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Induction of Apoptosis by Hypoxia in C-4 I Human Cervical Cancer Cells

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ABSTRACT

Hypoxia is defined as a hypobaric stress condition due to the atmospheric pressure in body cells and tissues. Hypoxia induces the formation of free radicals and causes damage to the cell. This study aimed to investigate the antiproliferative effects of hypobaric stress conditions and determine whether hypoxia could induce apoptosis in C-4 I cervical cancer cells. Human cervical carcinoma C-4 I cell line was used in this study. Cells have passaged twice a week and hypoxic conditions were performed in a specially prepared hypobaric cabinet with a rate of 98% N_2 and 2% O_2. Fractional hypobaric hypoxia conditions were applied 3 times for 3 hours at 24- and 48-hours intervals. MTT assay was used to measure C-4 I cell proliferation under hypobaric stress conditions. Morphological changes and cell density were investigated under a phase contrast microscope. Apoptotic Index (AI) and apoptotic morphological parameters were assessed by fluorescent microscopy using DAPI stain. Statistical significance was determined using one-way ANOVA. Hypobaric stress conditions inhibited the proliferation of C-4 I cells in a time-dependent manner and induced apoptosis in C-4 I cells. These results provide evidence that hypoxia induces antiproliferative effects and apoptosis in C-4 I cells. Thus, the results of this study will be the basis for further studies of hypobaric stress conditions to develop new alternative cancer treatment methods.

Keywords: Hypobaric Conditions, Hypoxia, Apoptosis, Cervical Cancer

INTRODUCTION

Oxygen molecules (O_2) sustain intracellular bioenergetics and are consumed by many biochemical reactions. O_2 is essential for most living organisms on earth. ATP produced by mitochondria is released as a result of metabolic pathways. It can provide the energy required for the cellular process [1,2]. Oxygen deficiency in cells and tissues is defined as hypoxia. When oxygen is not enough in the cells and tissues, ATP level decreases, cellular function cannot be controlled, and DNA replication stops because the enzymes required for DNA synthesis are inactivated under hypoxic conditions. Additionally, apoptosis could be a response to severe hypoxic and anoxic conditions. Severe hypoxia in the presence of energy exposes cells to apoptosis [3-5]. If the cells have too low ATP levels to undergo apoptosis, cell death is induced by necrosis [6]. Hypoxia causes inflammation and can be associated with the formation of reactive oxygen species in endothelial cells [7,8].

Hypoxia is a serious hallmark of solid tumors, which enhance metastasis, glycolytic metabolism, cell survival, cell migration/penetration, and angiogenesis [9-11]. Long-time adaptation to hypoxia involves alterations in gene expression. It is primarily stimulated by the hypoxia-inducible transcription factor 1 (HIF-1) [12,13]. HIF-1 is involved in the malignant progression of various tumors and is associated with tumor growth [14,15], metastasis [16,17], and apoptosis [18-21]. Moreover, HIF-1a is an important mediator of hypoxia signals in the inhibition of mitochondrial function [22-25]. Ischemic or hypoxic cell damage usually results from structural changes, malfunction of mitochondria [26], and damage in mitochondrial DNA [27]. Besides, the expression of mitochondrial and nuclear genome-encoded proteins can be downregulated by hypoxia [11, 28].

Hypoxia promotes apoptosis in several different ways. Oxygen deficiency prevents the transport of protons and reduction of mitochondrial-derived ATP causes activation of Bax or Bak that can disrupt the permeability of the mitochondrial membrane and release cytochrome c into the cytosol [6]. Additionally, radical formation and reactive oxygen

species generation stimulate hypoxia induced apoptosis [5]. Oxygen levels above 0.5% allow cell survival by preventing apoptosis [4,29].

To the best of our knowledge, hypoxia has not yet been studied for their antitumoral activity against C-4 I cervical cancer cells. Therefore, this study aimed to investigate the antitumoral effect and apoptosis induction in C-4 I cancer cells exposed to hypobaric stress conditions. Thus, the results of this study will contribute to a better understanding of the hypobaric stress conditions that can be further researched for the development of a potential therapeutic anticancer strategy.

METHODS

Cell Line

Human cervical carcinoma C-4 I cell line (ATCC), were used in this study. Cells were passaged twice a week and maintained at 37°C in Waymouth's MB 752/1 (Sigma) supplemented with 10% FBS, 100 IU/ml penicillin (İ.E. Ulagay) and 100 µg/ml streptomycin (İ.E. Ulagay) in a humidified atmosphere of 5% CO_2.

Application of hypobaric hypoxia conditions

Hypoxic conditions were performed in a specially prepared hypobaric cabinet with a rate of 98% N_2 and 2% O_2. Fractional hypobaric hypoxia conditions were applied 3 times for 3 hours at 24- and 48-hours intervals [30].

MTT Assay

Cell proliferation was evaluated using the 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-Diphenyltetrazolium bromide (MTT; Sigma) cell viability assay, as previously described [30]. Cells were seeded at a density of 3×10^5 cells/well in 96-well plates, then incubated for 24 hours in a 5% CO_2 incubator. After the incubation period, hypobaric hypoxia conditions were applied. At the end of the experiment period, the medium was removed and 40 µl MTT were added. After incubation for 4 hours, 160 µl DMSO was added and incubated overnight. The samples were read by ELISA reader at 570 nm wavelength, with the reference wavelength of 690 nm.

Phase Contrast Microscopy

Morphological changes, cell density, and antiproliferative effect of groups were investigated under a phase contrast microscope at × 200 magnification.

AI assay

AI method was used for cellular analysis. This method is based on the ratio of apoptotic cell count to normal cell numbers and the percentage of apoptosis in the cells. For this method, cells were stained with DAPI and examined with a fluorescent microscope. Normal nuclei and apoptotic nuclei were scored and AI was determined according to the following formula as previously described [31].

*AI = Apoptotic Cells / Total cells * 100*

Statistical Analysis

Statistical analysis was done using Prism 6.0 software (GraphPad Software, San Diego, California, USA). Statistical significance was determined with ANOVA. $p < 0.05$ was considered statistically significant.

RESULTS

The antiproliferative effect was measured with the MTT assay after hypobaric hypoxia conditions were applied to C-4 I cells. The viability values and statistical analyzes of hypobaric stress at 0, 24- and 48-hours post-treatment (PT) are given in Table 1. Antiproliferative effect of hypobaric stress conditions on C-4 I cells was found to be 47%, 55%, and 36% at 0h, 24h, and 48h PT respectively as shown in Table 1. These results showed a significant decrease in the rate of growth of hypoxia condition treated cells compared to the control cells ($p < 0.05$).

Table (1): Absorbance values of C-4 I cells treated with hypobaric hypoxia at 0, 24, and 48 hrs PT using MTT assay.

Absorbance Values (570 – 690 nm) Avg ±SD			
Groups	**0 h PT**	**24 h PT**	**48 h PT**
Control	328.966 x 10^{-3} ± 0.06	462.566 x 10^{-3} ± 0.03	1069.793 x 10^{-3} ± 0.07
Hypobaric Hypoxia	153.466 x 10^{-3} ± 0.03*	254.133 x 10^{-3} ± 0.01*	392.266 x 10^{-3} ± 0.06*

$p<0.01$: * (Statistical significance); PT: Post Treatment; 0 h: immediately after hypobaric stress.

Figure 1. showed that C-4 I cell survival rate decreased to 50% at 0- and 24-hours PT time, while the cell survival at 48 hours PT time was much lower and reached 40% compared with hypoxia untreated control group.

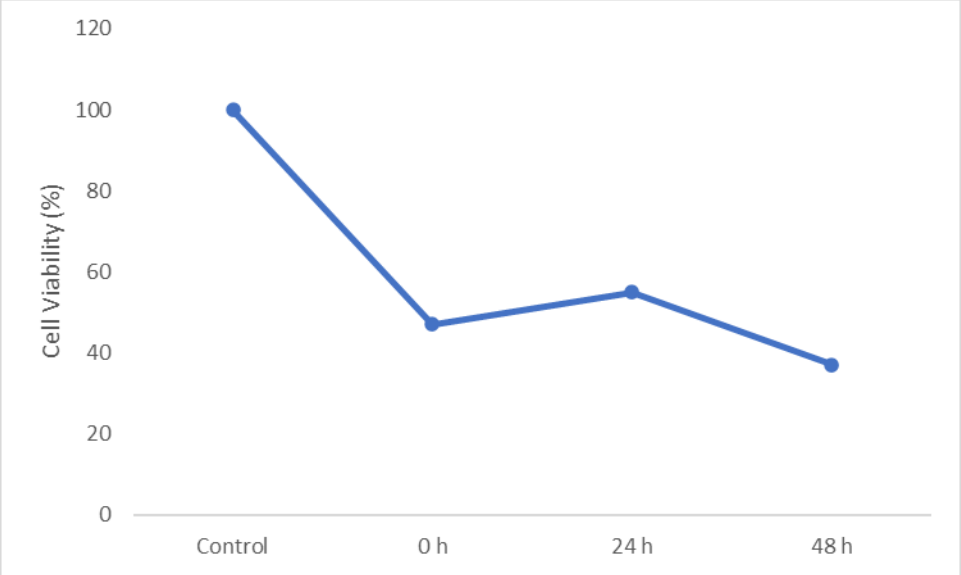
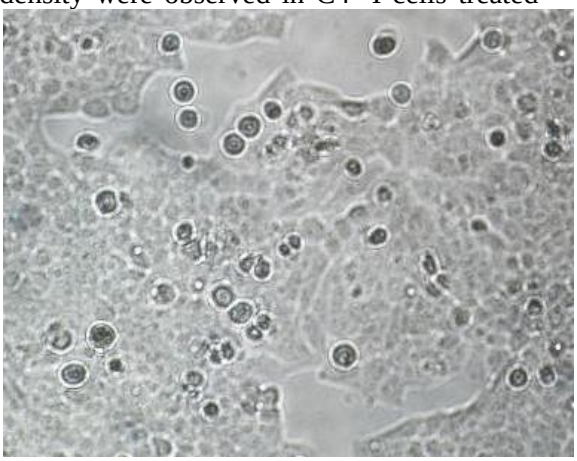
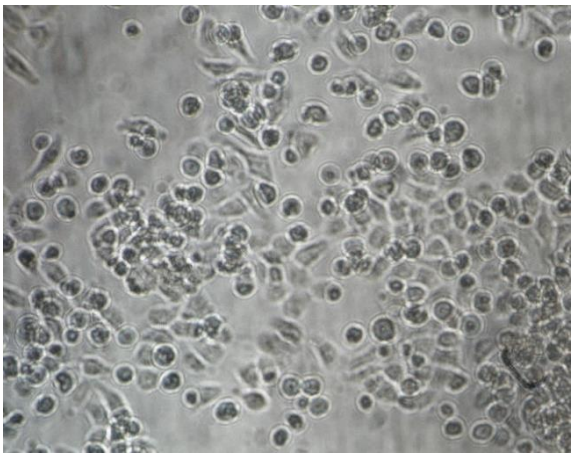


Figure (1): Cell viability (%) values of C-4 I cells treated with hypobaric hypoxia at 0, 24, and 48 hrs PT.

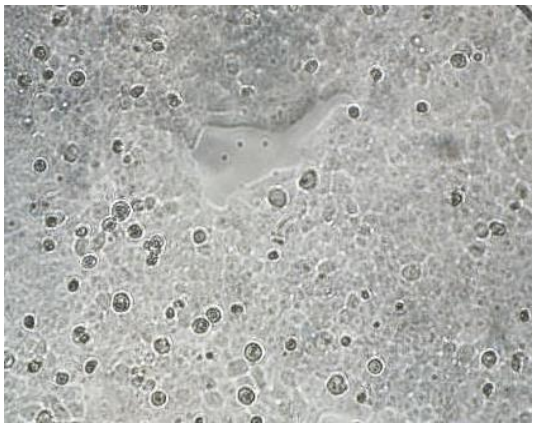
Morphological changes were determined by phase contrast microscope, granule formation, and changes in cell shape, and cell density were observed in C4- I cells treated with hypoxia conditions after 0, 24, and 48 hrs. Incubation compared with control untreated cells as shown in Figure 2.



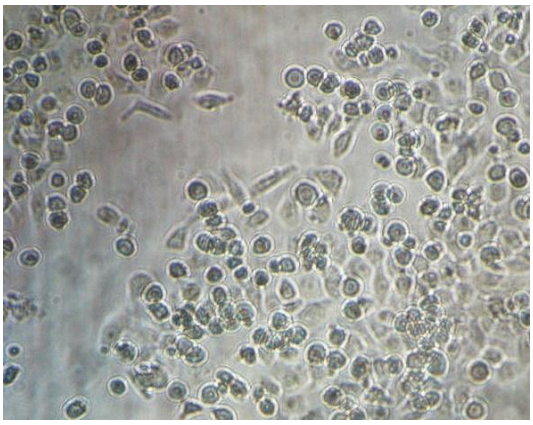
C-4 I 0h Control



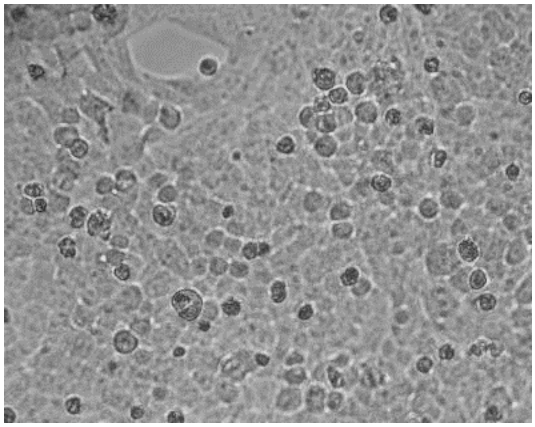
C-4 I 0h Hypobaric Hypoxia PT



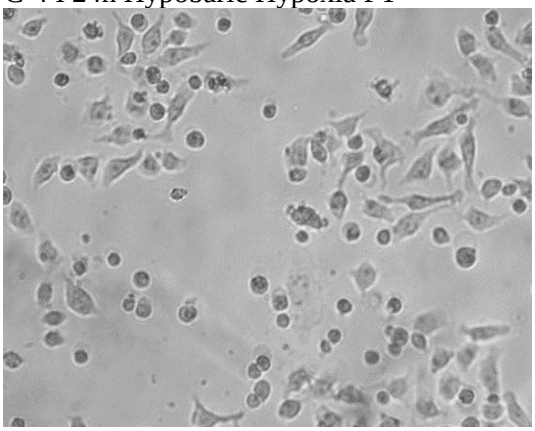
C-4 I 24h Control



C-4 I 24h Hypobaric Hypoxia PT



C-4 I 48h Control



C-4 I 48h Hypobaric Hypoxia PT

Figure (2): Phase contrast microscope images of C-4 I cells in the control and all hypobaric hypoxia (0-,24 - and 48-hours PT) groups (x200).

Results of this study showed AI value was induced by hypoxia stress and increased in a time-dependent manner. The highest AI values at 48 hours PT time, were reached more than 80%. While, at 0- and 24-hours PT time, AI values were 25% and 51%, respectively (Figure 3).

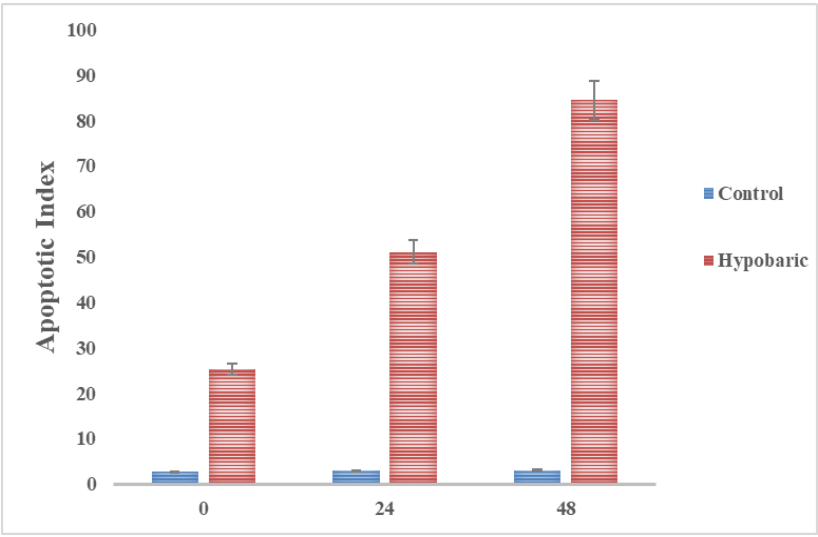
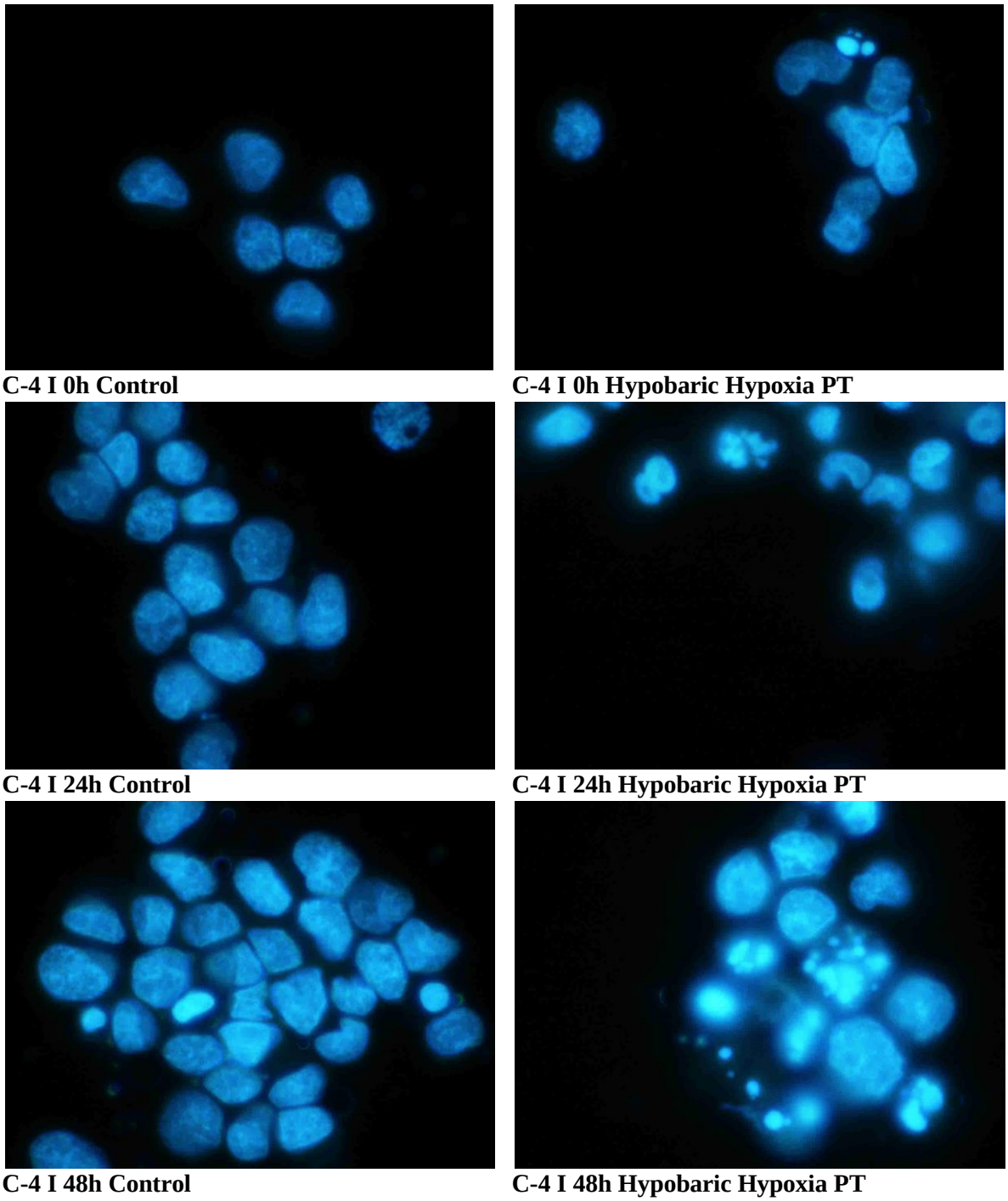


Figure (3): Percent of AI by counting 100 of C-4 I cells in the control and hypoxia stress groups at 0, 24, and 48 hours PT time, following DAPI staining under a fluorescence microscope (×1000). The data shown represents the mean of three independent experiments.

Figure 4 showed fluorescent images of the nuclear morphology of C-4 I cells in all hypoxia stress-treated groups (0-, 24- and 48-hours PT time) and in the control untreated group after DAPI staining. The cellular nuclear chromatin distributed homogeneously and nuclei seemed normal in the control group; whereas the nuclei became condensed and/or fragmented in all hypoxia treated groups. And apoptotic bodies increased in a time-dependent manner in hypoxia treated cells.



C-4 I 0h Control **C-4 I 0h Hypobaric Hypoxia PT**

C-4 I 24h Control **C-4 I 24h Hypobaric Hypoxia PT**

C-4 I 48h Control **C-4 I 48h Hypobaric Hypoxia PT**

Figure (4): Detection of AI via DAPI staining fluorescent images of the nuclear morphology of C-4 I cells in the control and hypoxia treated groups at 0-,24-, and 48-hrs PT time, following DAPI staining observed by a fluorescence microscope (X1000).

DISCUSSION

In this present study, C-4 I cervical cancer cell growth was depressed by hypoxia stress conditions at 0, 24, and 24h. In addition to decreasing cell proliferation and viability, hypoxia stress induced apoptosis in C-4 I cells. Our results indicated that the percentage of condensed or/and fragmented nuclei increased with time. These results indicate that hypoxia primarily affects the C-4 I cells proliferation by inducing cell apoptosis. These results are consistent with several previous studies, which showed that hypoxia was associated with apoptosis in various cancer cell lines [32-35].

Elkashef *et al*. [36] have found that the percentage of viable cells in the hypoxic environment was less than that of normoxic cells. In addition to this, cells in the early and late apoptotic stages were more likely to be present in the cells in the hypoxic environment than the cells in normoxia. In a study conducted by Wang *et al*. [37] showed that hypoxia stimulation decreased cell viability whereas induced apoptosis in a time-dependent manner in PC-12 cells. Western blot analysis showed that after hypoxia treatment for 24 h, Bax, c/pcaspase 3, and c/p-caspase 9 pro-apoptosis factor expression levels were increased, while Bcl-2 expression was decreased. In another study, Yin *et al*. [30] reported that there was no difference in the percentage of apoptotic cells between hypoxic and normoxic cells at 24 hours in GC-2 cells. However, at 48, 60, and 72 hours, they showed a significant increase in the apoptotic rate of hypoxia-treated cells. These results are similar to the Yao *et al.* [38] study that demonstrated that hypoxia inhibited cell viability, promoted apoptosis, and reduced migration in H9c2 cells. Moreover, these results are agreed with a study conducted by Zhang *et al.* [39] that showed that hypoxia treatment for 24 or 48 h reduced the viability of C28/I2 cells. In addition, the apoptotic ratio in C28/I2 cells treated with hypoxia is higher than in normoxic C28/I2 cells. Besides, they showed that hypoxia increased mitochondrial dysfunction in chondrocyte C28/I2 cells.

From this study, it was found that hypobaric stress conditions have a significantly antiproliferative effect on C-4 I cells compared to the control group ($p<0.05$). In addition, it has been shown that cells are induced to apoptosis by hypobaric stress conditions. Based on this research result, hypobaric stress conditions inhibited the proliferation of C-4 I cells and induced apoptosis in these cancer cells. Thus, hypoxia can be expected as an alternative method in cancer treatment and forms the basis for future studies.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS

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