Effects of hyperbaric oxygen treatment on gastric cancer cell line SGC7901

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Abstract. Hyperbaric oxygen (HBO) has been previously identified as an effective adjunct treatment option for the management of brain injury, diabetic ulcers and chronic wounds. However, the roles of HBO as an adjunctive therapy for tumors remain controversial. The present research project was performed to explore the effects of HBO treatment on proliferation, autophagy and endoplasmic reticulum stress response of the gastric cancer cell line, SGC7901. The present study demonstrated that, after subjecting SGC7901 cells to HBO treatment, the increase in cell proliferation was significant, compared with that of the control group. In addition, there was a significant increase in LC3-phosphatidylethanolamine conjugate (LC3-II) level, as well as binding immunoglobulin protein level, and a significant decrease in CCAAT-enhancer-binding protein homologous protein level. These suggested that hyperbaric oxygen treatment alone may promote proliferation and cell survival of gastric cancer cell SGC7901, and inhibit apoptosis through regulating cell autophagy and oxidative stress.

Introduction

Widely used in the clinic, hyperbaric oxygen (HBO) treatment has be shown to be an effective adjunct to management of brain injury (1), diabetic ulcers (2) and chronic wounds (3). However, the roles of HBO as an adjunctive therapy for tumors remains controversial. Some previous studies suggest that HBO therapy may improve postoperative outcome and outcome of radiotherapy and chemotherapy, especially proving beneficial for the treatment of radiotherapy (4,5). A combinatorial approach using chemotherapeutic drugs with HBO may enhance sensitivity of tumor cells to chemotherapeutic agents (6,7) and the application of HBO in chemotherapy for malignant lymphoma, brain tumor, lung cancer, gastric cancer and breast cancer may increase chemotherapeutic efficacy while decreasing treatment related toxicity (8). However, HBO treatment alone may stimulate the proliferation of tumor tissues (9). Other studies indicate that cancer cells in hypoxic conditions are more likely to metastasize and thus be more deadly (10-12). Meanwhile, hypoxic cells undergo a high rate of mutation to become a treatment-resistant genotype, and HBO treatment may improve it (13). The current study is to explore the effects of hyperbaric oxygen treatment alone on proliferation, autophagy and oxidative stress response of gastric cancer SGC7901 cells.

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Materials and methods

Cell culture. Gastric cancer SGC7901 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA), inoculated in Dulbecco's modified Eagle's (high glucose) culture medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (Clark Bioscience, Richmond, VA, USA) and cultured in a 5% CO_2 incubator at 37˚C.

HBO treatment. Prior to the experiment, the hyperbaric chamber was disinfected using UV techniques for 20 min, and cleaned using pure oxygen for 10 min. The cells were placed flat in the chamber under aseptic conditions for 90 min each time. The pressure of HBO was increased slowly to 0.2 MPa within 15 min and, after 60 min, the pressure was decreased to normal pressure within 15 min. Then, the cells were taken out of the chamber and cultured in the CO_2 incubator. During the experiment, the HBO chamber was kept ventilated with 95% oxygen at a flow rate of 2 l/min.

Cell grouping. The cells were split into two groups. HBO group: Cells at log phase were subjected to HBO treatment once a day. Control group: SGC7901 cells at a log phase of their growth were cultured in the CO_2 incubator without HBO treatment. When cells of HBO group were receiving HBO

treatment, this group of cells were removed from the incubator and maintained at room temperature.

SGC7901 cell proliferation measured using an MTT assay following HBO treatment. Following trypsinization, cells at log phase were resuspended and seeded in a 96-well cell culture plate at a density of $5x10^3$ cells/well. Following treating the cells with HBO, 20 μl MTT (5 mg/ml, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) solution was added into each well after 48 h. The cells were cultured for a further 4 h in the incubator. Then, the supernatant was abandoned and 100 μl DMSO (Sigma-Aldrich; Merck KGaA) was added to each well. Following oscillation in a constant temperature culture vibration machine at 37˚C for 10 min until the crystal was completely dissolved, the absorbance value (A value) at the wavelength of 490 nm was measured at a enzyme-linked immunosorbent assay reader (ELX800; BioTek Instruments, Inc., Winooski, VT, USA). The experiments were performed in triplicate and results are represented as the average value of three independent experiments.

Expression levels of cell autophagy and of oxidative stress associated proteins measured by western blot analysis. Following trypsinization, cells at log phase were resuspended and seeded in a 6-well cell culture plate at a density of $3x10^5$ cells per well. At 48 h following HBO treatment, radioimmunoprecipitation assay buffer (25 mM HEPES, 1.5% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.5 M NaCl, 5 mM EDTA, 50 mM NaF, 0.1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride and 0.1 g/l leupeptin, pH 7.8) was used for cell lysis and total protein extraction. The quantitative determination of total protein concentration of each group was made by the bicinchoninic acid assay method (cat. no. P0009; Beyotime, Institute of Biotechnology, Haimen, China). The same amount of protein (50 μg) of each group was loaded onto 12.5% SDS-PAGE gels. Electrophoresis separated proteins were then transferred onto polyvinylidene difluoride membranes. Following blocking with 5% skimmed milk, membranes were incubated overnight with the primary antibody against LC3-phosphatidylethanolamine conjugate (dilution, 1:500; cat. no. sc-134226; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), binding immunoglobulin protein (BiP; dilution, 1:400; cat. no. sc-1051; Santa Cruz Biotechnology, Inc.) or CCAAT-enhancer-binding protein homologous protein (CHOP; dilution, 1:500; cat. no. sc-7351; Santa Cruz Biotechnology, Inc.) at 4˚C. Membranes were then washed at room temperature and incubated for 2 h with IgG-horseradish peroxidase conjugate (dilution, 1:10,000; goat anti-mouse IgG; cat. no. AP124P), goat anti-rabbit IgG (dilution, 1:10,000; cat. no. AP132P) and rabbit anti-goat IgG (dilution, 1:10,000; cat. no. AP106P) all obtained from EMD Millipore (Billerica, MA, USA). Following another washing step (any unbound secondary antibody was removed by washing), membranes were treated with ECL visualization reagents (Thermo Fisher Scientific, Inc.) in the dark room. β-actin was used as the internal control in the experiment. Protein band intensities were determined by using the Quantity One software (version, 4.6.2; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The experiment was repeated three times and results were averaged. The ratio of the target protein band intensity to that of the internal control in each group was also calculated.

Statistical analysis. All statistical analysis was calculated using SPSS 16.0 statistical analysis software (SPSS, Inc., Chicago, IL, USA). The difference between control group and treatment group was evaluated using Student's t-test. $P<0.05$ was considered to be statistically significant.

Results

Effects of HBO treatment on SGC7901 cell proliferation. An MTT assay was used for measuring cell proliferation following HBO treatment. The result indicated that the OD_{490} value of SGC7901 cells following HBO treatment was significantly higher than that of control group with statistical difference (Fig. 1; $P<0.05$), which suggested that HBO treatment may promote the proliferation of gastric cancer SGC7901 cells.

Effects of HBO treatment on autophagy of SGC7901 cells. As presented in Fig. 2, the expression level of autophagosome marker protein LC3-II following HBO treatment was significantly increased when compared with the control group (Fig. 2B; $P<0.05$). The result demonstrated that HBO treatment may significantly stimulate the autophagy of gastric cancer cells SGC7901.

Effects of HBO treatment on the expression levels of oxidative stress-associated proteins, BiP and CHOP. Western blot analysis was used to evaluate the expression levels of oxidative stress-associated proteins BiP and CHOP following HBO treatment. The results are presented in Fig. 3. The expression level of BiP was significantly increased when compared with the control group ($P<0.05$). Conversely, the level of CHOP was significantly decreased ($P<0.05$). These data suggested that HBO treatment may promote SGC7901 cell survival and inhibit cell apoptosis caused by oxidative stress.

Discussion

HBO therapy provides 100% oxygen in a chamber with increased pressure. This treatment provides extra oxygen to support the growth of new blood vessels at the hypoperfusion area and is beneficial to help treat conditions such as wounds, carbon monoxide poisoning and soft tissue infections (14). Typically, tumor cells are less well-oxygenated than normal tissues, and tumor hypoxia often leads to rapid tumor growth as well as resistance to radiotherapy and anticancer chemotherapy (15). Therefore, HBO may theoretically be used as an effective therapy for tumor treatment by reversing tissue hypoxia (16), as tumor cells can be stimulated resulting in an increased sensitivity to chemo- and radiotherapy, and the effect of chemo- and radiotherapy can be enhanced by HBO (17-20). However, the effect of hyperbaric oxygen treatment alone on tumor treatment remains controversial (21). Some studies suggested that HBO may possess tumor-inhibitory effects (22-25), while others indicated that HBO treatment alone may stimulate tumor growth and metastasize (8,26,27).

In addition to the mitochondrial apoptosis pathway, previous findings have demonstrated that autophagy and endoplasmic reticulum (ER) stress can be also involved in apoptosis (28,29). As a highly conserved self-digestion process, autophagy serves as a 'battery' to promote cell survival in response to

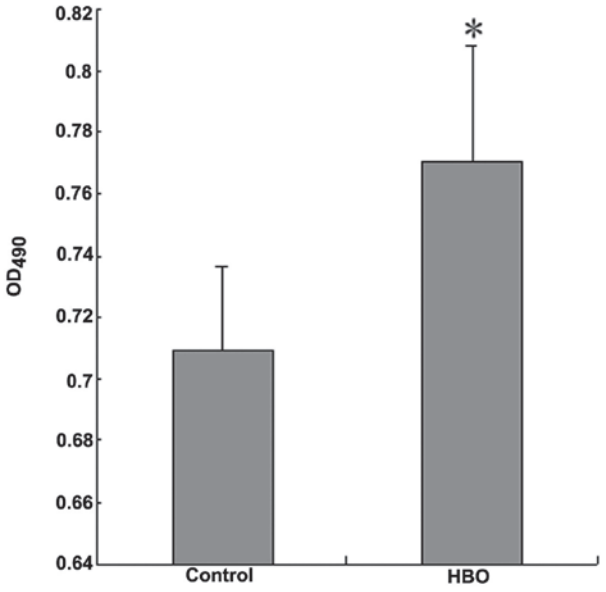

Figure 1. Effects of HBO treatment on SGC7901 cells proliferation. The SGC7901 cells proliferation following HBO treatment was higher than that of the control group. *P<0.05 vs. control. HBO, hyperbaric oxygen.

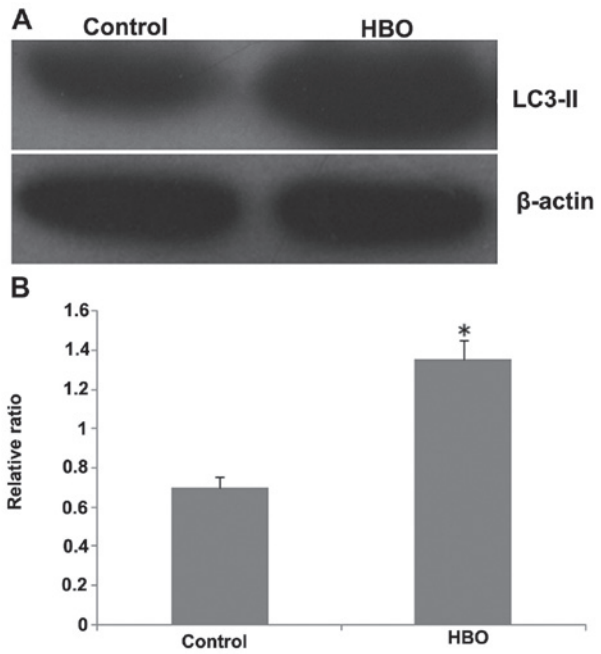

Figure 2. LC3-II expression in SGC7901 cells was upregulated by HBO treatment. (A) Western blot assay results for LC3-II protein detection in the cell lysates. (B) Quantification of these data. The gray-scale value of each band was read using Quantity One software. The relative ratio of LC3-II band intensity to that of β-actin was used to construct the Bar graph. *P<0.05 vs. control. HBO, hyperbaric oxygen; LC3-II, LC3-phosphatidylethanolamine conjugate.

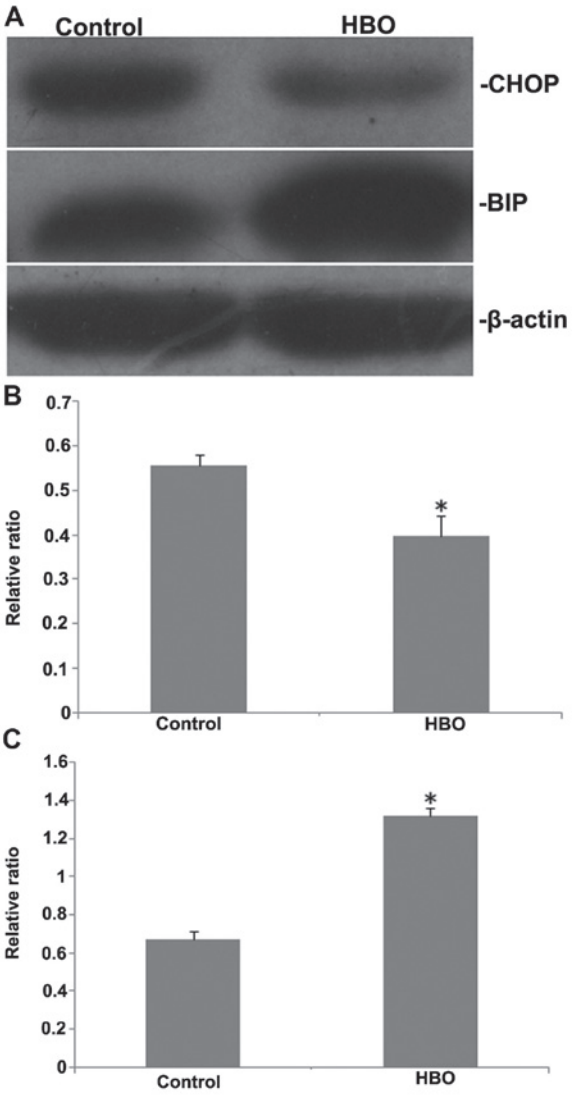

Figure 3. The change expression levels of oxidative stress-associated proteins BiP and CHOP following HBO treatment. (A) Western blot assay results for BiP and CHOP expression in the cell lysates (n=3). (B) Quantification of the western blot analysis from Fig. 3B. The gray value of each band was read using Quantity One software. The relative ratio between CHOP and β-actin was used to construct the Bar graph. *P<0.05 vs. control. (C) Quantification of Fig. 3C. The gray value of each band was read using Quantity One software. The relative ratio between BiP and β-actin was used to construct the bar graph. *P<0.05 vs. control. CHOP, CCAAT-enhancer-binding protein homologous protein; HBO, hyperbaric oxygen; BiP, binding immunoglobulin protein.

nutrient starvation, hypoxia and other metabolic stresses until the stress subsides. However, excessive or sustained autophagy has the potential to induce cell death (autophagic cell death), which makes autophagy a double-edged sword that could be either protective or detrimental to cells (30-32). LC3 can be used as a reliable autophagosome marker for monitoring autophagy. During autophagy, a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes (33). ER stress-induced apoptosis can be mediated by the expression/activation of apoptosis-related molecules (such as CHOP and caspase-12) or pro-survival molecules (such as glutamate decarboxylase and BiP) (34,35). ER stress and autophagy can both be seen as compensatory roles important for tumor cells under chronic metabolic stress to survive in the harsh environment and there are findings that certain anticancer drugs can induce autophagy and ER stress at the same time (36,37).

The present study sought to explore the effects of HBO treatment alone on proliferation, autophagy and ER stress of

gastric cancer cell line SGC7901. The results indicated that, following HBO treatment, the increase in SGC7901 cell proliferation was significant compared with that in the control group, and in addition, there was a significant increase level in autophagosome marker LC3-II, as well as prosurvival molecule BiP level. However, there was a significant decrease in the levels of apoptosis-related molecule, CHOP. Changes in expression levels of CHOP and BiP suggest cells adaptation to stress conditions following HBO treatment. These factors have indicated that HBO treatment may induce both autophagy and ER stress, and promote cell survival by regulating cell autophagy. Due to vigorous cell proliferation situations, it can be concluded that HBO treatment alone *in vitro* may promote tumor cell proliferation and enhance cell survival. However, it does not mean that tumor growth and metastasize would be stimulated if tumor patients receive HBO treatment alone, as cultured tumor cells *in vitro* are not deprived of oxygen, this is different from the hypoxic conditions *in vivo*. Therefore, the fact of tumor cell proliferation and inhibition of apoptosis *in vitro* after HBO does not suggest the same results on tumor patients who receive HBO treatment. For further research in the future, the authors intend to mimic the *in vivo* hypoxic conditions of tumor cells *in vitro* and investigate hypoxic cell survival effect following HBO treatment to further evaluate the promotion or inhibition effect of HBO treatment on gastric cancer and provide experimental evidence for the clinical treatment of gastric cancer.

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