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| **Authors:** | Zeynep AKBULUT<br>Ayşe Ozge KAVAKLI<br>Ayca KARAGOZ KOROGLU<br>Hatice MARAS<br>Ranan Gülhan AKTAS |

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# THE EFFECTS OF TEMPERATURE AND CARBONDIOXIDE CHANGES RELATED TO TYPE I COLLAGEN PRESENCE ON HEPATOCELLULAR CARCINOMA CELLS

**Zeynep AKBULUT**[1]
**Ayşe Ozge KAVAKLI**[2]
**Ayca KARAGOZ KOROGLU**[3]
**Hatice MARAS**[4]
**Ranan Gülhan AKTAS**[5]

**ABSTRACT:**
*THE EARLY PROGNOSIS OF HEPATOCELLULAR CARCINOMA (HCC) IS EXTREMELY DIFFICULT AND TREATMENT OPTIONS ARE STILL VERY LIMITED. THE STUDY AIMS TO EXAMINE THE EFFECTS OF TEMPERATURE AND CARBONDIOXIDE CHANGES RELATED TO THE PRESENCE OF TYPE I COLLAGEN, ONE OF THE MOST COMMON COMPONENTS OF THE EXTRACELLULAR MATRIX. TWO-DIMENSIONAL HEPG2 CELL CULTURES WERE MAINTAINED IN BOTH CLASSICAL CULTURE CONDITIONS AND TYPE I COLLAGEN-COATED CULTURE VESSELS AT +4C°, +24C°, AND +37C°. A COMPARATIVE EXAMINATION OF PHENOTYPIC CHANGES WAS PERFORMED AFTER 24, 48, 72, 96, AND 120 HOURS. THE CELLS THAT ARE IN THE INCUBATORS AT 37C° WITH 5% $CO_2$ DEMONSTRATED NORMAL GROWTH PATTERN. ALBUMIN EXPRESSION WAS HIGHER IN THE CELLS ON TYPE I COLLAGEN COATED SURFACES. CARBONDIOXIDE DEPLETION CAUSED MORPHOLOGICAL CHANGES AND A GRADUAL DECREASE IN VIABILITY. THE CANCER CELLS ON TYPE I COLLAGEN-COATED SURFACES PRESENTED SIGNIFICANT PHENOTYPIC CHANGES IN ALL GROUPS. THE RESULTS OF THE STUDY SUGGEST THAT THE CELLS ON TYPE I COLLAGEN-COATED SURFACES WERE MORE RESISTANT TO THE TEMPERATURE AND THE CARBONDIOXIDE CHANGES IN THE MICROENVIRONMENT. FURTHER STUDIES RELATED TO THE MECHANISM(S) THAT LEADS TO THIS REZISTANCE IN CANCER CELLS MIGHT BE INSIGHTFUL FOR THE DEVELOPMENT OF NEW THERAPEUTICS.*

**KEYWORDS:** CANCER, EXTRACELLULAR MATRIX, HEPG2, COLLAGEN TYPE I

[1] Ph.D. Maltepe UniversityCancer and Stem Cell Research Center (MUKKAM), Turkey zeynep.akbulut@maltepe.edu.tr
*Corresponding Author: Ph.D. Zeynep Akbulut
[2] Student, Maltepe University Faculty of Medicine, Turkey, kavakliozge@gmail.com
[3] Research Assistant, Istinye University Faculty of Medicine, Histology and Embryology Department, Turkey, ayca.koroglu@istinye.edu.tr
[4]M.Sc.Maltepe University Cancer and Stem Cell Research Center (MUKKAM), Turkey, haticemaras@maltepe.edu.tr.
[5] Prof. Dr. Maltepe University Faculty of Medicine, Embryology and Histology Department, Cancer and Stem Cell Research Center (MUKKAM), Turkey, ranan.aktas@maltepe.edu.tr

## INTRODUCTION

Hepatocellular Carcinoma (HCC) is the fifth common cancer type between the men, while it is 3rd common cancer cause between women. One of the most common causes of deaths from cancers is liver cancer[6]. HepG2 cells are derived from a patient who has been diagnosed as hepatocellular carcinoma. Those cells mimic many functional features of normal hepatocytes. Therefore, they are commonly used for studies related to healthy liver tissue as well as cancer researches. HepG2 cells present advantages to the scientists since they have differentiation capacity and also they proliferate in many different in vitro conditions[7]. In vitro cell, culture conditions are designed to mimic in vivo conditions in the human body. Incubators are the appliances that provide body temperature, humidity, and 5-6% carbondioxide for the cells. Differences at those cell culture conditions create stress for the cells and this end swith the production of reactive oxygenspecies (ROS) and induction of oxidative stress. Cell death or abnormal cell functionality might be results of this stress on the cells[8]. Hepatocytes live in a mildly hypoxic environment in the liver because of the porto-venous circulation. It has been found that the semildhypoxicconditions have rotective effects on hepatocytes. However, reports are stating the harmful effects of low oxygen in culture conditions[9].

Optimum microenvironment conditions are crucial for the maintenance of normal cell functions. Both normal cells and cancer cells are being affected by the microenvironmental conditions The extra cellular matrixis one of the most important components of this microenvironment[10]. Collagen is the major content of the extracellular matrix. When hepatocytes cultured either on Type I collagen or on other basement membrane extracts, cells start to differentiate and express more liver-specific genes. In contrast, the hepatocytes that are cultured on plastic surfaces. Express less hepatic specific genes and demonstrate different phenotypic pattern[11]. HCC cases demonstrate more Type I collagen when compared with normal liver tissue. Additionally, it has been shown that cell proliferation rate under the effect of Type I collagen is higher when it was compared with the effects of Type 4 collagen or fibronectin[12].

Microenvironment and cancer relationship is one of the hot topics for recent studies. The studies related to the effects of different components of microenvironment on cancer cells present promising results about the therapy and prognosis. Another group of scientists is being focused on the differentiation of cancer cells.

Collagen also stimulates differentiation potential on the cells. However, studies investigating the response of different cancer cells to the changes in heat and carbon dioxide

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[6] Burley, Michelle, R.; Roth, Charles, M. *Effects of Retinoik Acid on Proliferation and Differention of HepG2 Cells*, The Open Biotechnology Journal:2017, p. 47

[7] Brueckl, Corrina at al. *Hyperoxia-induced reactive oxygen species formation in pulmonary capillary endothelial cells in situ*, Am. J. Respir. Cell Mol. Biol:2006, p.453

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[12] Lillegard, Joseph, B. et al. *Normal Atmospheric Oxygen Tension and the Use of Antioxidans Improve Hepatocyte Spheroid Viability and Function*, J. Cell Physiol: 2011, p. 2987

in the microenvironment are limited. No study in the literature shows how cancer cells, which enter the differentiation process in both classical culture and collagen-coated environments, will be affected by the heat change and carbon dioxide deprivation.

In this study, we aim to examine how HepG2 cells will be affected by heat and carbondioxide changes in the microenvironmentwhere differentiation with collagen is stimulated, the effects of microenvironment change on cell proliferation, cell death.

## MATERIAL AND METHODS

### Cell culture and study design

The human hepatocellular carcinoma (HepG2, ATCC, USA) cell line was maintained in Dulbecco's modified Eagle's medium (DMEM; Pan Biotech, Germany) supplemented with L-glutamine, 10% (v/v) heat-inactivated fetal bovine serum (FBS; Pan Biotech, Germany) and 100 IU/ml penicillin and 100 µg/ml streptomycin (Pan Biotech, Germany) at 37C° in ahumidified atmosphere of 5% $CO_2$ in a Panasonic incubator.The cells were seeded into either un-coated or Type I collagen-coated in vitro culture plates. The cells were plated for at a density of 115,000 / $cm^2$ for all experiments for 12 well plates and 400,000/ $cm^2$ for 6 well plates. After 24 hours cultivation cells were performed within different culture conditions: (i) classical culture (control group-37C°-5% $CO_2$), (ii) cold condition (+4C°), (iii) room temperature (24C°), (iv) 37C° but without $CO_2$. Both culture cells were performed within five different periods; 24th, 48th, 72nd, 96th, and 120th hour.

### Assessment of Morphological Changes

A- Live-cell images were recorded under an inverted phase microscope (Primovert, Zeiss) for the mutual evaluation of microenvironment temperature and $CO_2$ changes for the death and morphology of HepG2 cells at mentioned above time.

B- H&E staining: HepG2 cells were seeded on coverslips in 12 well plates in eight different microenvironments. Then the cells were fixed with 4% formaldehyde (room temperature, 20min.) and rinsed three times in phosphate-buffered saline H&E staining was performed at a specifiedtime. Photographs were captured under a Zeiss Primovert light microscope (Jena, Germany). Nucleus structure, acidic, and basic changes of the cytoplasm of cells were evaluated after this staining.

C- Cell counting: HepG2 cells were trypsinized, washed twice in PBS (w/o Ca and Mg), and centrifuged by pipetting. After centrifugation, removed the supernatant and the dilution rate was determined and cell counts were performed under the light microscope (Zeiss) on the Thoma slide.

### Analysis of immunohistochemical

Coverslips were autoclaved and placed into the wells of a 12-well plate. HepG2 cells were grown on either non-treated (un-coated) or Type I collagen-coated coverslips in the different microenvironments. At the specified time, the cells were washed twice with PBS and fixed in Kwik-Diff™ Reagent 1, Fixative (Thermo Fisher Scientific, USA) for 20 min. Fixed cells were washed three times with PBS, blocked in 2% bovine serum albumin for 10min at RT, and immunostained with primary albumin or alfa-fetoprotein mouse monoclonal antibodies (Biocare Medical, USA) overnight at 4C°. Cells were washed three times and probed with secondary goat anti-mouse IgG (H+L) antibody DyLight® 488 conjugate (Thermo Fisher Scientific) for 1 h at 37C°. The cells were counter-stained with Hoechst 33258 and observed under the Zeiss LSM 700 confocal laser scanning microscope (Germany).

**Statistical Analysis**

Cell counting data are expressed as mean ± standard error of the means (SEM). Statistical evaluation was performed by a two-way ANOVA test using GraphPad Prism V.8.01 and Sidak's multiple comparisons or Dunnett's multiple comparisons tests were carried out to find groups whose mean differences were significant.

**RESULT**

**The effect of heat and carbon dioxide changes on cell death**

The number of cells which are un-coated (classical) or with Type I collagen-coated in normal culture conditions (37C° and 5%$CO_2$), increased as the culture time progressed. The number of HepG2 cells in the Type I collagen-coated environment increased significantly compared to the un-coated culture medium at the end of the 120th hour of the culture period ($p<0,005$). Also,the number of HepG2 cells in the un-coated culture medium increased significantly at 120th hour compared to un-coated culture cells after 24th hour ($p<0.01$) (Figure 1a).

The lack of $CO_2$ did not affect HepG2 cells with Type I collagen-coated in 37C°, whereas the cells in classical culture were unable to resist the $CO_2$ shortage and died. While it was seen microscopically that the cells in the classical culture decreased significantly after 96th hour, there was no statistically significant difference between the cell numbers ($p>0.05$) (Figure 1b).

In 24C° and absent of $CO_2$, the effect of heat exchange on HepG2 cells was found to be greater than that of $CO_2$. Accordingly, after 96 hours, it was demonstrated that all cells died in both the culture environment. Also, when we compared the results for up to 72 hours, it was seen that the cells in the collagen environment are more resistant and continue proliferation (Figure 1c).

When we look at the culture group at +4C°, while no living cells were in classical culture whereas the number of cells with Type I collagen-coated environment decreased approximately ten-fold at the end of the 24th hour ($p<0.001$). Also, there were not demonstrate any alive cells in other culture periods (Figure 1d).

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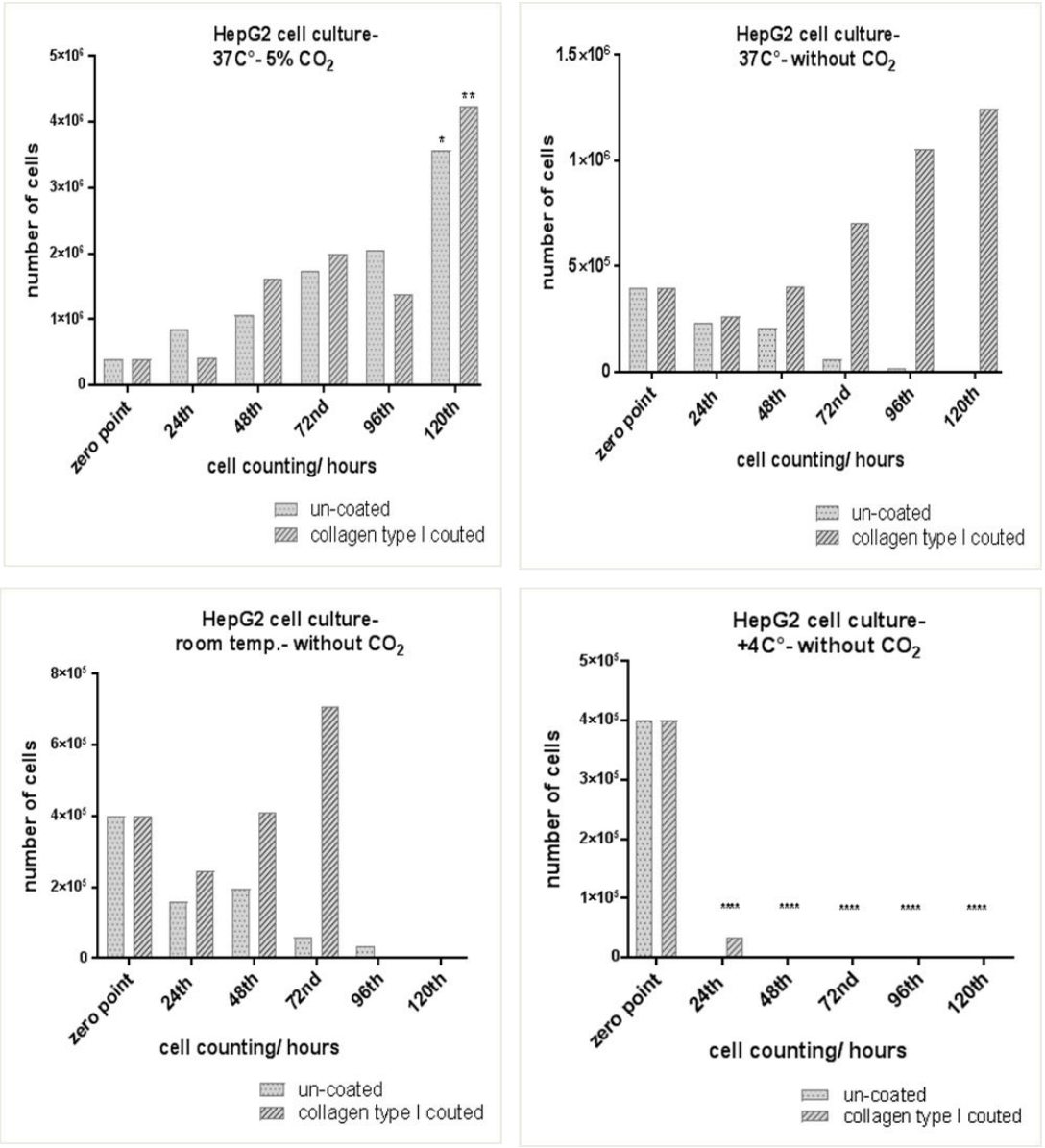


*Figure 1: The Effect of microenvironment temperature and carbondioxide changes on cell death a) comparison of un-coated (control) and collagen type I coated cell counts under normal culture conditions (37C°- 5% $CO_2$), b) comparison of cell counts under 37C° and without $CO_2$ culture condition, c) comparison of cell counts under room temperature and without $CO_2$ culture conditions, d) comparison of cell counts +4C° and without $CO_2$ culture conditions. * p<0.01, **p<0.005, ****p<0.0001.*

**Morphological Analysis**

When comparing alive cell images under an inverted microscope, it was observed that the morphology of HepG2 cells cultured in Type I collagen changes towards a spindle structure and the cells approach each other with these extensions (Figure 2a,b). According to H&E staining; cell structure deterioration was observed due to heat exchange in both un-coated and Type I collagen-coated culture environments. HepG2 cells were not visualized after 24 hours in both culture groups cultured at +4C°. And also disorders of the morphological structure of the cells monitored at room temperature were visualized under an inverted microscope (Figure 2c).

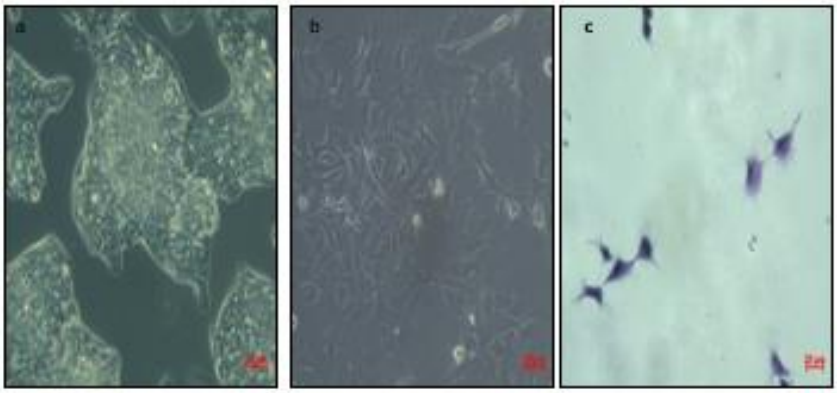

*Figure 2: Morphological analysis; a) image of HepG2 cells in un-coated culture under inverted microscope (20X), b) image of HepG2 cells in collagen type I coated under inverted microscop (20X), c) H&E staining of HepG2 cells in collagen type I coated culture in RT and without $CO_2$ after 24 hours seeding (40X).*

**Immunofluorescence staining**

**Expression of albumin**

At the microscopic level, labeling of the cells with the specific immunofluorescent antibody demonstrated the upregulation of albumin in the presence of Type I collagen at 24th hour but a decrease in un-coated culture environment in 37C° with 5% $CO_2$. A significant increase in expression was also found in both un-coated and Type I collagen-coated culture at 48th hour, 72nd, 96th, and 120th hour (Figure 3a). Cells have been observed to have a normal morphological structure.

In the groups followed in the 37C° and $CO_2$free culture environment, an increase in albumin expression was observed at the 72nd hour in the Type I collagen-coated group, while a decrease was observed in the following hours (96th and 120th hours). A significant disruption in cell structures, shrinkage, and decrease in viability was observed at 120th hour (Figure 3b).

In the groups followed in the RT and without $CO_2$ environment, morphological structure degradation of the cells depending on the time, dispersion in the cytoskeleton, as well as the decrease in viability and albumin expression decreased compared to other environmental conditions (Figure 3c).

Since cells could not be detected in cell counting at +4C° and H&E staining, no immunohistochemical analysis was performed on this group of cells.

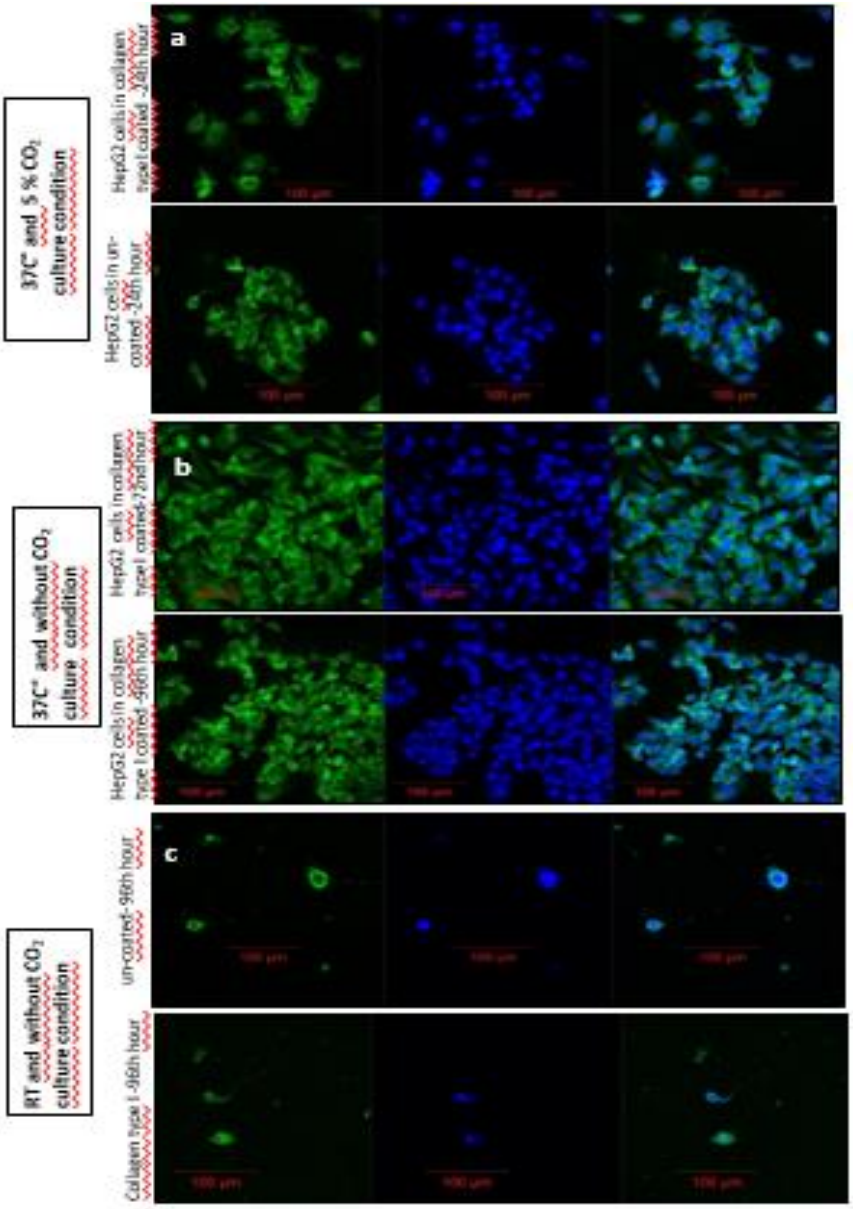


*Figure 3: Confocal microscopic images of HepG2 cells labeled with the albumin antibody. DyLight® 488 was used as the secondary antibody for the groups; a) un-coated and collagen type I coated culture conditions in 37C° and 5% $CO_2$ after 24th hour, b) collagen type I coated culture condition in 37C° and without $CO_2$ after 72nd and 96th hours, respectively, c) un-coated and collagen type I coated culture conditions in RT and without $CO_2$ after 96th hour. Nuclei were counter stained blue with Hoechst 33258. Images merged (right).*

**Expression of alfa-fetoprotein (AFP)**

At the microscopic level, labeling of the cells with the specific immunofluorescent antibody demonstrated the upregulation of AFP in the presence of Type I collagen at 24th hour but a decrease in un-coated culture environment in 37°C with 5% $CO_2$. But after 24th hour, there was a significant increase in both un-coated and Type I collagen-coated culture at the other analysis periods (Figure 4a). Cells have been observed to have a normal morphological structure.

In the groups followed in the 37C° and without $CO_2$ culture environment, morphological structures of the cells deteriorated depending on the time, viability decreased

and there was a tendency to decrease in AFP expression compared to the group preserved in the 37C° and with 5% $CO_2$. A significant disruption in cell structures, shrinkage was observed at the 120th hour. Significantly, the cells with Type I collagen-coated culture are more resistant to different environmental conditions compared to the cells in the classical culture medium, the disruptions in the cell structures are less and the vitality is higher (Figure 4b).

Depending on the time, the morphological structure of the cells maintained at RT and without the $CO_2$ environment, the disruption in the cytoskeleton, as well as the liveliness and AFP expression, decreased compared to the other groups (Figure 4c).

Since cells could not be detected in cell counting at +4C° and H&E staining, no immunohistochemical analysis was performed on this group of cells.

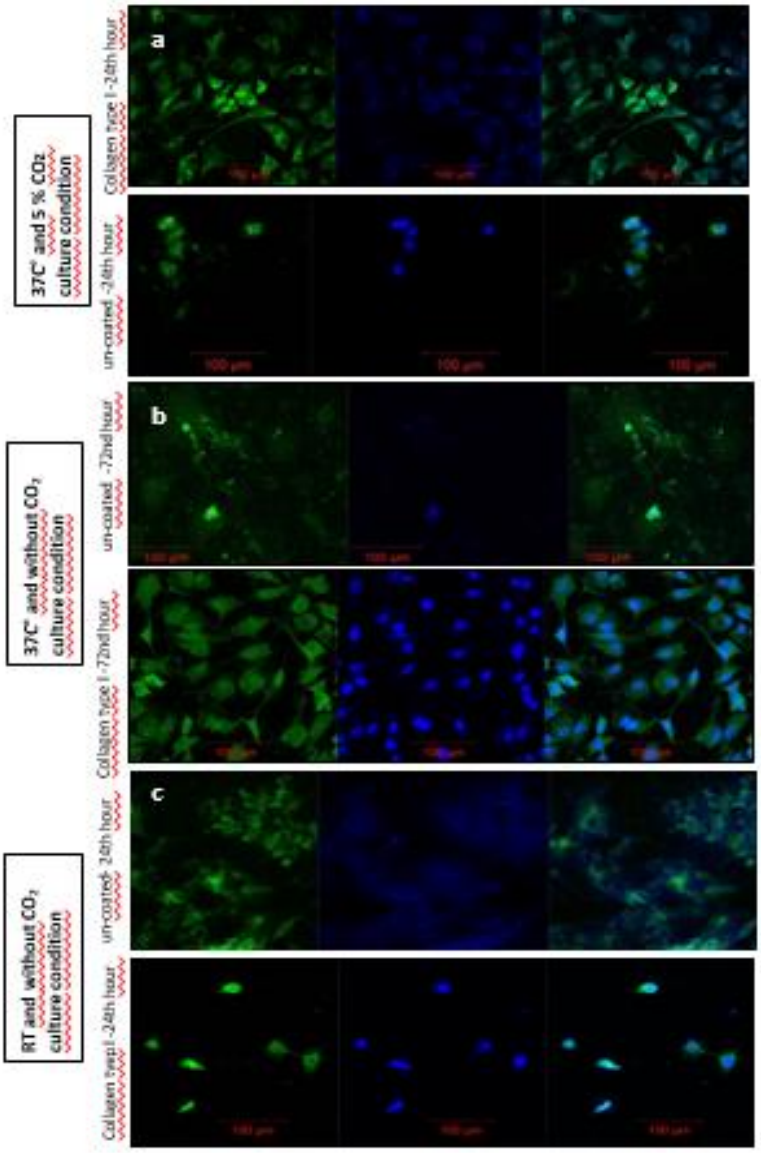


*Figure 4: Confocal microscopic images of HepG2 cells labeled with the AFP antibody. DyLight® 488 was used as the secondary antibody for the groups a) un-coated and collagen type I coated culture conditions in 37C° and 5% $CO_2$ after 24th hour, b) un-coated and collagen type I coated culture condition in 37C° and without $CO_2$ after 72nd hour, c) un-coated and collagen type I coated culture conditions in RT and without $CO_2$ after 24th hour. Nuclei were counterstained blue with Hoechst 33258. Images merged (right).*

## DISCUSSION

The tumor microenvironment is formed of different cell types together with soluble proteins and the extracellular matrix (ECM). The physical and biochemical properties of the tumor-associated ECM are essentially different from that of the normal tissue stroma, e.g. altered ECM composition, increased collagen cross-linking and further structural and rigiditychanges of the ECM[13].

The microenvironment is very important for both normal and cancerous tissue. Changes in the composition or architecture of ECM have been shown to promote tumor growth, angiogenesis, and metastatic progression[14]. Collagen as the most dominant component of the in vivo ECM is likewise used to mimic the ECM in vitro. Type I collagen is also very important among liver extracellular matrix components[15].

In our study, it was observed that type I collagen makes liver cancer cells more resistant to stress environments created by heat and carbon dioxide changes. It is noteworthy that HepG2 cells in a Type I collagen-coated culture are more resistant to different environmental conditions than cells in a classical culture medium, there is less disruption in cell structures and greater viability. This suggests that the presence of Type I collagen in the experimental environment increases cell strength against microenvironment change.

Zheng et al. demonstrated that the growth rates of HepG2 in Type I collagen, coated culture medium were higher than that of Type I collagen and fibronectin-coated culture media, as a result of molecular analysis, they triggered the type I collagen by regulating the integrin β1 / FAK pathway[16]. Furthermore it has been reported that breast cancer cells shift their functional metabolic activity in the response of collagen matrix density[17]. Another study, Zhang et al. demonstrated that Type I collagen could enhance the aggressive progression of residual HCC cells after suboptimal heat treatment[18]. Although mammals are very sensitive to hypothermia, in vitro studies that are often exposed to non-optimal temperatures over time lead to the formation of significantly cold-tolerant lines, and the optimal growth of mammalian cells occurs at 35-37C°, culture cells can continue to grow and divide at 25-33C°[19]. Hunt et al. expose various mammalian cell lines to an experimental setup that lasts for days and weeks at 4-24C° temperature intervals and then follows the normal culture temperature (37C°) that living cells continue to divide, including gene expression. They found that they became suitable for routine tests[20].

Mammalian cells remain at 37C° body temperature, most likely resulting in very different molecular adaptations that affect many aspects of cellular activity. However,

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[13] Moghe, Prabhas, V. et al. *Culture Matrix Configuration and Composition in the Maintanence of Hepatocyte Polarity and Function*, Biomaterials: 1996, p.3.

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[15] Morris, Brett, A. et al. *Collagen Matrix Density Drives the Metabolic Shift in Breast Cancer Cells*, EBioMedicine: 2016, p.1

[16] Zheng, Xinglong, et al. *Collagen 1 Promotes Hepatocellular Carcinoma Cell Proliferation By Regulating Integrin B1/FAK Signaling Pathway in Nonalcoholic Fatty Liver*, Oncotarge: 2017, p. 95586

[17] Morris, Brett, A. et al. *Collagen Matrix Density Drives the Metabolic Shift in Breast Cancer Cells*, EBioMedicine: 2016, p.1

[18] Zhang, Rui, et al.. *Extracellular matrix collagen I promotes the tumor progression of residual hepatocellular carcinoma after heat treatment*, BMC Cancer: 2018, p.1

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[20] Hunt, Lisa, et al. *Low-Temperature Pausing of Cultivated Mammalian Cells*, Biotechnology and Bioengıneerıng: 2004, p.157

periodic hypothermic exposure to mammalian culture cells may result in cold-tolerant cell lines. In in-vitro studies, when mammalian cells are exposed to cold, the ATP rate decreases, and membrane permeability increases. Mild hypothermia (25–33C°) reduces the rate of progression throughout the cell cycle, while moderate (16-20C°) or severe hypothermia (4-10C°) can block the cell cycle in the G2 phase or at the G1 / S limit, respectively[21].

In our study, when the stress caused by heat change and $CO_2$ change in cancer cells is compared, the effect of cells on heat change can be explained by the effect of hypothermia on cell functions. Cold-induced stress can trigger apoptosis, but this may depend on cell type and duration and variety of hypothermic conditions[22].

When we look at the effect of carbon dioxide deprivation on HepG2 cell cultures, it was seen that the $CO_2$ change in the environment where Type I collagen does not create significant stress on the liver cancer cells and the cells can continue to multiply, but the cells die due to this stress in the classical culture environment. This indicates that Type I collagen is effective in the resistance of cells to environmental stress in the cancer cell line. Molecular oxygen is one of the most important variables in modern cell culture systems. Fluctuations in concentration may affect cell growth, differentiation, signaling, and free radical production. To maintain culture viability, experimental validity, and reproducibility, oxygen levels must be constantly kept within physiological "normoxic" limits. The first variable that affects the oxygen concentration is atmospheric (gas) oxygen in cell culture. The gaseous oxygen concentration in cell culture is usually stated as 21%. Although it represents the volume of oxygen in 21% dry air, conventional cell culture incubators require additional volumes of both water vapor and carbon dioxide gas. herefore, the total oxygen concentration should be reduced to allow the presence of additional gases[23]. The most important factor affecting dissolved oxygen concentration in cell culture is temperature. As the environment cools, oxygen solubility increases significantly. As the temperature passes from 37C° to 5C°, the equilibrium concentration of dissolved oxygen in the culture medium almost doubles[24]. Theoretically, the increased oxygen capacity of the cold environment exposes the cells to high oxygen levels[25].

Collective cell migration is an important mechanism for both normal epithelial development and cancer invasion. During this cell migration, cells move in coordinated and maintain cell-cell adhesion. Nguyen-Ngoc et al. used basement membrane gels to model the normal microenvironment and Type I collagen to model the stromal ECM. Their results showed that breaks in the basement membrane could stimulate invasion and spreading via the resulting direct contact between cancer cells and Type I collagen[26].

In our study, the transformation towards a spindle structure was observed in the morphology of HepG2 cells in the collagen environment. This suggests that Type I collagen

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[21] Rieder, Conly, L.; Cole, Richard, W. *Cold- Shock and theMammalian Cell Cycle*, Cell Cycle: 2002, p. 169

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is effective on the differentiation of cell morphologies and this differentiation may also have a role in the interaction between cells and supports similar studies.

Roobol et al. investigated the effects of hypothermia on the cytoskeleton, protein synthesis and disruption, cell cycle and molecular functions by exposing cells that they cultured using various cell lines to temperatures ranging from 4-10C° to 6-30 hours, and showed that it is well preserved in mild hypothermia (27-32C°), and cell integrity deterioration increases as the temperature decreases. They also emphasized that the expressions of protein structures in cellular activities fluctuate in the form of an increase or decrease in low hypothermia[27]. In our study, although there was no significant difference in albumin expression in HepG2 cells, there was a decrease in the 37C° and without $CO_2$ and in cells followed at room temperature as time progressed. Depending on the change in physiological conditions due to hypothermia and carbon dioxide deprivation of HepG2 cells and the decrease in the number of living cells, it is expected that a decrease in albumin expression is also possible.

## CONCLUSION

The data obtained as a result show that the collagen in the cancer cell microenvironment affects the cell's vital activities in adverse conditions that may occur under environmental conditions. Furthermore, we think that these data will be the basis for further studies to answer the questions below, which are; can new cell death experimental models be created by using heat change or carbon dioxide change? or can the change in collagen-heat-carbon dioxide combinations, three important factors in the cancer microenvironment, provide new data for new treatment development research?

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All authors equally contributed in the research and drafting of this paper.

All authors report no potential conflict of interest.

[27] Zheng, Xinglong, et al. *Collagen 1 Promotes Hepatocellular Carcinoma Cell Proliferation By Regulating Integrin B1/FAK Signaling Pathway in Nonalcoholic Fatty Liver*, Oncotarge: 2017, p. 95586

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