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Effects of hyperbaric oxygen therapy on the morphological characteristics and survival of MCF-7 breast cancer cells

Hiperbarik oksijen tedavisinin MCF-7 meme kanseri hücrelerinin morfolojik özellikleri ve sağkalımı üzerindeki etkileri

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ABSTRACT

Aim: This study aims to determine the effects of hyperbaric oxygen therapy at different pressure values on cell morphology and cell survival in the MCF-7 breast cancer cell line.

Materials and Methods: The experimental groups were formed by applying 100% oxygen to MCF-7 breast cancer cells at 1.5, 2, and 2.5 atmospheres for 2 hours. The control group did not receive treatment. At the end of the experiment, cell survival was investigated by CCK-8 analysis, cell shapes were determined by cresyl violet staining, and cell surface morphologies were determined by scanning electron microscope.

Results: Cell viability was significantly reduced at atmospheric pressure of 1.5, 2, and 2.5 compared to the control group ($p < 0.005$). As pressure increased, the surface area of the cell decreased, nuclear condensation increased, and the cell borders became irregular. Cell membrane bleb and cell membrane porosity increased at 2 and 2.5 atmospheres.

Conclusion: Hyperbaric oxygen therapy severely reduces the viability of MCF-7 breast cancer cells under increased pressure. It can induce apoptosis and change the shape and surface morphology of MCF-7 breast cancer cells. Although further studies are needed, our study supports the potential use of hyperbaric oxygen therapy in the treatment of breast cancer.

Keywords: Breast cancer; MCF-7; Hyperbaric oxygen therapy; Scanning electron microscope; CCK-8

ÖZ

Amaç: *Bu çalışma, MCF-7 meme kanseri hücre hattına farklı basınçlar altında uygulanan hiperbarik oksijen tedavisinin hücre morfolojisi ve hücre sağkalımı üzerindeki etkilerini belirlemeyi amaçlamaktadır.*

Gereç ve Yöntem: *MCF-7 meme kanser hücrelerine 1,5, 2 ve 2,5 atmosfer basınç altında 2 saat boyunca %100 oksijen uygulanarak deney grupları oluşturuldu. Kontrol grubuna ise herhangi bir tedavi uygulanmadı. Deney sonunda; hücre sağkalımları CCK-8 analizi hücre şekilleri cristal viyole boyama ve hücre yüzey morfolojileri taramalı elektron mikroskobu ile belirlendi.*

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Bulgular: *Hücre canlılığı, kontrol grubuna kıyasla 1,5, 2 ve 2,5 atmosfer basıncında önemli ölçüde azaldı (p < 0.005). Basınç arttıkça hücre yüzey alanının azaldığı, nükleer yoğunlaşmanın arttığı ve hücre sınırlarının düzensizleştiği görüldü. İki ve 2,5 atmosfer basınçta, hücre zarının kabarcık şekilli protrüzyonu ve porasyonunda artış tespit edildi.*

Sonuç: *Hiperbarik oksijen tedavisi artan basınçla orantılı olarak MCF-7 meme kanseri hücrelerinin canlılığını ciddi şekilde azaltır. Hücre şeklini ve yüzey morfolojilerini değiştirir. MCF-7 meme kanseri hücrelerinde apoptozu tetikleyebilir. Daha ileri çalışmalara ihtiyaç duyulmasına rağmen, çalışmamız meme kanseri tedavisinde hiperbarik oksijen tedavisinin kullanım potansiyelini desteklemektedir.*

Anahtar Sözcükler: *Meme kanseri; MCF-7; Hiperbarik oksijen tedavisi; Taramalı elektron mikroskobu; CCK-8.*

INTRODUCTION

Breast cancer is the most common cancer diagnosed among women and represents more than one of the ten new cancers diagnosed each year. In 2020, approximately 2.26 million women have been diagnosed with breast cancer and 685 000 deaths were reported worldwide (1). Surgery is usually the first step in breast cancer treatment. It is followed by chemotherapy or radiation therapy or, in some cases, by hormones or targeted treatments (2). Researchers are looking for better ways of treating breast cancer and managing the side effects of treatment. Today, breast cancer research is focused on applications to induce apoptosis, reduce inflammation, and prevent metastasis. Cancer cell lines are widely used to study the biological mechanisms of cancer.

Hyperbaric oxygen therapy (HBOT) is a treatment that increases the amount of oxygen dissolved in tissues by breathing pure oxygen at higher than normal atmospheric pressure. Depending on the reason for HBOT, the treatment pressure can vary from 250 to 280 kPa and the duration of the treatment can vary from 45 to 300 minutes. Patients can receive up to 40 sessions (3). HBOT is used in the treatment of tissue damage, decompression sickness, carbon monoxide poisoning, and gas gangrene caused by insufficient oxygenation. In addition, potential research areas, including inflammation and systemic diseases, COVID-19, and cancer, will also be examined. HBOT is generally used as an adjuvant treatment along with other therapeutic applications, such as radiation therapy and chemotherapy. It is generally considered safe (4).

Cell morphology is an indicator that provides quantitative information on cancerous cells. It plays an important role in cell mobility and metastasis, as well as in tumor invasiveness (5).

The aim of this study is to investigate the effects of HBOT application under different pressures on the cellular morphology and viability of the MCF-7 breast cancer cell line. Recent studies have shown that HBOT in MCF-7 breast cancer cell culture decreased cell proliferation. However, the effect of HBOT on the surface morphology of MCF-7 breast cancer cells was not fully explained.

MATERIALS and METHODS

MCF-7 (HTB-22, ATCC, Rockville, Maryland, USA) is a human breast cancer cell line containing progesterone, estrogen, and glucocorticoids receptors. It is derived from a patient with metastatic adenocarcinoma (6). Cells were cultured in RPMI 1640 medium (Gibco, New York, USA) containing 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, St Louis, MO, USA), and 1% penicillin/streptomycin (Gibco, New York, USA) in a humidified atmosphere containing 5% CO_2 at 37° C. Growth was monitored daily by a microscope to ensure cell health, and the medium was changed every 2 days. Cells were passaged when they had reached >90% confluent monolayer, passage cells into new tissue culture flasks. Cells were harvested using a 0.25% trypsin-EDTA solution (w/phenol red) (Gibco, New York, USA) and centrifuged after the addition of medium for trypsin inactivation.

Hyperbaric oxygen treatment and experimental groups

MCF-7 breast cancer cells were cultured for 24 hours in a separate culture plate at 37 ° C in a humidified atmosphere with 5% CO_2 before the groups formed. HBOT treatment was performed in a small research hyperbaric chamber (Model R&D, Yaklaşım Makine, BaroxHBO, Istanbul, Turkey). The gas in the chamber was flushed 15 l/min for 5 min and then pressurized to 1.5 - 2.5 atmosphere (atm). Three experimental groups

were formed by exposing MCF-7 breast cancer cells to 1.5, 2, and 2.5 atm (~100% O_2), in a hyperbaric chamber for 2 hours. Cells from the control group were placed in a humidified atmosphere in a 37 ° C incubator at 21% O_2, 5% CO_2 and 1 atm.

CCK-8 assay for cell viability

The CCK-8 (water-soluble tetrazolium salt based colorimetric assay) (Boster Bio.Tech., Pleasanton, USA) analysis was used to determine the effect of HBOT treatment on the viability of MCF-7 breast cancer cells. Cells were seeded in 96-well plates at a density of $5x10^4$ cells/well/100µl medium. Twenty-four hours after HBOT, 10 µl of CCK-8 solution was added to the cells and the absorbance was measured after 3 hours at a wavelength of 450 nm using a microplate reader (7). The percentage of cell viability was determined with Microsoft Excel 2019 software (for Windows 10, Microsoft Corporation, Washington, USA).

Cresyl violet staining

Cresyl violet staining was used to investigate the morphological features of cells at the light microscopic level. Round coverslips (12 mm in diameter) were placed in 24-well culture plates, $2x10^5$ / well cells were seeded and incubated for 24 hours. After HBOT, cells were fixed in 4% paraformaldehyde at +4°C for 3 hours. The MCF-7 breast cancer cells were stained for 5 min with 0.5% cresyl violet. During the next stage, the cells were washed with dH_2O for 5 min and dehydrated using a series of increasing concentrations of ethanol (70%, 80%, 90%, 96%). Round coverslips were left in xylene solution for 5 min (8). The mounted slides were examined using a Nikon Eclipse 80i light microscope with a camera attachment and a Nikon image analysis system (Nikon Instruments Inc., New York, USA).

Scanning electron microscopy (SEM)

The effects of HBOT on the ultrastructural structure of MCF-7 breast cancer cells were observed by SEM. Round coverslips (12 mm in diameter) were placed in 24-well culture plates, $2x10^5$ / well cells were seeded and incubated for 24 hours. After hyperbaric oxygen therapy, cells were fixed in 2.5% glutaraldehyde at +4°C for 3 hours. The round coverslips were washed with PBS and dehydrated by increasing concentrations of ethanol (50%, 70%, 90%, 96%, 99.6%). The samples were dried with air and covered with gold (Emmitech K550, UK) before SEM examination (FEI, Quanta 650 Field Emission SEM, USA).

Statistical analysis

Statistical analysis was performed using SPSS 17.0. (SPSS Inc., Chicago, USA). All data are presented as arithmetic average ± Standart Error. Mann–Whitney U tests were used to compare variables between the groups. $p < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Effects of HBOT on Cell Viability

The viability of cells from the control group (untreated) was accepted as 100%. Twenty-four hours after HBOT, the cell viability of each group was determined as 64.64 % ± 3.11 (1.5 atm), 49.34 % ± 0.38 (2 atm), and 22.24 % ± 1.71 (2.5 atm) (Figure-1). A statistically significant difference was found between cell viability of the control group compared to the 1.5, 2, and 2.5 atm pressure groups ($p < 0.005$). Cell viability of the 1.5, 2, and 2.5 atm pressure groups were compared with each other, a statistically significant difference was observed ($p < 0.005$).

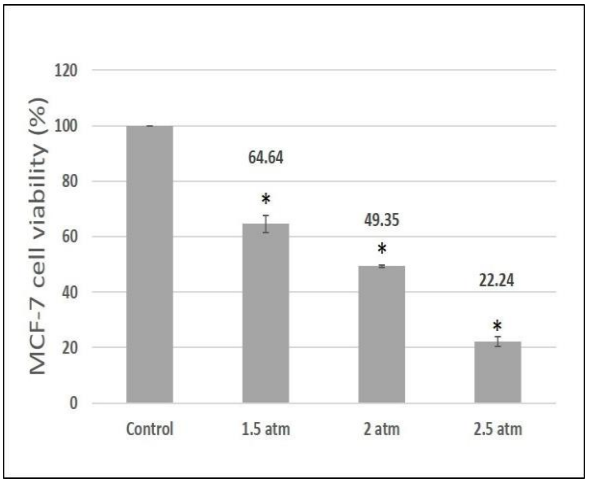


Figure-1. CCK-8 cell viability analysis. Cell survival decreased significantly at 1.5, 2, and 2.5 atmospheric pressures compared to control groups ($p < 0.005$) *.

Light microscopic changes of MCF-7 breast cancer cells

Control group cells (untreated) examined, epithelial-like morphology, and dome structures were determined. In the 1.5-atm pressure group, the morphology of most cells was similar to that of the control group. In addition, round cells with

condensed nuclei were observed. In the 2 atm and 2.5 atm pressure groups (i) the surface area of the cells decreased, (ii) the cell borders became irregular, (iii) the number of round apoptotic cells with nuclear condensation increased, and (iv) the density of the cells in the unit area decreased. Cellular shrinkage was observed in some cells in the 2 and 2.5 atm pressure groups (Figure-2).

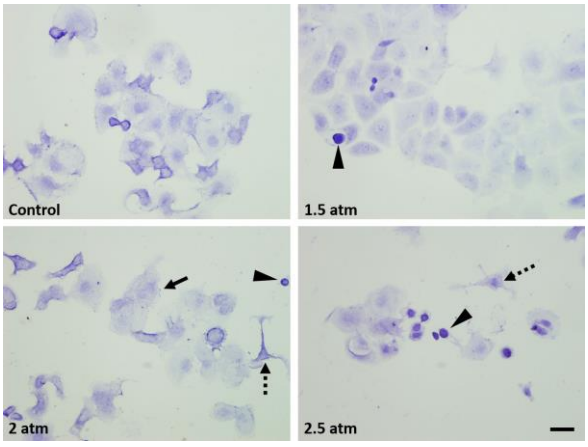


Figure-2. Cresyl violet staining of MCF-7 breast cancer cells in all experimental groups (X20). In the 2 atm and 2.5 atm pressure groups, the surface area of the cells decreased, the cell borders became irregular (arrow), the number of round cells with nuclear condensation (arrow head) increased, the density of the cells in the unit area decreased. Cellular shrinkage (dotted arrow) was observed in the 2 and 2.5 atm pressure groups. Scale bar: 30 µm

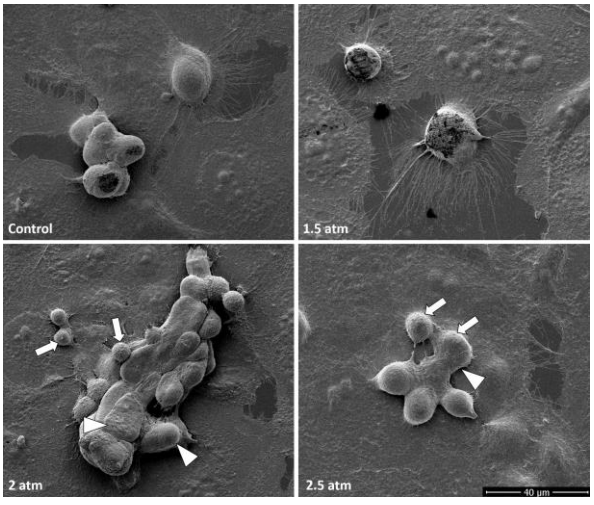


Figure-3. SEM images of MCF-7 breast cancer cells in all experimental groups (X2000). In the 2 atm and 2.5 atm pressure groups, cell membrane bleb (arrow) and cell membrane porosity (arrow head) increased.

Ultrastructural analysis with SEM

Control group cells (untreated) were found to spread on the glass surface with a large number of filopodia. They displayed epithelial-like morphology. The cell shapes in the other groups were similar to those of the control group, but the cell sizes were smaller than those of the control group. The bleb and porosity of the cell membrane increased in the 2 and 2.5 atm. Porous cells had irregular shapes. The epithelial dome structure in those cells had begun to deteriorate. The blebbing cells were round in shape and smaller than other cells (Figure-3).

DISCUSSION

The common features of most tumors are low levels of oxygen called hypoxia, whose severity varies depending on the type of tumor. It is generally recognized that oxygen concentrations in hypoxia tumor tissues are lower than normal tissues, with an average of 1 - 2 % O_2 below. Hypoxia plays a central role in many carcinogenic characteristics, such as angiogenesis, cell survival, metastasis, EMT-like cancer cell migration, and glycolytic metabolism. Hypoxic cancer cells are more aggressive and invasive with a better ability to metastasize. Limits the efficacy of radiotherapy, chemotherapy, and immunotherapy, affecting the prognosis by inducing an aggressive tumor phenotype (9). Patients with hypoxic breast tumors are at increased risk of recurrence and death from breast cancer (10). Today, targeting tumor hypoxia is proposed as a potential therapeutic approach in cancer management. For this reason, we examined the effects of HBOT on MCF-7 breast cancer cells. HBOT is suggested to break the hypoxic environment in the cancer microenvironment and inhibit tumor growth. The increased amount of oxygen combined with radiation therapy and chemotherapy can facilitate the killing of cancer cells (11). In our study, it was determined that cell viability decreased with an increase in pressure (1.5, 2 and 2.5) in oxygen-administered groups compared to hypoxia. Granowitz et al. (12) evaluated the effect of HBOT treatment at 2.4 atm (100% oxygen) for 20 hours in MCF-7 breast cancer cells. Similarly to our findings, it was observed that cell proliferation decreased dramatically. The decrease in the number of cells began in the seventh hour. However, apoptosis was not observed in MCF-7 breast cancer cells (12). Chen et al. (13) applied HBOT treatment from 2.5 to 3.5 atm to MCF-7

cells for 6 hours. Induction of apoptosis in MCF-7 cells has not been reported. HBOT alone did not induce apoptosis in pancreatic ductal tumor cells (14). However, it improved apoptosis in HL-60 leukemia cells, Jurkat cells and lymphocytes (hematopoietic cells) (15, 16). Light microscopy examinations of this study showed apoptosis-like changes in cell shapes. Blebbing and increased porosity were observed in cell surface morphologies. SEM analysis were used only to examine cell surface morphologies, not to determine the number of cells. For this reason, cells were imaged as soon as the HBOT application was finished. Flores-Romero et al. (21) reported that pyroptosis and necroptosis may cause an increase in the number and size of cell pores. There are studies suggesting that increased cell membrane permeabilization may be an effective adjuvant in new cancer treatments (22, 23). Bleb formation on the cell membrane usually indicates apoptotic cell death (24). Non-apoptotic blebs have been observed in various cellular processes, including mitosis, propagation, and migration (25). Our study showed that HBOT treatment can induce apoptosis in MCF-7 cells. Ganguly et al. reported that HBOT triggers apoptosis with intracellular H_2O_2 accumulation in hematopoietic cells (15). We believe that the inhibition of cell proliferation and induction of apoptosis revealed in our study is not only related to oxygenation. If cancer cells are exposed to high pressure alone, their proliferation may be inhibited. (12). Takano et al. showed that high pressure decreases cell viability by inducing apoptosis in human lenfoblast cells (17). The absence of a high-pressure group in a normoxic environment is the limitation.

Cancer and HBOT studies have recently focused on whether increased oxygen acts as a cancer promoter or not. In our study, hyperbaric oxygen therapy was observed to not induce MCF-7 cell proliferation. Studies examining the effects of HBOT in MCF-7 breast cancer cells (12), A549 lung cancer cells (18), and LNCaP prostate cancer cells (19) in the literature support our findings. Oi et al. (20) reported that HBOT increased cell proliferation and decreased apoptosis in the SGC7901 gastric cell line. Cancer types have different responses to oxygen. However, this should not lead to the exclusion of HBOT in cancer treatment research.

CONCLUSION

HBOT is generally used as an adjuvant treatment along with other therapeutic applications, such as radiation therapy and chemotherapy. The use of HBOT as part of cancer treatment has not yet been approved, although some promising results have emerged recently (5). We concluded that HBOT has potential in the treatment of breast cancer, but the effect of HBOT on breast cancer subtypes should be in investigated further clinical studies.

Conflict of interest: The authors declare that they have no conflict of interest.

References

1. Ferlay J, Colombet M, Soerjomataram I, et al. Cancer statistics for the year 2020: An overview. Int J Cancer 2021;Apr 5.
2. Trayes KP, Cokenakes SEH. Breast Cancer Treatment. Am Fam Physician 2021;Aug 1;104(2):171-8.
3. Leach RM, Rees PJ, Wilmshurst P. Hyperbaric oxygen therapy. BMJ 1998;Oct 24;317(7166):1140-3.
4. Ortega MA, Fraile-Martinez O, García-Montero C, et al. A General Overview on the Hyperbaric Oxygen Therapy: Applications, Mechanisms and Translational Opportunities Medicina (Kaunas). 2021;Aug 24;57(9):864.
5. Lyons SM, Alizadeh E, Mannheimer J, et al. Changes in cell shape are correlated with metastatic potential in murine and human osteosarcomas. Biol Open 2016;Feb 12;5(3):289-99.
6. Ishikura N, Sugimoto M, Yorozu K, Kurasawa M, Wakita D, Kondoh O. Effect of Bevacizumab on a Human Breast Cancer Model that Exhibited Palbociclib-resistance by RB Knockout. Cancer Diagn Progn 2022;Sep 3;2(5):533-41.
7. Tang W, Peng W, Zhang H, Zhang Y, Li B, Duan C. Period 3, a tumor suppressor in non-small cell lung cancer, is silenced by hypermethylation. Int J Clin Exp Pathol 2018;Jan 1;11(1):120-8.
8. Geserick P, Hupe M, Moulin M, et al. Cellular IAPs inhibit a cryptic CD95-induced cell death by limiting RIP1 kinase recruitment. J Cell Biol 2009;Dec 28;187(7):1037-54.
9. Muz B, de la Puente P, Azab F, Azab AK. The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. Hypoxia (Auckl) 2015;Dec 11;3:83-92.

10. Tutzauer J, Sjöström M, Holmberg E, et al. Breast cancer hypoxia in relation to prognosis and benefit from radiotherapy after breast-conserving surgery in a large, randomised trial with long-term follow-up. Br J Cancer 2022;May;126(8):1145-56.
11. Xue T, Ding JS, Li B, Cao DM, Chen G. A narrative review of adjuvant therapy for glioma: hyperbaric oxygen therapy. Med Gas Res 2021;Oct-Dec;11(4):155-7.
12. Granowitz EV, Tonomura N, Benson RM, et al. Hyperbaric oxygen inhibits benign and malignant human mammary epithelial cell proliferation. Anticancer Res 2005;Nov-Dec;25(6B):3833-42.
13. Chen YC, Chen SY, Ho PS, et al. Apoptosis of T-leukemia and B-myeloma cancer cells induced by hyperbaric oxygen increased phosphorylation of p38 MAPK. Leuk Res 2007;Jun;31(6):805-15.
14. G Bosco, L Guizzon, Z Yang, E Camporesi, Effect of hyperbaric oxygenation and gemcitabine on apoptosis of pancreatic ductal tumor cells in vitro. Anticancer Res. 2013 Nov;33(11):4827-32.
15. Ganguly BJ, Tonomura N, Benson RM, Osborne BA, Granowitz EV. Hyperbaric oxygen enhances apoptosis in hematopoietic cells. Apoptosis 2002; Dec;7(6):499-510.
16. Weber SU, Koch A, Kankeleit J, et al. Hyperbaric oxygen induces apoptosis via a mitochondrial mechanism. Apoptosis 2009;14, 97-107.
17. Takano KJ, Takano T, Yamanouchi Y, Satou T. Pressure-induced apoptosis in human lymphoblasts. Exp Cell Res 1997;Aug 25;235(1):155-60.
18. Chen SY, Tsuneyama K, Yen MH, Lee JT, Chen JL, Huang SM. Hyperbaric oxygen suppressed tumor progression through the improvement of tumor hypoxia and induction of tumor apoptosis in A549-cell-transferred lung cancer. Sci Rep 2021;Jun 8;11(1):12033.
19. Tang H, Zhang ZY, Ge JP, Zhou WQ, Gao JP. Effects of hyperbaric oxygen on tumor growth in the mouse model of LNCaP prostate cancer cell line. Zhonghua Nan Ke Xue 2009;Aug;15(8):713-6. Chinese
20. Qi Y, Ruan J, Wang M, et al. Effects of hyperbaric oxygen treatment on gastric cancer cell line SGC7901. Biomed Rep 2017;Apr;6(4):475-9.
21. Flores Romero H, Ros U, Garcia Saez AJ. Pore formation in regulated cell death. EMBO J 2020;Dec 1;39(23):e105753.
22. Aguilar AA, Ho MC, Chang E, et al. Permeabilizing Cell Membranes with Electric Fields. Cancers (Basel) 2021;May 10;13(9):2283.
23. He S, Singh D, Helfield B. An Overview of Cell Membrane Perforation and Resealing Mechanisms for Localized Drug Delivery. Pharmaceutics 2022;Apr 18;14(4):886.
24. Khajah MA, Luqmani YA. Involvement of Membrane Blebbing in Immunological Disorders and Cancer. Med Princ Pract 2016;25 Suppl 2(Suppl 2):18-27.
25. Aoki K, Satoi S, Harada S, Uchida S, Iwasa Y, Ikenouchi J. Coordinated changes in cell membrane and cytoplasm during maturation of apoptotic bleb. Mol Biol Cell 2020;Apr 1;31(8):833-44.