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Original Article
Özgün Araştırma

An evaluation of the effects of medium pH on the viability of the HepG2 cell line

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Abstract: Cancer cells are characterized by increased glucose uptake and the production of lactate, which leads to acidification of the tumor microenvironment. This acidification facilitates the development of invasiveness and metastasis. In this study, we investigated the effects of medium pH manipulation on the proliferation and viability of human hepatocellular carcinoma (HepG2) cells *in vitro*. HepG2 cells were grown in media with pH ranging from 6.0 to 8.5 for 24 h. The cells were then subjected to WST-1 and trypan blue exclusion assays to evaluate viability and cell proliferation, respectively. At pH 6.8 and 6.6, HepG2 cell viability was not significantly different from the control group (pH 7.2) ($p>0.05$) but there was a significant decrease at pH 6.4, 6.2 and 6.0 ($p<0.05$). Furthermore, there was a significant decrease in cell viability at pH's 7.8, 8.0 and 8.5 ($p<0.05$). Cell numbers decreased at pH 6.8 and increased at pH 6.6, although not significantly ($p>0.05$), and decreased at pH 7.6 and 7.8 (significant at pH 7.8; $p<0.05$). In acidic environments, the cells were spindle-shaped and formed islands but they became more spherical and had reduced adhesion capacity in alkaline media. In this study, an alkaline environment reduced the proliferation and viability of the cell line, HepG2. Therefore, after further investigations, in addition to current treatments, systemic alkalization that appropriately increases the pH of the tumor microenvironment may suppress the activity of tumor cells and increase the efficacy of normal HCC treatment.

Keywords: Alkali medium; HepG2; microenvironment; proliferation; Warburg effect

HepG2 hücre hattının canlılığı üzerine medium pH'sının etkisinin incelenmesi

Özet: Kanser hücreleri, tümör mikroçevresinin asitleşmesine neden olan artmış glikoz alımı ve laktat üretimi ile karakterizedir. Asidik mikro-çevre kanser hücrelerinin invazyon ve metastaz gelişimini kolaylaştırır. Bu çalışmada medium pH değişiminin, *in vitro* insan hepatoselüler karsinom (HepG2) hücrelerinin proliferasyon ve canlılığına etkisinin değerlendirilmesi amaçlanmıştır. HepG2 hücreleri pH değeri 6.0 ile 8.5 arasında değişen medium ortamlarında 24 saat boyunca inkübe edildi. Daha sonra hücre canlılığı ve proliferasyon değerlendirmeleri için sırasıyla WST-1 ve tripan mavisi hücre canlılık testleri uygulandı. HepG2 hücre canlılığında, medium pH'sı 6.6 ve 6.8 olan gruplar ile kontrol grubu (pH 7.2) karşılaştırıldığında önemli bir farkın bulunmadığı ($p>0.05$), ancak pH 6.4, 6.2, 6.0'da önemli oranda azalmanın olduğu saptandı ($p<0.05$). Ayrıca alkali gruplardan pH 7.8, pH 8.0 ve pH 8.5'te hücre canlılık oranında belirgin bir azalma gözlemlendi ($p<0.05$). Hücre sayısında pH 6.8'de artış ve pH 6.6'da azalmanın olduğu ancak istatistiksel olarak önemli fark olmadığı ($p>0.05$), pH 7.6 ve 7.8'de azalmanın bulunduğu ve bu azalmanın pH 7.8'de önemli olduğu saptandı ($p<0.05$). Asidik ortamlarda, iğ şeklindeki hücrelerin adalar oluşturduğu, alkali ortamda ise yapışma kapasiteleri azalmış hücrelerin yuvarlak şekilde olduğu gözlendi. Bu çalışmada, alkali mikro-çevrenin HepG2 hücre hattının canlılığını ve proliferasyonunu azalttığı tespit edilmiştir. Bu nedenle, daha ileri araştırmalardan sonra mevcut tedavi yöntemlerine ek olarak tümör mikro-çevre pH'sını uygun bir şekilde yükselten sistemik alkalizasyonun, tümör hücrelerinin aktivitesini baskılayabileceği ve normal HCC tedavisinin etkinliğini artırabileceği sonucuna varıldı.

Anahtar kelimeler: Alkali ortam; HepG2; mikro-çevre; proliferasyon; Warburg etkisi

Introduction

Cancer, which is defined by abnormal cell growth that leads to an imbalance between cell proliferation and cell death as a consequence of multiple changes in gene expression (Ruddon, 2007), is one of the most common causes of death worldwide (Torre et al. 2016) . The International Agency for Research on Cancer (IARC) reported that 18.1 million people were diagnosed with cancer in 2018, and of that number, 9.6 million died (Bray et al. 2018). Hepatocellular carcinoma (HCC), which originates from liver cells called hepatocytes, is the most common, primary malignant tumor of the liver (Rodríguez-Hernández et al. 2018).

The metabolism of cancer cells is particularly oriented to survival and proliferation. One of the

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most important changes is in energy metabolism (Hsu and Sabatini 2008). Many cancer cells convert glucose to lactate, although less energy is gained than through aerobic breakdown. Otto Warburg, in the 1920s, was the first person to report the occurrence of this metabolic process, even in the presence of sufficient oxygen. This phenomenon later became known as the "Warburg Effect" (Gatenby and Gillies 2004; Kato et al. 2013; Tian et al. 2019). Many studies have demonstrated that the pH of the extracellular fluids of cancer cells is acidic because of the Warburg Effect (Schwartz et al. 2017). The pH of the extracellular environment of tumor cells is generally 0.3 to 0.7 pH units lower than that of normal cells (Hao et al. 2018). While the extracellular pH of tumors ranges from 6.5 to 6.9, normal extracellular pH is between 7.2 and 7.5. An acidic microenvironment is a characteristic feature of the environment of cancer cells, such as HCC (Zhang et al. 2010; Chen et al. 2017). Moreover, the extracellular pH of malignant melanoma tissue is approximately 6.7 whereas the extracellular pH of normal skin tissue cells is approximately 7.3. Similarly, the extracellular pH of uterine cancer cells (6.9) is more acidic than normal uterine cells (7.6) (Hao et al. 2018).

The two main reasons why the pH of the microenvironment of cancer cells is acidic are the increased expression and activity of carbonic anhydrases and proton carriers which transport lactate and CO_2 to the outside of the cell, and the accumulation of CO_2 and lactate there due to poor perfusion (Zhang et al. 2010; Chen et al. 2017). Many recent studies have shown that the Warburg effect enhances the probability of: survival of tumor cells DeBerardinis et al. (2008); Lodish et al. (2016); Tuccitto (2018) tumor progression, metastasis and angiogenesis Chen et al. (2017); Zhang et al. (2017), suppression of the immune response in the tumor microenvironment Alfarouk et al. (2015), and resistance to the drugs administered during chemotherapy (Raghunand and Gillies 2000; Chen et al. 2017; Zhang et al. 2017).

Therefore, therapeutic interventions that target the acidity of the tumor microenvironment are being employed (Neri and Supuran 2011). For example, systemic alkalizers and alkalizing agents such as sodium bicarbonate have been successfully used to suppress the cell growth and metastasis of the human breast cell lines, MCF-7 and MDA-MB-321, and the human colon cancer cell line, HCT116, by increasing the extracellular pH (Robey et al. 2009; Silva et al. 2009; Ibrahim-Hashim et al. 2012; Estrella et al. 2013). Moreover, oral sodium bicarbonate treatment was reported to reduce the extent of metastasis of breast and prostate cancer in immune system-deficient mice (Robey et al. 2009). In addition, in a clinical study involving patients with small cell lung cancer, an alkaline diet administered in combination with EGFR inhibitors increased the average survival time from 18.6 months to 28.5 months (Hamaguchi et al. 2017). Moreover, the radiation-induced death rate of human colorectal cancer cells (RKO.C) is lower at a more acidic pH than at the normal pH (Park et al. 2000, 2003). Although the relationship between cancer and the Warburg Effect has been the subject of a substantial amount of research over the past decade, to the knowledge of the authors of the present study, the effects of changes in the pH of the medium on the proliferation and viability of the human hepatocellular carcinoma cell line, HepG2, have not been investigated. Therefore, in this study, we evaluated the effects of both acidification and alkalization of the tumor microenvironment on the proliferation and viability of HepG2 cells and, indirectly, tumor progression.

Materials and Methods

Chemicals and Medium: The sodium bicarbonate, hydrochloric acid, trypan blue, Minimum Essential Medium (MEM), L-glutamine, penicillin-streptomycin and sodium pyruvate were purchased from Sigma-Aldrich (St. Louis MO, USA). The WST-1 cell proliferation assay reagent was purchased from Roche (Basel, Switzerland), the essential amino acid was purchased from GIBCO (Grand Island, NY, USA), and the trypsin-EDTA was purchased from Biological Industries (Kibbutz Beit Haemek, Israel).

Cell culture: The human hepatocellular carcinoma HepG2 cells were provided by the American Type Culture Collection (ATTC, HB-8065, USA). The cell culture medium consisted of MEM supplemented with 10% FBS, 2mM L-glutamine, 1% antibiotics (penicillin and streptomycin), 1% sodium pyruvate, 1% Non-Essential Acid (NEAA) and 2.2% g/l sodium bicarbonate. The frozen vials of cells were stored in a freezer at −80°C (Nüve-DF 490) until the experiment was conducted, at which time they were thawed by immersing the vial in a shaking waterbath (Nüve-Nb 20) at 37 °C. After thawing, the vial was disinfected with 70% isopropanol. The cells were then transferred into a sterile 75 cm^2 tissue cell culture flask containing 20 ml of MEM (supplemented with 20% FBS) and incubated at 37 °C under a humidified atmosphere containing 5% CO_2 (Nüve-EC 160) until they reached 75-80% confluence. During the incubation process, the culture medium was re-

placed with fresh medium every second day. When the cells reached approximately 80% confluence, they were removed from the flasks by trypsinization with 0.25 trypsin-EDTA and then sub-cultured. Later, the number of viable cells was counted by using 0.4 % trypan blue (Sigma, St Louis, MO, USA) staining and a Bio-Rad TC10™ automatic cell counter. After counting, the cell suspension was diluted with the same medium (containing 10% FBS) to 10^8 cells per 75 cm^2 flask before seeding. Then, all the cells were maintained in a humidified incubator containing 5% CO_2 at 37°C for 72 h. All of the preceding procedures were carried out under a sterile laminar flow hood.

Cell Viability Assays: The WST-1(2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) assay and trypan blue dye exclusion method were used to evaluate the inhibitory effects of different pH environments on HepG2 cells.

WST-1 assay: Cell viability was measured spectrophotometrically with WST-1, a cell proliferation reagent (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's recommendations. For this purpose, HepG2 cells were transferred to a 96-well culture plate at a density of 3 x 10^4 cells per well and incubated for 24 h at 37°C under a 5% CO_2 atmosphere. Twenty-four hours later the medium in each well was replaced with a medium adjusted with HCl or NaOH to one of 11 different pH levels ranging from pH 6 to 8.5. The cells in all wells were then incubated under the same conditions for 24 h. After 24 h, 10 µL of WST-1 was added to each well. The contents of the wells were then mixed for two minutes in an orbital shaker (Wise-Stir-MSH-20A). Following that procedure, the plates were incubated in a 5 % CO_2 atmosphere at 37°C for 30 min. Each experiment described here was performed in triplicate. In the final step, to calculate cell viability, the absorbance was measured at a test wavelength of 450 nm and a reference wavelength of 620 nm by using a microplate reader spectrophotometer (Tecan, software Magellan, Switzerland). All experiments were performed in triplicate.

The inhibitory effects of the cellular microenvironment were evaluated according to the formula of Yin et al. (2018), as follows:

$$\text{Proliferation rate} = \frac{\text{Atest-Ablank}}{\text{Acontrol-Ablank}} \quad \text{And percentage viability} = \frac{\text{Atest-Ablank}}{\text{Acontrol-Ablank}} \times 100$$

(Atest: absorbance values of test group, Acontrol: absorbance values of control group (medium at pH 7.2), Ablank: absorbance value of blank sample)

Trypan blue exclusion assay: The trypan blue test was applied according to the manufacturer's instructions. In brief, 3 x 10^5 cells were inoculated into each well of a 6-well plate and incubated for 24 at 37°C under an atmosphere containing 5% CO_2. After 24 h of growth, the culture medium was removed and replaced with the test medium. For the trypan blue exclusion assay, the culture medium was removed from each well after 24 h. The cells were then washed with 1 ml trypsin-EDTA, after which 750 µL trypsin was added per well to remove the cells. The removed cells were then re-suspended in 750 µL of culture medium. The cell suspensions were then diluted 1:1 (v:v) with 0.4% trypan blue and then a 20 µL mixture was transferred to a cell count slide (Bio-Rad 145-0011). Cell counting was performed with an automated cell counter (Bio-RadTC20 Hercules, CA, USA). All experiments were performed in triplicate.

External cell morphology determination: Cells were seeded into 6-well plates at a seeding density of 3×10^5 cells per well in cell growth medium at pH 7.2, and then allowed to acclimate for 24 hrs. The medium was then replaced with a medium at pH 6.6, 6.8, 7.6, or 7.8. The morphological examination of the cells was carried out under a phase contrast inverted microscope (Zeiss-Vert.A1, Oberkochen, Germany) after the 24-hour incubation period.

Statistical analysis: The statistical analyses were performed using SPSS v 21 (SPSS Inc., Chicago, IL, USA). The logarithmic transformation of cell counts for trypan blue test results was performed (with logarithms base 10). The data were tested with the Shapiro Wilk test for normality. Since the data were normally distributed, the means were analyzed with the one-way analysis of variance test (ANOVA), with post hoc Tukey's and Dunnett's tests applied. All data are presented as mean ± standard deviation (SD), and $p<0.05$ was applied as the level of statistical significance. All experiments were replicated at least three times.

Results

WST-1 Cell Viability Assay: The WST-1 method was used to determine the inhibitory effects of pH on

the viability of HepG2 cells. The percentage of viable cells in the medium with pH 7.0 (101.52%) was very similar to that observed for the control group pH 7.2 (100%). However, the inhibitory effects of the media at pH 6.4, 6.2, and 6.0 were significantly higher than the control group ($p<0.05$). In the more alkaline section of the pH spectrum, at pH 7.4 the cell viability increased significantly in comparison to the control group ($p<0.05$), at pH 7.6 there was no difference ($p>0.05$), and at pH's 7.8, 8.0 and 8.5, there was a significant decrease in cell viability ($p<0.05$) (Figure 1). Based on the cell viability results, media at four different pH levels were selected for the trypan blue test for confirmation of the WST-1 test results and to examine cell morphology.

Trypan blue exclusion assay: The inhibitory effects of culture media at pH 6.6, 6.8, 7.6, and 7.8 on the proliferation of HepG2 cells was assessed with a trypan blue exclusion assay and the results are shown in Table 1.

The number of live cells decreased at pH 6.8 but increased at pH 6.6 in comparison to the control group, although neither difference was significant ($p>0.05$). When these results were compared with results from the WST-1 assay, cell viability at pH 6.6 (102.88%) was also higher than at 6.8 (99.71%). In addition, the numbers of live HepG2 cells decreased at both pH 7.6 and pH 7.8, but only at pH 7.8 was the difference significant ($p<0.05$), with cell viabilities of approximately 95% and 65% at pH 7.6 and 7.8, respectively (Figure 1).

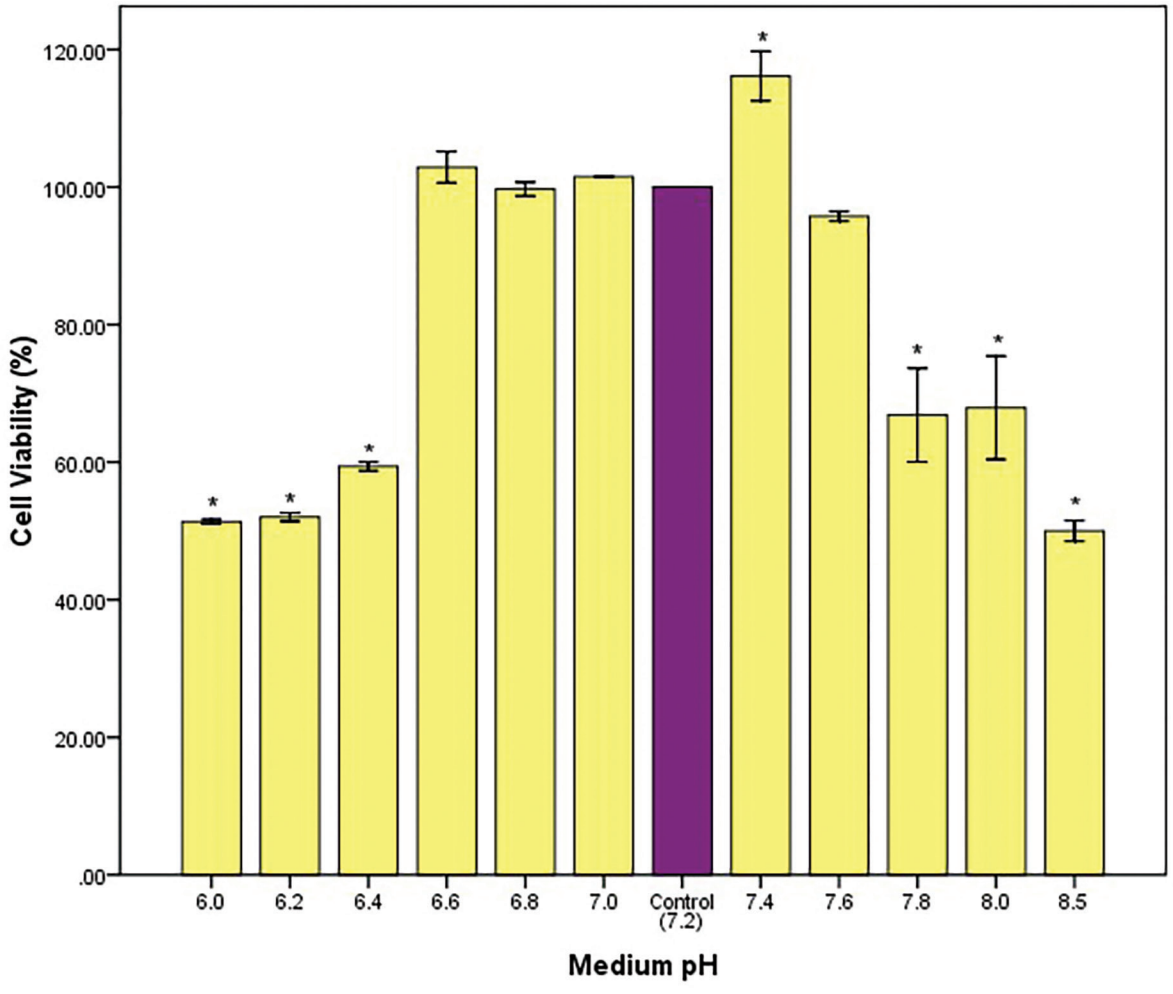


Figure 1. Effects of alteration of the pH level of the culture medium on the viability of HepG2 cells. Data are expressed as mean± SE; *indicates that the mean for the pH is significantly different from the control pH (7.2) ($p<0.05$).

Table 1. HepG2 cell proliferation in culture media at different pH values.

pH	Number of Live Cells/mm³ (logarithms base 10) (Mean ± SD)
6.6	6.29 ± 0.025[a]
6.8	6.25 ± 0.016[a,b]
Control (7.2)	6.27 ± 0.015[a,b]
7.6	6.19 ± 0.024[b]
7.8	6.02 ± 0.061[c]

[a,b,c] The mean number of live cells in the media marked with different superscripts is significantly different ($p<0.05$)

Characterization of the morphology of HepG2 cells cultured in media of different pH: At pH 6.2 and 6.8, the spindle-shaped HepG2 cells formed large islands but at pH 7.6 they formed small patches of round cells. In addition, HepG2 cells grown in the more alkaline pH 7.8 environment were also spherical but had lost their capacity to adhesion (Figure 2).

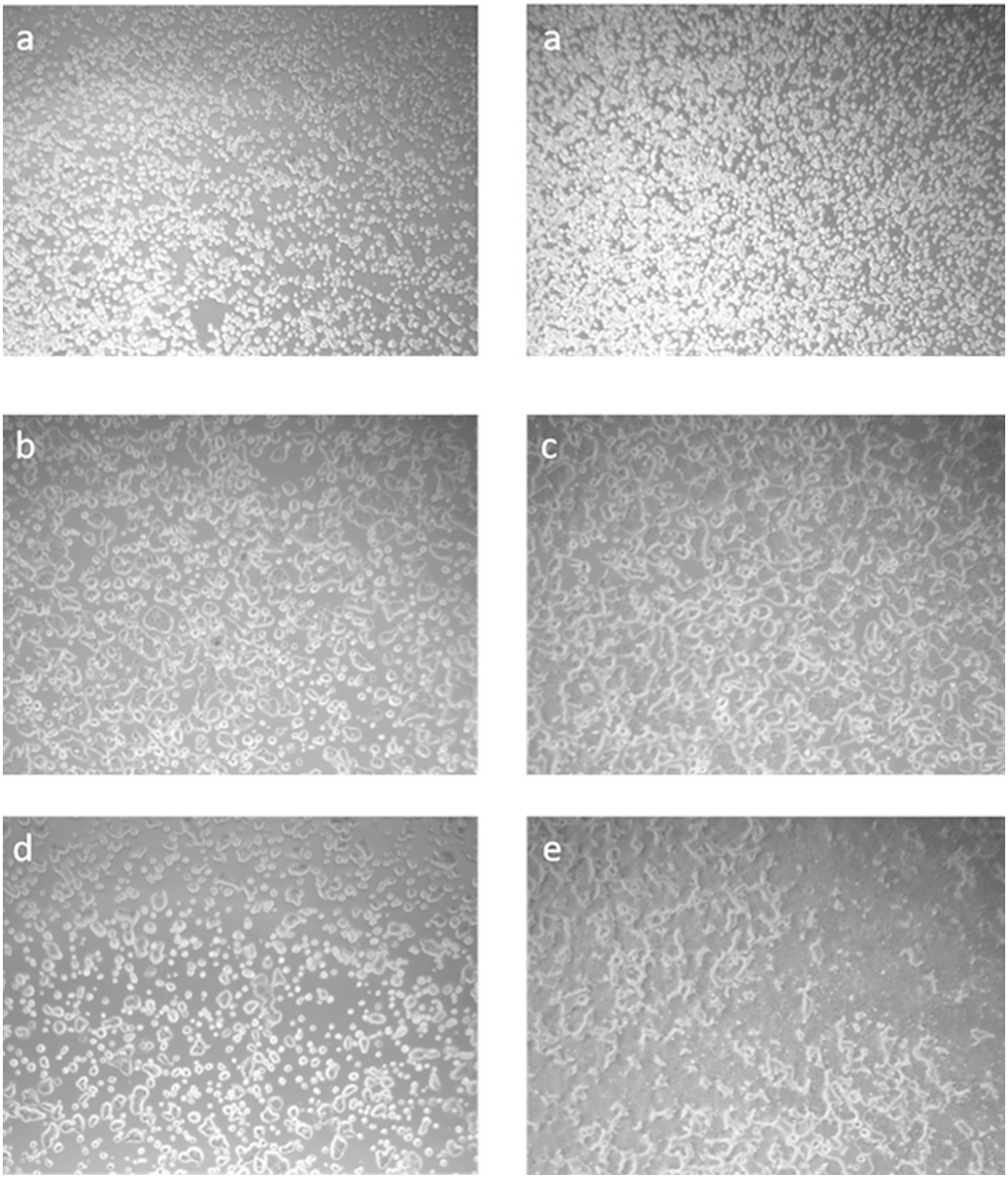


Figure 2. Microscope images at 80 × magnification of HepG2 cells cultured at pHs ranging from 6.6 to 7.8 pH for 24 hrs in a 6-well plate: (a) pH 7.2 (control group), (b) pH 6.6, (c) pH 6.8, (d) pH 7.6, (e) pH 7.8.

Discussion

The Warburg Effect (aerobic glycolysis) is a typical characteristic of cancer cells in that they exhibit abnormal metabolism due to an increase in the rate of glucose uptake and the production of lactate, even in the presence of oxygen, and is considered a distinctive feature of cancers (Silva et al. 2009). The metabolic consequences of this phenomenon are low glucose concentration, high lactate concentration, and lower extracellular pH in the tumor microenvironment. Lower extracellular pH and a hypoxic environment facilitate tumor growth and development and induce metastasis and invasion via multiple pathways. These pathways include point mutation, gene amplifications, inactivation of metastasis suppressor genes resulting from deletions, genomic instability, and overexpression by the genes involved (Rofstad et al. 2006; Swietach et al. 2007). In the clinical environment, the acidic tumor microenvironment reduces the anti-tumor immune response, the effectiveness of chemotherapy and radiotherapy, and ultimately the survival rate after surgical intervention. The tumor microenvironment and metabolic pathways inducing this microenvironment, which are fundamental to the cancer prognosis, mean that *in vivo* and *in vitro* studies on that microenvironment are gaining importance (Lacroix et al. 2018). Approaches that target this acidity, such as the direct neutralization of the acidic microenvironment, the development of acid-activatable drugs and nanoparticles, and the targeting of the metabolic pathways of tumor cells, have been reported (Pillai et al. 2019).

In the present study, the effects of manipulation of the normal acidic environment of HepG2 cells on their viability and proliferation were investigated. In the trypan blue exclusion assay, the number of live cells decreased at pH 6.8 and increased at pH 6.6 in comparison to the control group (pH 7.2), but the differences were not significant ($p>0.05$). Separately, the WST-1 test showed that cell viability in the range from pH 7.0 to 6.6 was close to or higher than in the control group, but the number of viable cells significantly decreased ($p<0.05$) at pH 6.4 and lower, with the proportion of live cells at approximately 60% or lower (Fig. 1, Table 1). It has been reported that the *in vivo* extracellular pH of many tumors ranges from 6.6 to 7.0 (Hao et al. 2018). The results of the WST-1 test in the present study are supportive of these data.

Extracellular acidity leads to a more malignant cell phenotype, the activity of which is mediated by signal proteins, transcription factors, cytokines, invasion-related receptors, and the modulation of the hundreds of genes that stimulate cell proliferation in the early stages of tumor progression (Rofstad et al. 2006; Moellering et al. 2008; Fukamachi et al. 2013). In this study, the metastatic activity and invasiveness of HepG2 cells cultured at pH 6.6 were likely increased by the inactivation of the tumor suppressing cell adhesion molecule, E-cadherine, as reported by Chen et al. (2009). Separately, when HS27 fibroblast cells were cultured in a high lactate (acidic) environment, the level of hyaluronan, which is responsible for rapid tissue growth, increased (Stern et al. 2002). Rofstad et al. (2006) reported that the melanoma cell lines, A-07, D-12 and T-22, when cultured in acidic media, and then injected into mice, increased pulmonary metastasis due to the overexpression of matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9), and cathepsin B and L, which are proteolytic enzymes.

In a holistic sense, the biology of cancers can be seen as the product of an evolutionary and ecological process. All tumor cell phenotypes potentially provide an evolutionary advantage that supports cell proliferation, especially increased glucose metabolism and, consequently, interstitial acidosis. Moreover, local acidosis, which is widely observed in nature, is seen as "niche engineering", a phenomenon by which plants and animals change their environment and consequently gain a competitive advantage that helps ensure their growth and survival (Gatenby et al. 2006; Gatenby and Gillies 2008). Based on the results of our study, the increased proliferation rate of HepG2 cells in acidic environments may be interpreted as the first step of "niche engineering".

In the current study, the proliferation and viability of HepG2 cells in media at pH 6.6, 6.8, and 7.0 did not differ significantly from the control (pH 7.2) ($p>0.05$), which provides indirect support for earlier reports that tumors generally have an acidic extracellular environment that, by inference, is supportive of their growth (Zhang et al. 2010; Chen et al. 2017; Hao et al. 2018). The present study also showed that the proportion of viable HepG2 cells was lower when they were cultured in alkali media, except at pH 7.4, and significantly lower at pHs of 7.8 and higher ($p<0.05$). The significant decrease in both cell viability and proliferation at pH 7.8 (Fig 1, Table 1) in comparison to the control group appears to be a key finding of the present study. These findings could support to create and maintain an alkaline microenvironment around tumor cells in

patients that would make a meaningful difference to their treatment outcomes.

Other evidence for the potential benefits of treatment with alkalization was provided by Trived et al. (2018) who reported that increasingly alkaline pH reduced the viability of the breast cancer cell line, MDA-MB-231. In addition, an extract from the neem tree, *Azadirachta indica*, in combination with alkaline pH treatment, caused a dose-dependent decrease in cell viability *in vitro*. Moreover, several recent preclinical and clinical studies have reported that tumor behavior was altered by the administration of sodium bicarbonate orally or other alkali diet treatment. Also, in a modeling study, orally administered bicarbonate therapy reduced the incidence of metastasis in breast and prostate cancer in immunity-deficient mice (Robey et al. 2009). In another study, patients with non-small cell lung cancer (NSCLC) and epidermal growth factor (EGFR) mutation were treated with an alkaline diet, in addition to the low-dose EGFR inhibitors, gefitinib, erlotinib, and afatinib, at the standard treatment doses. Patients were followed-up long-term and their survival was prolonged in comparison to patient populations receiving the standard treatment. The researchers stated that their results with advanced lung cancer patients constituted clinical evidence that there may be positive outcomes related to this treatment (Hamaguchi et al. 2017). In contrast, the activity of cytokine-induced killer cell's antitumor activity against HepG2 cells was reduced in a medium at pH 6.5 (Izumi et al. 2003). In addition, the intracellular accumulation of various lipophilic anticancer agents has been shown to be controlled by the cellular pH gradient. Therefore, studies on pH sensitive nanoparticles are being conducted for selective cancer chemotherapy (Feng et al. 2017; Xia et al. 2018).

Conclusion

An acidic tumor microenvironment can change the behavior of tumor cells and manifest as proliferation, invasion, and metastasis, and also reduce the immune system response to the tumor and the success of chemotherapy. The present study demonstrated that an alkaline medium can significantly reduce the proliferation and viability of HepG2 cancer cells *in vitro*. Therefore, in addition to the existing treatment strategies, the appropriate alkalization of the microenvironment of tumor cells may increase the probability of successful treatment. To further that objective, preclinical and clinical studies should be conducted into changes in the activity of receptors, signal proteins, transcription factors, cytokines, and cell cycle control genes resulting from the alkalization of the tumor microenvironment.

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Reference

Alfarouk KO, Stock CM, Taylor S, Walsh M, Muddathir AK, Verduzco D, Bashir AH, Mohammed OY, Elhassan GO, Harguindey S, Reshkin SJ, Ibrahim ME, Rauchet C. (2015). Resistance to cancer chemotherapy: failure in drug response from ADME to P-gp. Cancer Cell Int. 15,71 DOI: 10.1186/s12935-015-0221-1.

Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. (2018) Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68, 394-424 DOI: 10.3322/caac.21492.

Chen M, Chen C, Shen Z, Zhang X, Chen Y, Lin F, Ma X, Zhuang C, Mao Y, Gan H, Chen P, Zong X, Wu R. (2017). Extracellular pH is a biomarker enabling detection of breast cancer and liver cancer using CEST MRI. Oncotarget. 8, 45759- 45767 DOI: 10.18632/oncotarget.17404.

Chen Y, Chen CH, Tung PY, Huang SH, Wang SM. (2009). An acidic extracellular pH disrupts adherens junctions in HepG2 cells by Src kinases-dependent modification of E-cadherin. J Cell Biochem,851-859

DeBerardinis RJ, Sayed N, Ditsworth D, Thompson CB. (2008). Brick by brick: metabolism and tumor cell growth. Curr Opin Genet Dev. 18, 54-61 DOI: 10.1016/j.gde.2008.02.003

Estrella V, Chen T, Lloyd M, Wojtkowiak J, Cornnell HH, Ibrahim-Hashim A, Bailey K, Balagurunathan Y, Rothberg JM, Sloane BF, Johnson J, Gatenby RA, Gillies RJ et al. (2013). Acidity generated by the tumor microenvironment drives local invasion. Cancer Res. 73, 1524-1535 DOI: 10.1016/j.gde.2008.02.003

Feng Q, Shen Y, Fu Y, Muroski ME, Zhang P, Wang Q, Xu C, Lesniak MS, Li G, Cheng Y.(2017). Self-assembly of gold nanoparticles shows microenvironment-mediated dynamic switching and enhanced brain tumor targeting. Theranostics, 1875-1889 DOI: 10.7150/thno.18985.

Fukamachi T, Ikeda S, Wang X, Saito H, Tagawa M, Kobayashi H. (2013). Gene expressions for signal transduction under acidic conditions. Genes , 65-85

Gatenby RA, Gillies RJ. (2008). A microenvironmental model of carcinogenesis. Nat Rev Cancer, 56-61

Gatenby RA, Gillies RJ.(2004). Why do cancers have high aerobic glycolysis? Nat Rev Cancer 4, 891-899

Gatenby RA, Gawlinski ET, Gmitro AF, Kaylor B, Gillies RJ.(2006). Acid-mediated tumor invasion: a multidisciplinary study. Cancer Res, 5216-5223

Hamaguchi R, Okamoto T, Sato M, Hasegawa M, Wada H. (2017). Effects of an alkaline diet on EGFR-TKI therapy in EGFR mutation-positive NSCLC. Anticancer Res, 37, 5141-5145

Hao G , Xu ZP, Li L. (2018). Manipulating extracellular tumour pH: an effective target for cancer therapy. RSC Adv. 8, 22182-22192

Hsu PP, Sabatini DM. (2018). Cancer cell metabolism: Warburg and beyond. Cell 134, 703-707

Ibrahim-Hashim A, Cornell HH, Abrahams D, Lloyd M, Bui M, Gillies RJ, Gatenby RA. (2012). Systemic buffers inhibit carcinogenesis in TRAMP mice. J Urol, 188, 624-631

Izumi H, Torigoe T, Ishiguchi H, Uramoto H, Yoshida Y, Tanabe M, Ise T, Murakami T, Yoshida T, Nomoto M, Kohno K. (2003). Cellular pH regulators: potentially promising molecular targets for cancer chemotherapy. Cancer Treat Rev, 541-549

Kato Y,Ozawa S, Miyamoto C, Maehata Y, Suzuki A, Maeda T, Baba Y. (2013). Acidic extracellular microenvironment and cancer. Cancer Cell Int, 13, 89

Lacroix R, Rozeman EA, Kreutz M, Renner K, Blank CU. (2018). Targeting tumor-associated acidity in cancer immunotherapy. Cancer Immunol Immunother. 67, 1331-1348

Lodish H, , Berk A, Kaiser CA, Paul M. (2016) Molecular Cell Biology. W. H. Freeman, 5th edition, New York

Moellering RE, Black KC, Krishnamurty C, Baggett BK, Stafford P, Rain M, Gatenby RA, Gillies RJ. (2008). Acid treatment of melanoma cells selects for invasive phenotypes. Clin Exp Metastasis, 411-425

Neri D, Supuran CT. (2011). Interfering with pH regulation in tumours as a therapeutic strategy. Nat Rev Drug Discov. 10, 767-777

Park H, Lyons JC, Ohtsubo T, Song CW. (2000). Cell cycle progression and apoptosis after irradiation in an acidic environment. Cell Death Differ, 7, 729-738

Park HJ, Lee SH, Chung H, Rhee YH, Lim BU, Ha SW, Griffin RJ, Lee HS, Song CW, Choiet EK. (2003). Influence of environmental pH on G2-phase arrest caused by ionizing radiation. Radiat Res. 159, 86-93 DOI: 10.1667/0033-7587(2003)159[0086:ioepog]2.0.co;2.

Pillai SR, Damaghi M, Marunaka Y, Spugnini EP, Fais S, Gillies RJ. (2019). Causes, consequences, and therapy of tumors acidosis. Cancer Metastasis Rev. 38, 205-222 DOI: 10.1007/s10555-019-09792-7.

Raghunand N, Gillies RJ.(2000). pH and drug resistance in tumors. Drug Resist Upda,t 3, 39-47

Robey IF, Baggett BK, Kirkpatrick ND, Roe DJ, Dosescu J, Sloane BF, Ibrahim Hashim A, Morse DL, Raghunand N, Gatenby RA, Gillies RJ. (2009). Bicarbonate increases tumor pH and inhibits spontaneous metastases. Cancer Res. 69, 2260-2268 DOI: 10.1158/0008-5472.CAN-07-5575.

Rodríguez-Hernández MA, González R, de la Rosa ÁJ, Mauriz JL, Padillo FJ, Muntane J. (2018). Molecular characterization of autophagic and apoptotic signaling induced by Sorafenib in liver cancer cells: In vitro and in vivo studies. J Hepatol 68, 670-671 DOI:https://doi.org/10.1016/S0168-8278(18)31599-X

Rofstad EK, Mathiesen B, Kindem K, Galappathi K. (2006). Acidic extracellular pH promotes experimental metastasis of human melanoma cells in athymic nude mice. Cancer Res. 66, 6699-6707 DOI: 10.1158/0008-5472.CAN-06-0983.

Ruddon RW. (2007). Characteristics of Human Cancer. In: Cancer Biology, 4th Edition. Oxford University Press Inc, New York. p. 3-16

Schwartz L, Seyfried T, Alfarouk KO, Moreira JV, Fais S. (2017). Out of Warburg effect: an effective cancer treatment targeting the tumor specific metabolism and dysregulated pH. Semin Cancer Biol, 43, 134-138 DOI: 10.1016/j.semcancer.2017.01.005.

Silva AS, Yunes JA, Gillies RJ, Gatenby RA. (2009). The potential role of systemic buffers in reducing intratumoral extracellular pH and acid-mediated invasion. Cancer Res. 69, 2677-2684 DOI: 10.1158/0008-5472.CAN-08-2394

Stern R, Shuster S, Neudecker B, Formby B. (2002). Lactate stimulates fibroblast expression of hyaluronan and CD44: the Warburg effect revisited. Exp Cell Res, 24-31.

Swietach P, Vaughan-Jones RD, Harris AL. (2007). Regulation of tumor pH and the role of carbonic anhydrase 9. Cancer Metastasis Rev. 26, 299-310

Tian XP, Wang CY, Jin XH, Li M,Wang FW, Huang WJ, Yun JP,Xu RH, Cai QQ,Xie D. (2019) Acidic Microenvironment Up-Regulates Exosomal miR-21 and miR-10b in Early-Stage Hepatocellular Carcinoma to Promote Cancer Cell Proliferation and Metastasis. Theranostics. 9, 1965-1979

Torre LA, Siegel RL, Ward EM, Jemal A. (2016). Global cancer incidence and mortality rates and trends—an update. Cancer Epidemiol Biomarkers Prev 25, 16-27 DOI: 10.1158/1055-9965.EPI-15-0578.

Trivedi A, Ahmad R, Misra A.(2018). Effect of alkaline pH on cytotoxicity profile of neem (Azadirachta indica) ethanolic extract against human breast cancer cell line MDA-MB-231. Eur J Integr Med, 1-7

Tuccitto A. (2018). pH regulatory molecules in the tumour microenvironment: modulators of aggressiveness and immune profile of human hepatocellular carcinoma. The Open University, Faculty of Science Technology Engineering and Mathematics (STEM), PhD thesis, Milton Keynes (UK)

Xia F, Hou W, Zhang C, Zhi X, Cheng J, Fuente JM, Song J, Cui D. (2018). pH-responsive gold nanoclusters-based nanoprobes for lung cancer targeted near-infrared fluorescence imaging and chemo-photodynamic therapy. Acta Biomater, 308-319 DOI: 10.1016/j.actbio.2017.12.034.

Yin P, Yang L, Xue Q, Yu M, Yao F, Sun L, Liu Y. (2018). Identification and inhibitory activities of ellagic acid- and kaempferol-derivatives from Mongolian oak cups against α-glucosidase, α-amylase and protein glycation linked to type II diabetes and its complications and their influence on HepG2 cells' viability. Arab J Chem, 11, 1247-1259

Zhang X, Lin Y, Gillies RJ.(2010). Tumor pH and its measurement. J Nucl Med. 51, 1167-1170, DOI: 10.2967/jnumed.109.068981.

Zhang Y, Zhang T, Wu C, Xia Q, Xu D. (2017). ASIC1a mediates the drug resistance of human hepatocellular carcinoma via the Ca 2+/PI3-kinase/AKT signaling pathway. Lab Invest. 97, 53-69 DOI: 10.1038/labinvest.2016.127