonstrates the feature of senescence *in vitro*. Increased cell size in culture is one of the characteristic features of senescence (Cristofalo and Pignolo *et al.* 1993). The healtier cell morphology and lower percentage of apoptotic cells grown in HikaryoXL™ indicates that HikaryoXL™ provides protection against hypocapnic stress.

CONCLUSION

The results obtained in the present investigations suggest that HikaryoXL™ confers protection to mouse dermal fibroblasts from fluctuations in the pH due to disturbances in CO_2 tension.

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