Original Research Article

International Journal of Bioassays
ISSN: 2278-778X
www.ijbio.com
OPEN ACCESS

EFFECT OF ALKALINITY ON CANCEROUS CELLS AT DIFFERENT pH AND MORPHOLOGICAL VARIATIONS IN-VITRO

Mohsin Ali Khan[1], Aparna Misra[2]*, Anchal Trivedi[2] and AN Srivastava[3]
[1]Chairman Research, [2]Department of Biochemistry, [3]Department of Pathology,
Era's Lucknow Medical College & Hospital, Sarfarazganj, Hardoi Road, Lucknow-226003, India

Received for publication: May 27, 2014; **Revised:** June 12, 2014; **Accepted:** July 05, 2014

Abstract: The acidic environment due to excessive glycolytic activity and poor perfusion promote the cancerous cell growth. This condition also decreases the efficiency of chemotherapeutic drugs. The present study investigates whether alkalinity increment of the media can reduce the cancerous cell growth and improve the effect of chemotherapeutic drugs *in-vitro*. For this experiment, the vero cells were control whereas MDA-MB and IM cells were experimental cancerous cells. The alkaline pH of media reduces the cancerous cell growth up to 18% to 42%, but the exact mechanism is not known with certainty.

Key Words: Vero, IM 9, MDA-MB, Fig. No., In-vivo, In-vitro

INTRODUCTION

It has been shown by plenty of research that acidic pH has a clear link with cancer[1]. The external pH of solid tumors is acidic as a consequence of increased metabolism of glucose and poor perfusion[2]. Acidic pH has been shown to stimulate tumors cell invasion and metastasis[3].

Cancer progression is a multistep process, which is strongly influenced by the physical properties of the tumor micro environment[4]. The extra cellular pH of tumors is generally more acidic than Normal tissue[5]. This is probably due to the consequence of collaboration between eminent aerobic glycolysis and reduced blood flow[6]. Despite of, the acidity of tumors, most *in-vitro* assays of tumors cell functions are routinely performed at neutral to alkaline medium pH[7]. Schlappek *et al.,* observed that transient acidosis increased metastatic behaviors (colonization) in implanted murine sarcoma and lymphoma cells[8]. These findings strongly suggest a trophic effect of pH in metastasis and uncertainty of *in- vivo* growing conditions.

In culture, normal cells contrary to cancer cell or virus transformants show "contact inhibition" of growth; i.e. cell population density stabilizes relatively at low levels. The precise value is varying with the individual cell and serum[9]. This may be depend on DNA, RNA & protein synthesis. This contact inhibition of growth may be determined by population density and also vide artifactual variation in the pH of medium.

According to Obokata *et al.,* the stimulus-triggered fate conversion of somatic cells in to pluripotent cell and low pH can covert somatic cells to stem cells[10]. As per findings of another researcher, Bicarbonate increases pH of tumor and inhibits spontaneous metastasis[11]. External pH of solid tumors is acidic due to the consequence of increased metabolism of glucose and poor perfusion[12].

Intention of this instant study is to observe the effect of acidic and alkaline pH on cancerous cell lines *in-vitro*. By taking a glance of the various studies, it transpires that the cancerous cell can grow in acidic pH which inferences that alkaline pH inhibits the growth of these cancerous cells. It has been shown that the resistance to anti-cancer chemotherapies often leads to regional failure, and it may be caused by biochemical and/ or physiological mechanisms. Aim of the study is to compare the effect of pH on different cancerous cell lines, and determine the role of alkaline pH on the growth of cancerous cells.

MATERIALS AND METHODS

Cell lines

Vero (ATCC- CCL-81) normal kidney epithelial cell line, MDA-MB-231 human breast carcinoma cell lines and IM9-ATCC multiple myeloma cancer cell line were obtained from the NCCS, Pune. Cell lines were established in this laboratory by transfection of MCF-7 with ER. For the purpose of routine culture, all cell lines were maintained as monolayers at 37°C in an incubator gassed with an atmosphere of 5% CO_2 at 95% humidity, in advanced dulbecco's minimum essential medium (DMEM) containing phenol red as a pH indicator and supplemented with 5% fetal bovine serum (FBS), 600 µg/ml L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 6 ml/500 ml 100 x non-essential amino acids (all from sigma, CA, USA) (complete

***Corresponding Author:**
Professor Aparna Misra,
Department of Biochemistry,
Era's Lucknow Medical College & Hospital,
Sarfarazganj, Hardoi Road,
Lucknow-226003, India.

medium). This medium requires an atmosphere of 5% CO_2 to produce HCO_3 buffering capacity to maintain pH at 7.4 for normal cell growth.

Microscopic analysis of morphological changes in response to pH

For each cell line, approximately 10^5 cells were seeded into culture flasks containing medium of different pH i.e. pH 6.2, 6.5, 6.8(acidic PH), 7.1, 7.4, 7.7, 8.0, 8.3 and 8.6 (Alkaline pH) and allowed to settle at 37°C for 24 h. Culture flask of pH 7.4 has been taken as control flask. The maximum growth of the cell was found at 48hours of incubation. Flasks were then removed from the incubator (i.e. from the 5% CO_2 atmosphere needed to maintain the buffering capacity of the DMEM) and exposed to the normal atmospheric environment atmosphere to change. Resultant changes in cell size and shape (termed contractolation thereafter) in each photographed field were quantified using Adobe Photoshop CS4 Measuring Tool in terms of the field area occupied by cells.

Live cell microscopy

The general growth characteristics of cells were continuously monitored by time-lapse photography using a live cell imager (Cell Observer HS, Zeiss, Germany). Cell monolayer grown overnight in an atmosphere of 5% CO_2 inside a 25 cm^2 tissue culture flask containing 4 ml DMEM were placed inside the imaging chamber which was also maintained at 37°C with 5% CO_2 atmosphere. Flasks were positioned to enable photography of cell islands composed of 3-4 cells with images being recorded at 20X magnification every 5 min over a 72 h period. The AxioVision software (Zeiss) was used to combine all the pictures to generate a video of 14 h which was then speeded up to a few minutes using Windows Movie Maker software (Microsoft). For pH induced effects, cells were observed for shorter periods in Petri dishes left under normal atmospheric conditions or, for recovery, back in the 5% CO_2 atmosphere.

RESULTS

Initially, the cells having the divergence of 0.3 in pH of acidic and alkaline medium were cultured normally. The pH 7.4 is the optimum normal pH was the control for experiment. Fig.1A shows photograph of vero cells snatched immediately after removing the flask from incubator when the cell medium was at pH7.4, after 48hrs. of incubation. The cells were healthy in shape and good confluency. Fig.1B is at 7.7, the status of cells were almost same but density was a little less. (Fig.1B). At pH 8.0, cells start shrinking with lesser confluency (Fig.1C) whereas at pH 8.3, cells became rounded and they start detaching from the flasks (Fig. 1D).

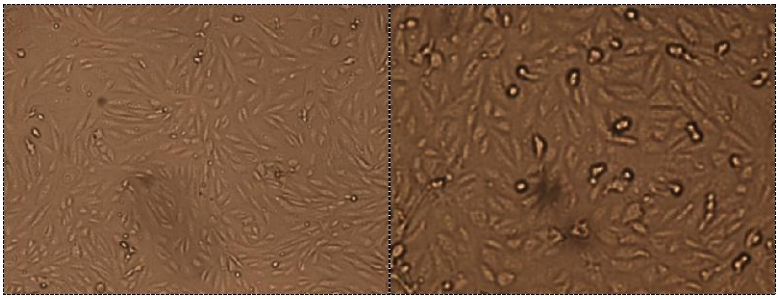

Figure 1A: Vero cells at pH 7.4 **Figure 1B:** Vero Cells at pH 7.7

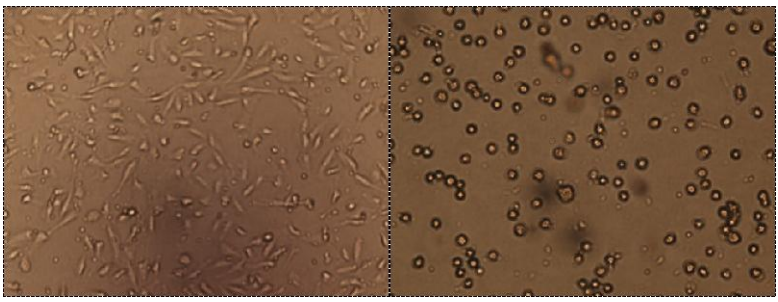

Figure 1C: Vero Cells at pH 8.1 **Figure 1D:** Vero cells at pH 8.4

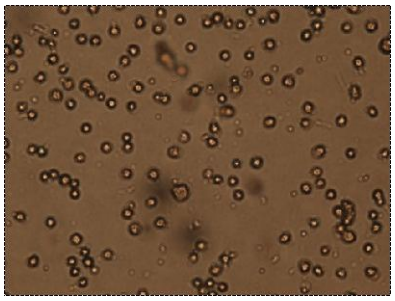

Figure 1E: Vero cells at pH 8.1

When we move towards acidic pH (7.1) cell shape and size are same as 7.4 (Fig. 2A). Further increase of pH towards acidity shows drastic change in morphology at pH 6.8, cells start rounded and detached from the surface (Fig.2B). At pH 6.5 cells became fully rounded and detached from the surface of flask (Fig. 2C).

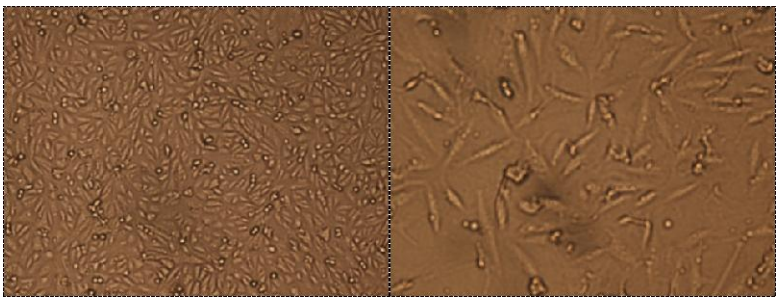

Figure 2A: Vero cells at pH 7.1 **Figure 2B:** Vero cells at pH 6.8

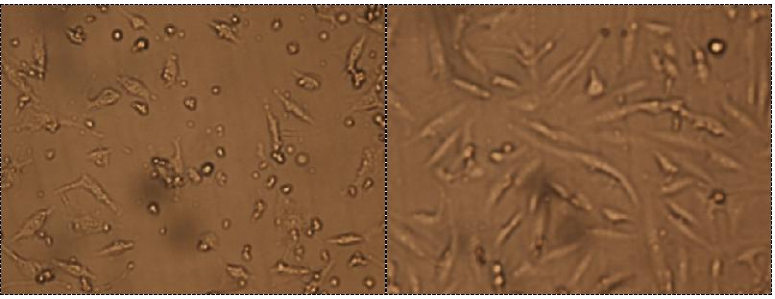

Figure 2C: Vero cells at pH 6.5 **Figure 2D:** Vero cells at pH 6.2

In Fig.3A MDA MB-231 breast cancer cell appear phenotypically as spindle shaped cells at pH 7.4 after 48 hours of culture and full confluence of cells are seen. At pH 7.7, (Fig. 3B), the cell shape are maintained whereas Numbers of viable cells decreased. At pH 8.0 (Fig. 3C) and at pH 8.3 cell shape and viability both are deteriorated. (Fig.3D). At pH 8.7, numbers of viable cells decreased about 40%.

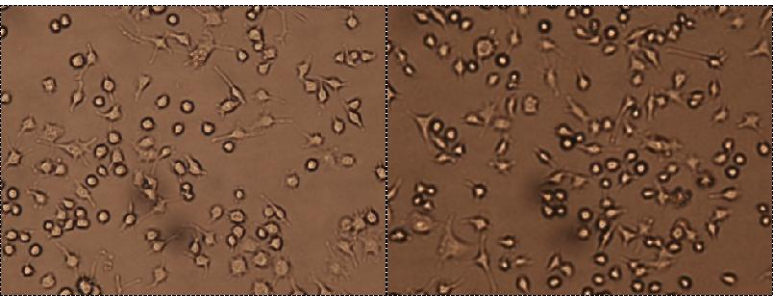

Figure 3A: MDA-MB cells at pH 7.4 **Figure 3B:** MDA-MB cells at pH 7.8

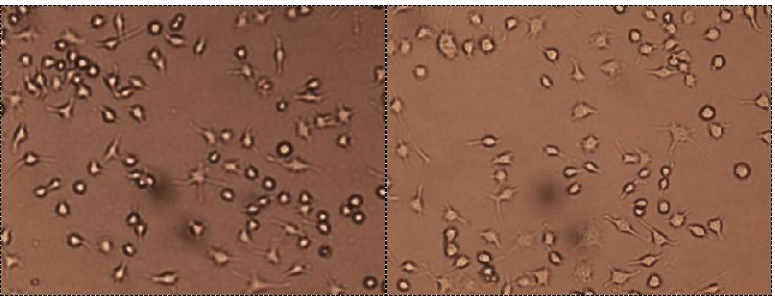

Figure 3C: MDA-MB cells at pH 8.0 **Figure 3D:** MDA-MB cells at pH 8.3

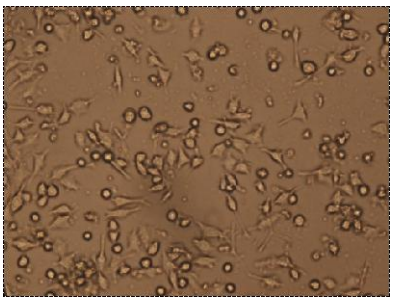

Figure 3E: MDA-MB cells at pH 8.6

At acidic pH, growth of MDA cells are increased. As the pH of medium decreased, the growth of cells increased. Spindle shape cells are found with full confluency. (Fig. 4A-4C). At pH 6.2, few cells start to contralocate. (Fig. 4D.)

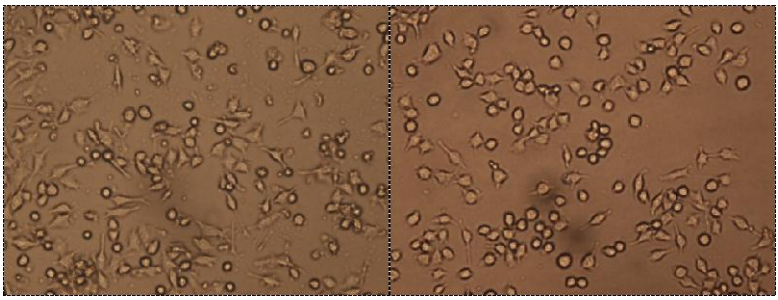

Figure 4A: MDA-MB cells at pH 7.1 **Figure 4B:** MDA-MB cells at pH 6.8

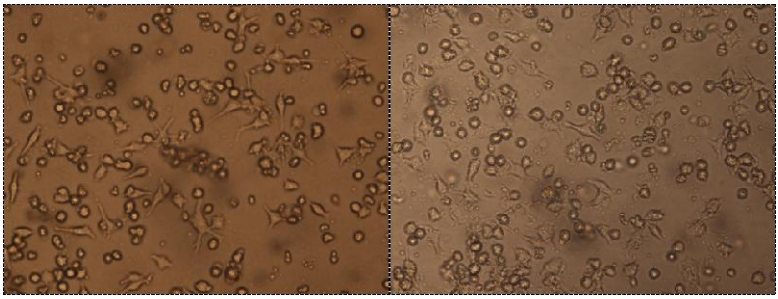

Figure 4C: MDA-MB cells at pH 6.5 **Figure 4D:** MDA-MB cells at pH 6.2

Fig.5A are IM 9 cells which are multiple myeloma cells and they make suspension in medium. At pH 7.4 cells are rounded and shiny under the microscope. As the pH goes towards alkalinity, cell shape and size alters with the decreased viability of cells.

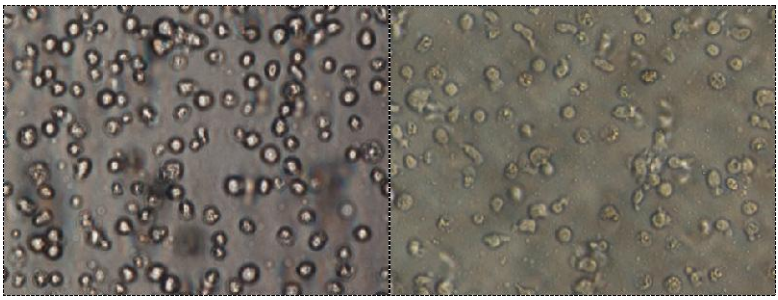

Figure 5A: IM9 cells at pH 7.4 **Figure 5B:** IM9 cells at pH 7.7

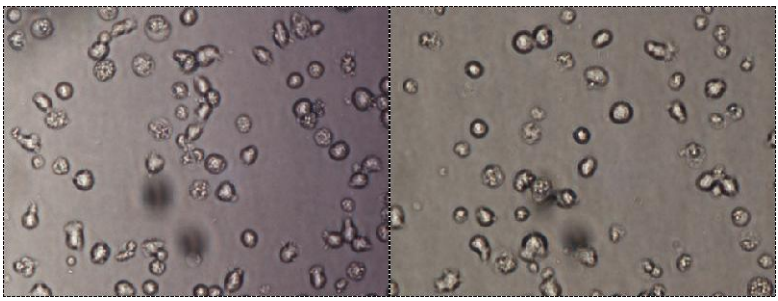

Figure 5C: IM9 cells at pH 8.0 **Figure 5D:** IM9 cells at pH 8.3

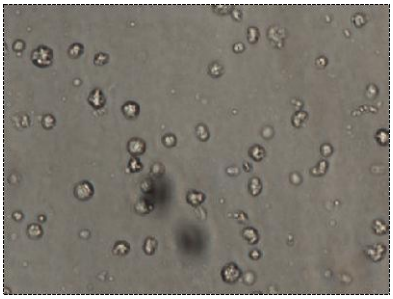

Figure 5E: IM9 cells at pH 8.6

At pH 7.7, cell growth decreased and the clumping of cells are clearly seen. (Fig. 5B). At pH 8, further increment of cell death are found (Fig. 5C) whereas at pH 8.4, IM9 cell are not growing (Fig. 5D)

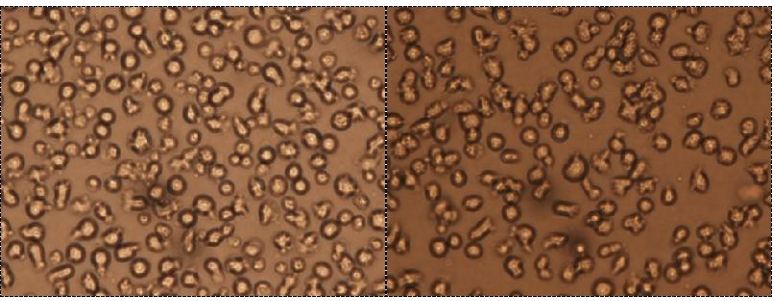

Figure 6A: IM9 cells at pH 7.1 **Figure 6B:** IM9 cells at pH 6.8

At pH 7.1, growth of IM9 cells are good. Shape and size are normal, cell numbers are almost same as at pH 7.4 (Fig. 6A). At pH 6.8, the growth of cells is increased. Shape and size are normal. Whereas numbers of live cells increased (Fig. 6B). At pH 6.5, cell are properly adhered to the flask. Number, shape and size are increased (Fig. 6C). At pH 6.2, contact inhibition start showing the decrement of cell size (Fig. 6D).

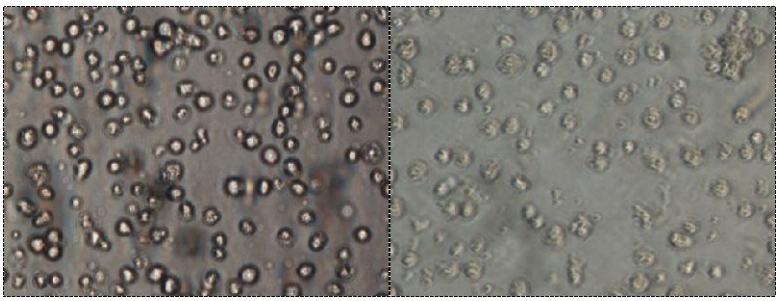

Figure 6C: IM9 cells at pH 6.5 **Figure 6D:** IM9 cells at pH 6.2

Table 1 shows that MDA are showing maximum cell death at pH 8.6 after 24 hours of culture as well as IM9 are showing maximum cell death at pH 8.0. Whereas in the normal cells i.e. vero cells are presenting cell death in both acidic and alkaline pH. Therefore, according to this study, different cells can show the variations in the pH at which they are showing maximum cell death.

Table 1

SR. NO.	PH	VERO CELL LINE				MDA MB 231				IM9 CELL LINE			
		Total no. of cell	Live cell	Dead Cell	% of Dead cells	Total no. of cell	Live cell	Dead Cell	% of Dead cells	Total no. of cell	Live cell	Dead Cell	% of Dead cells
1	6.2	1.0600000	8.630000	1.940000	18%	2.7400000	2.5100000	2.340000	9%	1.6200000	1.320000	0.30000	18%
2	6.5	1.9900000	1.0300000	9.540000	48%	2.3600000	2.2200000	1.480000	6%	1.7000000	1.150000	5.500000	32%
3	6.8	1.7200000	1.0100000	7.030000	41%	2.8500000	2.7100000	1.380000	5%	1.3000000	1.020000	2.800000	21%
4	7.1	1.8000000	1.1400000	8.020000	40%	8.630000	7.860000	0.760000	9%	1.4500000	1.3000000	0.150000	10%
5	7.4	1.9600000	1.0800000	7.020000	39%	2.4600000	2.1900000	2.760000	11%	1.0200000	8.360000	7.340000	7%
7	7.7	2.300000	1.300000	1.000000	43%	1.0700000	9.860000	0.080000	11%	1.0400000	9.370000	1.010000	10%
8	8.0	2.410000	1.110000	1.000000	52%	1.3100000	3.860000	0.092000	14%	1.9800000	1.040000	0.940000	48%
9	8.3	2.810000	1.330000	1.480000	47%	1.0600000	1.810000	1.800000	16%	1.6500000	1.180000	4.700000	29%
10	8.6	1.430000	1.310000	0.210000	22%	1.0200000	1.770000	1.920000	18%	1.6900000	1.020000	4.90000	28%

Group Statistics:

Comparison of vero cell with MDA MB

T-Test (Unpaired)

	GRP	N	Mean	Std. Deviation	Std. Error Mean
Total no. of cell	Vero Cell (exp1)	10	2.0330	.59388	.18780
	MDA MB 231 (exp2)	10	5.3720	1.68578	.53309
Live cell	Vero Cell (exp1)	10	2.8210	3.34218	1.05689
	MDA MB 231 (exp2)	10	4.9230	1.69647	.53647
Dead Cell	Vero Cell (exp1)	10	6.9200	3.27102	1.03439
	MDA MB 231 (exp2)	10	3.5430	1.71562	.54253
% of Dead cells	Vero Cell (exp1)	10	34.70	8.667	2.741
	MDA MB 231 (exp2)	10	8.80	4.940	1.562

Independent Samples Test

		T	df	p-value	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
							Lower	Upper
Total no. of cell	Equal variances assumed	-5.908	18	**<.001**	-3.3390	.56520	-4.52645	-2.15155
Live cell	Equal variances assumed	-1.773	18	.093	-2.1020	1.18525	-4.59212	.38812
Dead Cell	Equal variances assumed	2.891	18	**.010**	3.3770	1.16803	.92306	5.83094
% of Dead cells	Equal variances assumed	8.210	18	**<.001**	25.90	3.155	19.272	32.528

DISCUSSION

The enhancement of metabolism of glucose and poor perfusion transforms the external pH of solid tumors, which may lead towards acidic in nature. The instant work unfolds that, in a controlled Vero cell line, the effect of pH *in-vitro* is somewhat contraindicated with cancerous cell lines, i.e. MDA & IM9. Thus, at the basic as well as acidic pH, normal cultured cells line were not survived well. Conversely, in the cancerous cell lines, acidic pH increases the growth *in-vitro,* whereas at basic pH, cancerous cells get destroyed about up to 18 to 42%. So by regulating the pH towards alkaline, growth of cancerous cell can be inhibited about 48% in IM 9 and 18% of MDA cells.

As a consequence elevated acid production, the extracellular pH (pH^{ex}) of tumors is generally acidic[13]. Despite this, most in vitro experiments are still performed at the relatively alkaline pH^{ex} of 7.4. This is significant, because slight changes in pH^{ex} can have profound effects on cell phenotype. Yang J, Mani SA, Donaher JL, Observed in their study that culturing of melanoma cells at mild acidic pH 6.8 causes dramatic enhancement in both migration and invasion[14]. The cells cultured at acidic pH were more aggressive than controlled cells. So this study discloses that culturing of melanoma cells at acidic pH are more invasive[15].

As per findings made by Maithan A Khajal *et al.,* extracellular pH is an important factor controlling cell behavior, motility and matastasis[16]. Association of contralocation with activation of intracellular signaling molecules has been shown by several researchers[17-18]. It has been also investigated that the bicarbonate also increases the pH of tumor which inhibits the further metastasis in mouse model.

The pH induced contralocation involves an apparent shrinking of cells in to a more spherical shape with considerable ruffling of membrane and cells may revert completely to their original morphological upon return to pH 7.4[19].

Whereas general consensus favors, an acidic tumor micro environment, due in large measure to the extrusion of accumulation of lactic acid and protons produced excessive glycolytic activity to be conducive to metastasis[20]. This acidic environment encourages cell proliferation, metabolic adaptation and envasion through various mechanisms such as enhanced activity of CDC 42[21] de novo assembly of actin filaments and various actin binding proteins[22].

In this study, we are reporting that alkaline pH induces marked morphological changes in cancerous cell *in-vitro*. This study is also demonstrated the change in viability of these cells at different pH. These observations may have important implication not only on the control of cancerous cells but also for the understanding the environment responsible for metastasis of cells.

It has also been shown that low pH in tumors can diminish the effectiveness of some chemotherapeutic drugs. So this study is conducted to determine the effect of extracellular alkaline pH can inhibit the cancerous cells growth *in-vitro.* In conclusion, we find that the normal cells growth is inhibited in acidic pH and *vice versa* in cancerous cells but the effect of pH varies for various cell to cell.

ACKNOWLEDGEMENTS

The authors are thankful to Prof. Ajanta Roy, Head, Department of Biochemistry for permitting the experimental work in the Research laboratory. Special thanks are due to Prof.Farzana Mahdi, Director Academics, for her valuable suggestions.

REFERENCES

1. Wike-Hooley JL, Hoveman J and Rein hold HS. The relevance of tumor pH to the treatment of malignant disease. Radiother oncol, 1991, 2, 343. 66.

2. Wike-Hooley, JL, Haveman J and Reinhold HS, The relevance of tumour pH to the treatment of malignant disease. Radiother Oncol, 1991, 2, 343–66.

3. Grifiths JR. Are cancer cells acidic? Br J Cancer, 1991 64,425–7.

4. Sevick EM and Jain RK. Blood flow and venous pH of tissue-isolated Walker 256 carcinoma during hyperglycemia. Cancer Res, 1988, 48, 1201–7.

5. Vaupel P, Kallinowski, F and Okunieff P, Blood flow, oxygen and nutrient supply, and metabolic microenvironment. Cancer Res, 1989, 49, 6449–65.

6. Stolk JA, Bhujwalla ZM, Blackband SJ, Shungu DC, Wehrle JP and Glickson JD, Quantitative in vivo measurement of blood flow in RIF-1 tumors by 1H/2H NMR spectroscopy. Proc 10th Ann Mtg Soc Magn Reson Med1991, p. 984.

7. Bhujwalla, ZM, Tozer, GM, Feld, SB, Maxwell, RJ and Grifths, JR, Energy metabolism of RIF-1 tumors following hydralazine. Radiother Oncol, 1990, 19, 281–91.

8. Schlappack, OK, Zimmermann A and Hill, RP, Glucose starvation and acidosis: effect on experimental metastasic potential, DNA content and MTX resistance of murine tumour cells. Int J Cancer, 1991, 64, 663–70.

9. Eagle H and EM Levine, Species Specificity in Growth Regulatory Effects of Cellular Interaction, Nature 1967, 213, 1102.

10. Haruko Obokata, Teruhiko Wakayama, Yoshiki Sasai, Koji Kojima, Stimulus-triggered fate conversion of somatic cells into pluripotency Nature, 2014, 505,641.

11. Robey IF, Bagett BK, Kirkpatric ND, Cancer Res. Bicarbonate increases tumor pH and inhibits spontaneous metastases 2009 Mar 15; 69 (6):2260-8. doi: 10.1158/0008-5472.CAN-07-5575. Epub 2009, Mar 10.

12. Al Saleh S, Al Mulla F, Luqmani YA Estrogen receptor silencing induces epithelial to mesenchymal transition in human breast cancer cells. PLOS ONE, 2011, 6: 21.

13. Khandjian EW, Acidic extracellular environment induces only a subset of heat-shock proteins in primary mouse kidney cell cultures. Biochem Cell Biol, 1990, 68, 804–7.

14. Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA *et al.,* Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell, 2004, 117: 927-939. doi:10.1016/j.cell.2004.06.006.

15. Luqmani YA, Al Azmi A, Al Bader M, Abraham G, El Zawahri M Modification of gene expression induced by siRNA targeting of estrogen receptor alpha in MCF7 human breast cancer cells. Int J Oncol, (2009) , 34: 231-242.

16. Khajah MA, Al Saleh S, Mathew PM, Luqmani YA Differential effect of growth factors on invasion and proliferation of endocrine resistant breast cancer cells. PLOS ONE, 2012, 7: 30. doi: 10.1371/journal.pone.0041847.

17. Stock C, Schwab A. Protons make tumor cells move like clockwork. Pflugers Arch, 2009, 458: 981-992. doi:10.1007/s00424-009-0677-8.

18. Gatenby RA, Gawlinski ET, Gmitro AF, Kaylor B, Gillies RJ Acid-mediated tumor invasion: a multidisciplinary study. Cancer Res, 2006, 66: 5216-5223. doi:10.1158/0008-5472.CAN-05-4193.

19. Maulik G, Kijima T, Ma PC, Ghosh SK, Lin J *et al.,* Modulation of the c-Met/hepatocyte growth factor pathway in small cell lung cancer. Clin Cancer Res, 2002, 8: 620-627.

20. Stella MC, Comoglio PM HGF: a multifunctional growth factor controlling cell scattering. Int J Biochem Cell Biol, 1999, 31: 1357-1362. doi:10.1016/S1357-2725(99)00089-8.

21. Hung CM, Kuo DH, Chou CH, Su YC, Ho CT *et al.,* Osthole suppresses hepatocyte growth factor (HGF)-induced epithelial-mesenchymal transition via repression of the c-Met/Akt/mTOR pathway in human breast cancer cells. J Agric Food Chem, 2011, 59: 9683-9690. doi:10.1021/jf2021489.

22. Helmlinger G, Sckell A, Dellian M, Forbes NS, Jain RK. Acid production in glycolysis-impaired tumors provides new insights into tumor metabolism. Clin Cancer Res, 2002, 8: 1284-1291.

23. Garamszegi N, Garamszegi SP, Shehadeh LA, Scully SP, Extracellular matrix-induced gene expression in human breast cancer cells. Mol Cancer Res, 2009, 7: 319-329. doi:10.1158/1541-7786.MCR-08-0227.

Source of support: Nil
Conflict of interest: None Declared