RESEARCH ARTICLE

Effects of Hypobaric Conditions on Apoptosis Signalling Pathways in HeLa Cells

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Abstract

Nowadays increasing effectiveness in cancer therapy and investigation of formation of new strategies that enhance antiproliferative activity against target organs has become a subject of interest. Although the molecular mechanisms of apoptosis can not be fully explained, it is known that cell suicide program existing in their memory genetically is activated by pathophysiological conditions and events such as oxidative stress. Low pressure (hypobaric) conditions that create hypoxia promote apoptosis by inhibiting cell cycling. In this study, determination of the effects of fractional hypobaric applications at different times on HeLa cells at cellular and molecular levels were targeted. Experiments were carried out under hypobaric conditions (35.2 kPa) in a specially designed hypobaric cabin including 2% O_2 and 98% N. Application of fractional hypobaric conditions was repeated two times for 3 hours with an interval of 24 hours. At the end of the implementation period cells were allowed to incubate for 24 hours for activation of repair mechanisms. Cell kinetic parameters such as growth rate (MTT) and apoptotic index were used in determination of the effect of hypobaric conditions on HeLa cells. Also in our study expression levels of the Bcl-2 gene family that have regulatory roles in apoptosis were determined by the RT-PCR technique to evaluate molecular mechanisms. The results showed that antiproliferative effect of hypobaric conditions on HeLa cells started three hours from the time of application and increased depending on the period of exposure. While there was a significant decrease in growth rate values, there was a significant increase in apoptotic index values ($p<0.01$). Also molecular studies showed that hypobaric conditions caused a significant increase in expression level of proapoptotic gene Bax and significant decrease in antiapoptotic Bfl-1. Consequently fractional application of hypobaric conditions on HeLa cell cultures increased both antiproliferative and apoptotic effects and these effects were triggered by the Bax gene.

Keywords: HeLa - hypobaric conditions - in vitro - Bcl-2 gene family

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Introduction

Cancer encountered in the point that of the balance between cell proliferation and cell death is uncontrolled. Cancer causes about 13% of all annual deaths worldwide (Karimi et al., 2014). Nowadays although there are a wide range of treatment options in cancer including surgery, radiotherapy, chemotherapy, immunotherapy, gene therapy and even alternative treatments, there is no definite form of cancer treatment. Using cell culture and detecting apoptosis are the best solutions to observe the response of cancer cells to drugs. Absence of apoptosis might cause uncontrolled cell proliferation and this is known as the characteristics of the cancer cells (Cooper and Hausman, 2006; Wang et al., 2014; Wen et al., 2014).

Apoptosis emerges in both physiological and pathological conditions in organism and it is the selective removal processes of the cells which are dangerous for organism or cells are not require. Nowadays studies intended for activation or inhibition of apoptosis revives new treatment possibilities in many diseases such as cancer, AIDS and autoimmune disorders with the elucidation of the biochemical and genetic components that have role in apoptosis process (Cacciapaglia et al., 2009).

Apoptosis regulatory genes have been the subject of many studies. Cell suicide process initiates with removal of the apoptosis repressor molecules and antiapoptotic agents have effort to prevent activation of this suicide process. Currently it is known that apoptosis has both activators and repressors. Both groups of molecules are balanced in the cell and they determine whether the cell enters apoptosis. Recent studies indicate that some oncogenes and tumor suppressor genes control programmed cell death. Bcl-2, p53, and c-myc are known as apoptosis regulatory genes in vertebrates (Murphy et al., 1996; Vikhanskaya et al., 1998; Chen et al., 2014). Also, while Bcl-2, Bcl-xL, Bcl-W, Mcl-1 can suppress

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apoptosis, Bad, Bid, Bim, Bmf, Bik, Hrk, Noxa and Puma can induce apoptosis (Zhou et al., 2010).

Hypobaric environment conditions were first tested in the United States Human Space Program; structural and physiological effects of hypobaric conditions were investigated. The results showed that hypobaric conditions increased oxidative stress occurring in cells and disrupted mitochondrial function (Jayalakshmi et al., 2005; Ramanathan et al., 2005; Maiti et al., 2006).

The evaluation of molecular mechanism of the effect of hypobaric conditions on cancer cells, investigation of apoptosis-related genes and development of new strategies to increase antitumor effect against target organs are important study fields at the present time.

In this study, creation antiproliferative effect on HeLa cell line under hypobaric conditions and determination of signaling pathways expected to promote apoptosis in cancer cells with this effect were purposed. For this purpose the results of this study will contribute to better understanding of the hypobaric conditions effects and development of new therapies based on the molecular mechanisms.

Materials and Methods

Cell line

Tumor cell line used in our experiments is HeLa (CCL-2) cells which are originated from cervical carcinoma. Minumum Essential Medium (MEM, Gibco) containing 10% Foetal Bovine Serum (FBS, Gibco Lab.), 100 IU/ml penicillin (Pronapen, Pfizer) and 100 µg/ml streptomycin (streptomycin sulfate, I.E. Ulagay) was used as standart cell culture medium for these cells. These cells are grown in a humid atmosphere containing 5% CO_2 and 95% air at 37°C in monolayer culture dishes. When the cells used in our experiments reach sufficient intensity in culture dishes, their passaging are done.

Application of hypobaric conditions

Hypobaric conditions that were used in this study were determined based on literatures. In our experiments, hypobaric conditions were administered for 3 hours with an interval of 24 hours fractionally. Survival curves that belongs HeLa cells used in experiments and period of times were obtained and molecular analysis were carried out with the period that induces apoptosis optimum level.

Mitochondrial dehydrogenase enzyme activity

Growth rates of the experimental groups were determined with MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method which detects cell viability (Mosmann, 1983; Denizot and Lang, 1986).

Apoptotic Index

At the end of the experimental period, culture medium of the cells were removed and were fixed with mixture of 200 µl methanol:FTS (1:1). After removing of the fixative, cells suspended 200 µl pure methanol were seeded on wet slide and were allowed to dry and were stained with DAPI (Diaminophenyliodide) in the dark for 20 minutes. Then after the rinse in PBS for 20 minutes, coverslip was closed and examined with fluorescence microscope. Apoptotic index values were determined by scoring normal nucleus and apoptotic nucleus in preparations prepared according to experimental groups.

Total RNA Isolation

In the first stage to determine the cells have entered apoptosis at the molecular level, RNA isolation was carried out using RNA isolation kit (Total RNA Kit, invitrogen) from control and 48 hours experiment group applied hypobaric with an interval of 24 hours fractionally.

Reproduce of Bcl-2 gene family using RT-PCR

Genes belonging to Bcl-2 gene family are translocated genes in human follicular lymphoma. It is known that this gene family inhibits apoptosis. The expression levels of Bcl-2 gene wer investigated against the apoptosis inducing effect of hypobaric pressure in control and 48 hours experimental group. For this purpose, 7 genes (Mcl-1, Bfl-1, Bax-α, Bcl-2, Bak, Bik and Bcl-x) belonging to Bcl-2 gene family reproduced with RT-PCR Bcl-2 kit (ApoPrimer Set Bcl-2 family, TAKARA) and (RT-PCR Kit, Promega) using isolated total RNAs and differences in expression levels of these genes were investigated.

Statistical Analysis

ANOVA and Dunnett's test was applied to all experimental groups. Statistically $p<0.05$ significance level was based for evaluation of the results.

Results

In our study, in the light of the evaluated parameters, cytotoxicity values occurred as a result of applying hypobaric conditions on HeLa cell cultures for 0, 3, 24 and 48 hours are shown in Table 1. In our experiments antiproliferative effects occurred as a significant decrease according to control in growth rate ($p<0.01$). Cytotoxicity graph based on these values is shown in Figure 1. As shown in Figure 1, antiproliferative effects caused by the hypobaric conditions increased in time dependent manner.

In our experiments % viability graphics prepared according to control group which was considered as 100% was shown in Figure 2. As seen in the figure the cytotoxicity caused by the hypobaric conditions increased in time dependent manner. Determined % viability values were 100%, 77.25%, 69.9% and 84.145% respectively according to control group.

In our experiments, apoptotic index occurred as a result of applying hypobaric conditions on HeLa cell cultures for 0, 3, 24 and 48 hours are shown in Table 2. As shown in Figure 3 apoptotic index values were determined as 2.25567, 2.13033, 2.85933 and 3.68367 respectively in control group. Determined apoptotic index values after treatment of hypobaric conditions for 3, 24, 48 hours were 3.72333, 6.6 and 6.41433 respectively.

In experimental groups applied 48 hours hypobaric conditions have maximum apoptotic index values, representation of expression of 7 genes belong Bcl-2 gene family with RT-PCR was shown in Figure 4.

It was observed that in control group Bfl-1 which

Table 1. Absorbance values of Mitochondrial Dehydrogenase Activity of HeLa Cell Cultures Applied Hypobaric for 0-48 h

Experimental Groups	Absorbance Values (570-690 nm) Control	Hypobaric
0 h	$604,892\times10^{-3}$	
3 h	$604,775\times10^{-3}$	$467,208\times10^{-3}$ (1)
24 h	$921,79\times10^{-3}$	$644,29\times10^{-3}$ (2)
48 h	$1298,575\times10^{-3}$	$1092,69\times10^{-3}$ (3)

*p < 0,05: (1) Significance according to control for 3 h; (2) Significance according to control for 24 h; Significance according to control for 48 h

Table 2. Apoptotic Index values of HeLa Cell Cultures Applied Hypobaric for 0-48 h

Experimental Groups	Apoptotic Index Values Control	Hypobaric
0 h	225,567	
3 h	213,033	3,72333 (1)
24 h	285,933	6,6 (2)
48 h	368,367	6,41433 (3)

*p < 0,05: (1) Significance according to control for 3 h; (2) Significance according to control for 24 h; Significance according to control for 48 h

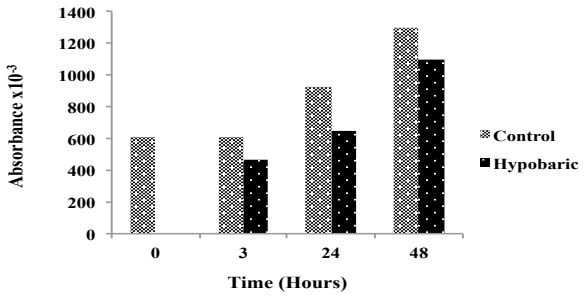

Figure 1. Cytotoxicity Graph of HeLa Cell Cultures Applied Hypobaric Conditions for 0-48 hours (570-690 nm)

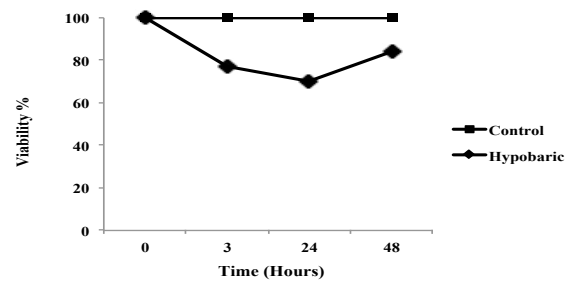

Figure 2. Viability% values of HeLa Cell Cultures Applied Hypobaric Conditions for 0-48 hours

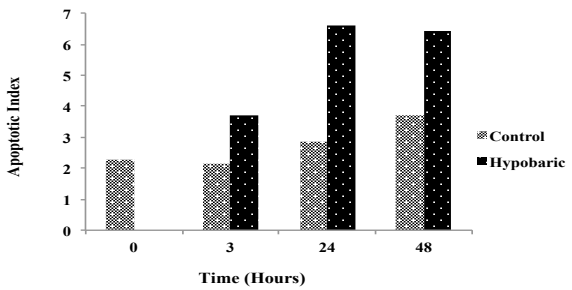

Figure 3. Apoptotic Index values of HeLa Cell Cultures Applied Hypobaric Conditions for 0-48 hours

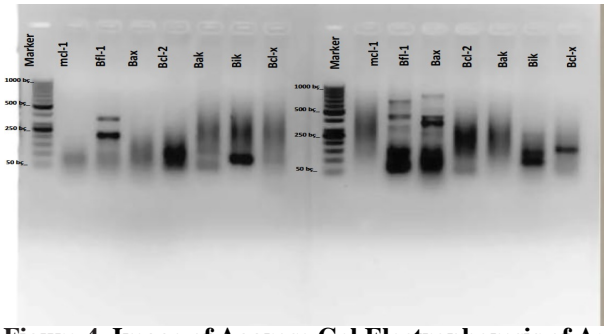

Figure 4. Image of Agarose Gel Electrophoresis of A: Mcl-1, Bfl-1, Bax, Bcl-2, Bak genes and B:Bik, Bcl-x, β-actin genes which is Bcl-2 gene family members expression of HeLa cells treated with hypobaric conditions. M:1,5 kb Marker, C:Control

is an antiapoptotic gene was more expressed than experimental group. While expression of Bax gene which is proapoptotic gene was not seen in control group, it was determined that Bax had expression level in experimental group which was exposed to hypobaric conditions. There was not a significant expression level of antiapoptotic Mcl-1 and Bcl-2; proapoptotic Bak and Bik the group applied hypobaric conditions.

Discussion

As increasing studies about the discovery of extraterrestrial atmosphere, response of the biological systems to these environmental conditions are understood better. In the light of this information, different atmospheric pressure systems have been developed which are known to be effective on animal and plant physiology and evolution (Massimo, 1992). Along with the idea of establishment of greenhouses on Mars and the moon, low atmospheric pressure engineering and at the same time the system installation challenges have emerged (Baker, 1981; He et al., 2003).

As shown in in vitro and in vivo experiments, hypobaric pressure increases cellular oxidative stress and to promote hypoxia suggests that especially impairs function in mitochondria (Costa et al., 1988). As a result of this lipids, proteins and DNA damaged and irreversible damage occurs for cells frequently (Magalhaes et al., 2005).

Mitochondria, which is exist in the center of the special type of cell death, apoptosis, with the effect of the of hypobaric pressure organelles in the cell to be most affected, has been the starting point of this study. From this point of view, in our study, apoptosis stimulating effect of hypobaric conditions on mammals and the elucidation of the molecular mechanism have been studied.

In this study, apoptotic death mechanisms that occurred in HeLa cell lines as a result of hypobaric pressure were evaluate via various cell kinetics and molecular biological methods. Bcl-2 gene is one of the important regulators of apoptosis and integral membrane protein of Bcl-2 function is to inhibit cell apoptosis (Song et al., 2014). When Bcl-2 gene is activated and overexpressed, an increase in the amount of protein inhibits apoptosis and leads to cell proliferation. While investigating the

molecular mechanisms of hypobaric pressure in this study, interruption of cell cycle and cell death plays a key role in cancer treatment.

Bcl-2 protooncogene is protective against biological and environmental factors which are induced apoptosis. Bcl-2 protect cells from apoptosis mechanism, to stabilize the mitochondrial membrane, the formation of free radicals and apoptosis block the effects to prevent ingress of regulatory molecules selectively. These effects arise by binding of a protein called Bax to Bcl-2 (Haldar et al., 1998). Bcl-2 and Bax are two important proteins and play role in mitochondrial-mediated pathway (Huang et al., 2014). The ratio between Bcl-2 and Bax expression level determines whether cells respond to an apoptotic signal (Liu et al., 2014). In contrast to Bcl-2, Bax protein accumulation in high amounts in the cell initiates apoptosis. Protein of Bax are made these effect by creating holes in the membrane of mitochondria and evading cytochrome c from the mitochondria, with this escape as triggering apoptosis by activating proapoptotic caspases (caspase family). Thus, inhibition of apoptosis by Bcl-2, occur with prevention of homodimers and pores formation (Blagosklonny et al. 1997).

In recent years, researches about the cell's life or death draw attention to mitochondria which is centre of apoptosis mechanism. Mitochondria are double membrane organelles. Bcl-2 is a protooncogene that encoding a 24-26 kDa protein, and this protein located on the outer membrane of mitochondria facing cytoplasm and the nucleus membrane which is part of the endoplasmic reticulum. These proteins, regulates ion exchanges and protective effect against membrane degradation. Another interesting feature of the Bcl-2 family, control the effects of reactive oxygen levels on apoptosis acting as prooxidan. In addition, caspases are the central component of the apoptotic program. Caspase activation is specific to cell and caspase inhibitors (IAP) prevent apoptosis by inhibiting effector caspases have been shown (Ling et al., 1998).

Bax is a proapoptotic member of the Bcl-2 family and involved in promoting apoptosis (Huckelhoven, 2004; Lockshin and Zakeri, 2004; Pecorino, 2008; Henke et al., 2011). Its expression is induced by radiation, chemotherapeutic drugs and other genotoxic stress forms (Kitada et al., 1996; Thomas et al., 1996). There is an amount of evidence has indicated that the expression of the Bax gene, parallels p53-mediated apoptosis (Miyashita et al., 1994). And also Bax gene was directly activated by p53 (Miyashita, 1995).

The mechanism of apoptosis shows differences according to stimulation and cell type. Intracellular stimuli affecting apoptosis in general can be grouped into three main groups: growth factors, oncogenes and tumor suppressor genes. Also, hyperthermia, radiation, cytotoxic anticancer drugs and capable to forming necrosis factors like hypoxia, generate apoptosis at low doses.

Historically, the low pressure system tested in the United States human space program for the first time and specialized vehicles have been developed in order to reduce the structural and physiological effects of hypobaric conditions. For example, Mercury, Gemini and Apollo vehicles have been produced to form a pressure of 34 kPa (pure oxygen) for astronauts in space. Also, Skylab has been produced pressure of 34 kPa (70% O_2/30% N_2 gas mixture) (Waligora et al., 1991; Massimo, 1992; Paul et al., 2004).

The results that found in this study were shown that hypobaric conditions revealed the antiproliferative effect begins at 3th hours from the time of application, and increased depending on the application time to HeLa cell culture. In our experiments, we determined a significant reduction in proliferation rate in parallel to a significant increase in apoptotic index value. One of the Bcl-2 gene family member proapoptotic Bax gene expression wasn't observed in the control group, while Bax gene expression was increased significantly in 48 hours experimental group which were exposed to hypobaric conditions. As a result, we concluded fractional administration of hypobaric conditions to HeLa cell cultures increase both antiproliferative and apoptotic effect.

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