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Leukemia Research 31 (2007) 805–815
Leukemia Research
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Apoptosis of T-leukemia and B-myeloma cancer cells induced by hyperbaric oxygen increased phosphorylation of p38 MAPK

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Received 18 July 2006; received in revised form 11 September 2006; accepted 13 September 2006
Available online 24 October 2006

Abstract

Tumor cells with different origins have different threshold to apoptosis. Hematopoietic (Jurkat, NCI-H929) cells and non-hematopoietic (A549, MCF-7) cells were received hyperbaric oxygen (HBO_2) treatment from 2.5 to 3.5 atmosphere absolute (ATA) of 100% oxygen for 6 h, and a significant percentage of apoptosis were shown only in hematopoietic Jurkat and NCI-H929 cells by either Annexin V or TUNEL assay. Oxidative stress was illustrated higher in HBO_2-treated hematopoietic cells by superoxide fluorochrome detectors. HBO_2 treatment leads to caspase-3, caspase-7 activation and further cleavage of PARP within cells. Furthermore, the increased phosphorylation of p38 MAPK was demonstrated in both Jurkat and NCI-H929 cells.

Keywords: Hyperbaric oxygen; Leukemia; Multiple myeloma; Oxidative stress; Apoptosis; p38 mitogen activated protein kinase

1. Introduction

Hyperbaric oxygen therapy (HBOT) is a medical treatment using oxygen administered at a greater than normal atmospheric pressure. Several benefits of HBOT have been demonstrated, including inhibition of toxin formation by certain anaerobes, increasing the flexibility of red cells, reducing tissue edema, preserving intracellular adenosine triphosphate, and maintaining tissue oxygenation in the absence of hemoglobin [1,2]. Therefore, hyperbaric oxygen (HBO_2) has been used to treat different clinical diseases, such as necrotizing soft tissue infections, compromised skin grafts and flaps, acute traumatic ischemias, and exceptional blood loss [3–6]. Although the partial pressure of oxygen in tissues can be increased under conditions of HBO_2, oxygen radicals and their derivatives may also be produced [7]. Oxidative stress caused by the metabolites of oxygen radicals and their derivatives may induce cells to undergo apoptosis, which is considered to be an adverse or beneficial effect of HBO_2 depending on the physiological conditions [8,9].

Malignant tumors have been recognized as a relative contraindication to HBO_2. It was concerned that HBO_2 might have cancer growth enhancing effects. However, recent clinical studies strongly suggest no more than a neutral effect of HBOT on tumor growth [10,11]. Our previous experiments demonstrated that exposure to early HBO_2 therapy attenuated the severity of disease progression in autoimmune NZB/W F1 mice [12]; proposing a targeted apoptosis to hyperproliferating lymphocytes. *In vitro* models are simplified systems that may aid the understanding of treatment mechanisms

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doi:10.1016/j.leukres.2006.09.016

of HBO_2. The aims of our studies herein are to compare the threshold to oxidative stress induced by hyperbaric oxygen treatment between hematological and non-hematological cells and to further clarify the potential signaling pathway in HBO_2-induced apoptosis of cancer cells. The experimental profile included assessment of reactive oxygen species, the mitochondrial membrane potential, the caspases and mitogen activated protein kinases (MAPKs).

2. Materials and methods

2.1. Cell culture

Human leukemia Jurkat cell line, human multiple myeloma NCI-H929 cell line, human lung carcinoma A549 cell line and human breast adenocarcinoma MCF-7 cell line were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). The cells were cultured at 10^6 cells/ml in RPMI 1640 (Gibco Invitrogen Ltd., Taiwan) with 10% fetal calf serum (Gibco Invitrogen Ltd., Taiwan), 2 mM L-glutamine (Gibco Invitrogen Ltd., Taiwan), 1 mM sodium pyruvate (Gibco Invitrogen Ltd., Taiwan), 4.5 g/l glucose (Gibco Invitrogen Ltd., Taiwan), 10 mM HEPES (Gibco Invitrogen Ltd., Taiwan), and 1.5 g/l sodium bicarbonate (Gibco Invitrogen Ltd., Taiwan). Individual cultures were maintained for no more than 2 months.

2.2. Hyperbaric oxygen treatment

HBO_2 treatment was performed in a small research hyperbaric chamber (HVOAN-400, Yilin Enterprise Company Ltd., Taipei, Taiwan, ROC). The gas in the chamber was flushed 15 l/min for 5 min and then pressurized to 2.5 ATA or reach higher pressure. Cells in groups were exposed to either HBO_2 (98% O_2, 2% CO_2 at 2.5 ATA or 98.6% O_2, 1.4% CO_2 at 3.5 ATA) or HBA (hyperbaric air) (8.4% O_2, 2% CO_2, 89.6% N_2 at 2.5 ATA or 6% O_2, 1.4% CO_2, 92.6% N_2 at 3.5 ATA) (Longshin Gas Ltd., Taipei, Taiwan, ROC) in a hyperbaric chamber for 6 h. Control cultures for each experiment were placed in an incubator at 37 °C, 21% O_2, 5% CO_2 at 1 ATA.

2.3. Flow cytometry for Annexin V/propidium iodide (PI)

Cells were resuspended in media at 5×10^5 cells/ml and transferred to 12-well plates. Annexin V/propidium iodide (PI) was examined at 8 h after HBO_2 or HBA treatment. Briefly, cells were collected by centrifugation, washed twice in PBS, and incubated for 15 min with Annexin V-FITC (BD Pharmingen, San Diego, CA, USA) and PI (Sigma Chemicals, St. Louis, MO, USA) in the appropriate binding buffer. Cells were electronically gated, and phosphotidylserine (PS) exposure and PI permeability determined by FACScan (Becton Dickinson, Richmond, CA) [13] were analyzed in both populations.

2.4. Cell cycle detection

Cell cycle was examined at 8 h after HBO_2 or HBA treatment. Cells (3×10^6) were fixed and permeabilized in 70% ethanol (4 °C) for at least 2 h in order to preserve GFP fluorescence. Cytosolic DNA fragments were then extracted by incubation with DNA extraction buffer (0.2 M Na_2HPO_4, 1 mM citric acid, 10 min). Finally, cells were resuspended in DNA staining solution (PBS, 100 μg/ml RNase A, 20 μg/ml PI) before analysis by flow cytometry. DNA degradation was determined as the percentage of DNA located in the sub-G_0/G_1 peak of the cell cycle.

2.5. TUNEL (TdT-mediated dUTP nick end labeling) assay

Cells were placed in 37 °C CO_2 incubator for further 48 h after HBO_2 exposure detection and quantification of apoptosis was determined using the In Situ Death Detection Kit (Roche Molecular Biochemicals, Germany), which labeled DNA strand brakes, following the manufacturer's instructions. Apoptotic ratio was determined by measuring numbers of TUNEL positive cells in 300 total cells.

2.6. Measurement of intracellular hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet -}$)

Cells were measured for intracellular H_2O_2 and $O_2^{\bullet -}$ immediately after HBO_2 or HBA exposure. To this end, cells were either labeled with 20 μM cell-permeable 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma Chemicals, St. Louis, MO, USA) for 15 min or 10 μM dihydroethidium (DHE) (Sigma Chemicals, St. Louis, MO, USA) for 5 min and washed twice with PBS before resuspension in 1 ml PBS buffer. The fluorescence corresponding to the oxidized probe was followed by measuring the green (510–550 nm; FL1) fluorescence population. The mean fluorescence intensity was measured by CELLQuest software.

2.7. Treatment with antioxidants

Superoxide dismutase (SOD) (Sigma Chemicals, St. Louis, MO, USA) and catalase (Sigma Chemicals, St. Louis, MO, USA) were freshly prepared each time due to their unstable nature in aqueous solution. The stock catalase and SOD solution was membrane-filtered and diluted with DMSO/medium to working concentrations of 1000 and 500 U/ml. Jurkat and NCI-H929 cells were pretreated with catalase and SOD for 30 min before exposure to HBO_2.

2.8. Detection of mitochondrial transmembrane potential

Flow measurement of the mitochondrial transmembrane potential ($\Delta\Psi_m$) was conducted by labeling groups of cells with 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6[3]$) (40 nM) (Molecular probes, Eugene, OR, USA) for 15 min after 8 h of HBA or HBO_2 exposure. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 50 μM) was used as a positive control for disruption of $\Delta\Psi_m$. Processing of the substrate was evaluated by flow cytometry [14].

2.9. Analysis of signaling protein by western blotting

Equivalent amounts of protein were mixed with an equal volume of 2× Laemmli buffer, boiled, and resolved on 10% SDS-PAGE gels. After the protein was transferred to nitrocellulose membranes by electroblotting (Bio-Rad, Richmond, CA), the blots were blocked by overnight incubation with 5% nonfat dry milk in Tris/boric acid/sodium chloride/Tween 20 and subsequently probed with the specific primary antibodies for the Bcl-2 family (Cell Signaling Technology Inc., Boston, MA, USA), the caspases (Cell Signaling Technology Inc., Boston, MA, USA) and the MAPKs (Cell Signaling Technology Inc., Boston, MA, USA). Immunoreactive proteins were visualized using horseradish peroxidase-linked secondary antibodies and further with ECL (Enhanced-chemiluminescence western blotting kit) (Amersham Biosciences, Pittsburgh, PA, USA).

2.10. Statistical analysis

Experimental data was calculated from six individual samples and expressed as the mean ± S.D. One-way ANOVA were used to compare differences between means of groups and statistical analysis was performed using SPSS. One-Dscan analysis software was used to analyze the expression contents of proteins in western blotting.

3. Results

3.1. The kinetics of HBO_2-induced apoptosis of T-leukemia Jurkat cells

To determine the maximal effects of the duration and pressure of HBO_2 on apoptosis, Jurkat cells were exposed to HBO_2 (100% O_2) or HBA (hyperbaric air) (21% O_2, 79% N_2) with elevated pressures from 2.5 to 3.5 ATA or different durations ranging from 2 to 12 h, respectively. The most significant observation of apoptosis in Jurkat cells after HBO_2 exposure compared with HBA exposure or non-exposure cells was demonstrated when the oxygen pressure reached 3.5 ATA for 6 h (Fig. 1) by Annexin V/propidium iodide staining ($P < 0.001$). We chose the atmospheric pressure of 3.5 and duration of 6 h throughout our following experiments to investigate the signaling pathway involved in HBO_2-induced apoptosis. With the observation that the ceiling effect of apoptosis in T-leukemia Jurkat cells occurred under HBOT, we also investigated whether other cell lines had a similar response (Table 1). Primary cells and transformed tumor cell line were treated with either 2.5 or 3.5 ATA of 100% oxygen for 6 h, and a significant percentage of apoptosis was compared with control cells that were either non-treated or treated with HBA. Control cells for HBA exposure are in purpose to compare the effect of pressures during oxygen supply. The percentage of apoptosis from different origins of cells was detected by Annexin V/PI staining. HBO_2 induced apoptosis was not observed in non-hematopoietic derived cancer cell line, such as A549, MCF-7 or PC-12 cells.

3.2. HBO_2 induced apoptosis of Jurkat and NCI-H929 cells

After exposure to HBO_2, Jurkat leukemia cells were arrested in the sub-G_1 phase; the amount of hypodiploid DNA was also increased after HBO_2 treatment compared with control groups (Fig. 2A). Furthermore, Jurkat cells were placed in 37 °C CO_2 incubator for further 48 h after HBO_2 exposure to detect the fragmented DNA (Fig. 2B), which occurred close to the final step in the apoptotic process. The percentage of TUNEL-positive cells was demonstrated significantly higher ($P < 0.001$) in both HBO_2-treated Jurkat and NCI-H929 cells compared with control cells (Fig. 2C).

3.3. Preventing HBO_2-induced intracellular hydrogen peroxide and superoxide accumulation inhibits apoptosis in Jurkat and NCI-H929 cells

To ascertain whether HBO_2-induced apoptosis is associated with increases of intracellular ROS, Jurkat and NCI-H929 cells were measured for intracellular hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet -}$) immediately after HBO_2 or HBA treatment. Both intracellular hydrogen peroxide and superoxide were significantly increased in HBO_2-treated groups compared with control groups ($P < 0.001$) (Fig. 3A). To further clarify whether HBO_2-induced intracellular ROS is required for apoptosis, both cells were pretreated with catalase and SOD for 30 min before exposure to HBO_2. The enhancement in intracellular ROS and HBO_2-induced apoptosis were inhibited by antioxidants such as SOD and catalase ($P < 0.01$) (Fig. 3B and C).

3.4. Collapse of the mitochondrial transmembrane potential ($\Delta\Psi_m$) in HBO_2 treated Jurkat and NCI-H929 cells

The involvement of mitochondria in HBO_2-mediated apoptosis was determined by assessing mitochondrial

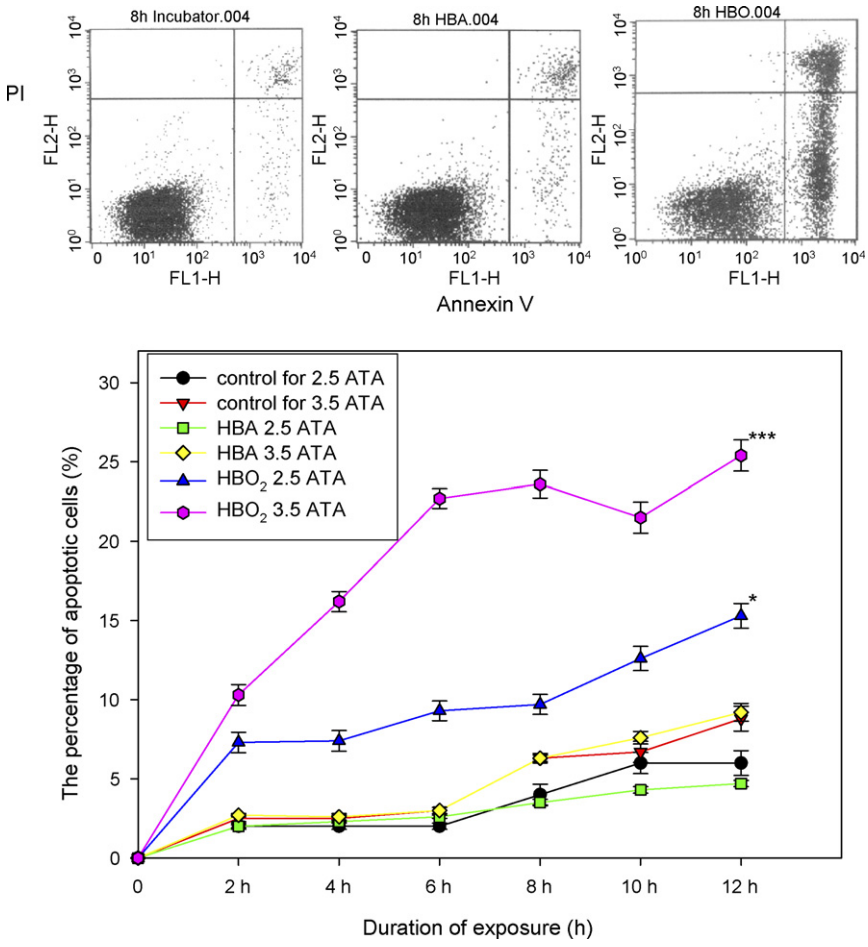

Fig. 1. Fluorescence dot-blot analysis of Jurkat cells in control, HBA-treated and HBO_2-treated groups by Annexin V/PI staining. The kinetics of HBO_2-induced apoptosis of Jurkat cells were examined at the pressure of 2.5 or 3.5 ATA with 0, 2, 4, 6, 8, 10, or 12 h of exposure, respectively. Results for each time points are shown as the mean ± S.D. of samples from six independent trials. The three "2.5 ATA" conditions (control for 2.5 ATA, HBA 2.5 ATA, HBO_2 2.5 ATA) were performed concurrently; while the three "3.5 ATA" conditions (control for 3.5 ATA, HBA 3.5 ATA, HBO_2 3.5 ATA) were studied simultaneously. $^{***}P < 0.001$ when comparing HBO_2-exposed cells to HBA cells. $^{*}P < 0.05$ when comparing 2.5 ATA HBO_2-exposed cells to 3.5 ATA cells.

Table 1
Cell line tested in HBOT-induced apoptosis by Annexin V flow cytometry assay

	Percentage of apoptosis (mean ± S.D.)	
	HBO_2 at 2.5 ATA for 6 h	HBO_2 at 3.5 ATA for 6 h
Primary cell culture		
Murine BALB/C splenocytes	3.6 ± 0.5	6.7 ± 1.5
NZB/WF1 splenocytes	10.3 ± 2.1*	16.2 ± 2.7**
NOD splenocytes	8.1 ± 1.4*	12.2 ± 3.3**
Human PBMC (peripheral blood mononuclear cells)	<3.0	<3.0
Cell line		
Human T-leukemia Jurkat cell line	20.7 ± 4.3***	30.5 ± 3.1***
B-myeloma NCI-H929 cell line	15.6 ± 2.4***	20.4 ± 3.7***
Lung carcinoma A549 cell line	<3.0	<3.0
Breast adenocarcinoma MCF-7 cell line	<3.0	<3.0
Murine pheochromocytoma PC 12 cell line	<3.0	<3.0

Groups of cells were either cultured in the incubator or exposed to hyperbaric oxygen (HBO_2) or hyperbaric air (HBA) in a hyperbaric chamber for 6 h. Apoptosis was measured by Annexin V/PI at 8 h after HBO_2 exposure, and the percentage of apoptotic cells was calculated. Results are shown as the mean ± S.D. of samples from three individual experiments. $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$ were indicated in this table when comparing HBO_2-exposed cells to control cells in the incubator. The percentage of apoptosis for the cells maintained in the incubator or exposed to HBA is below 3%.

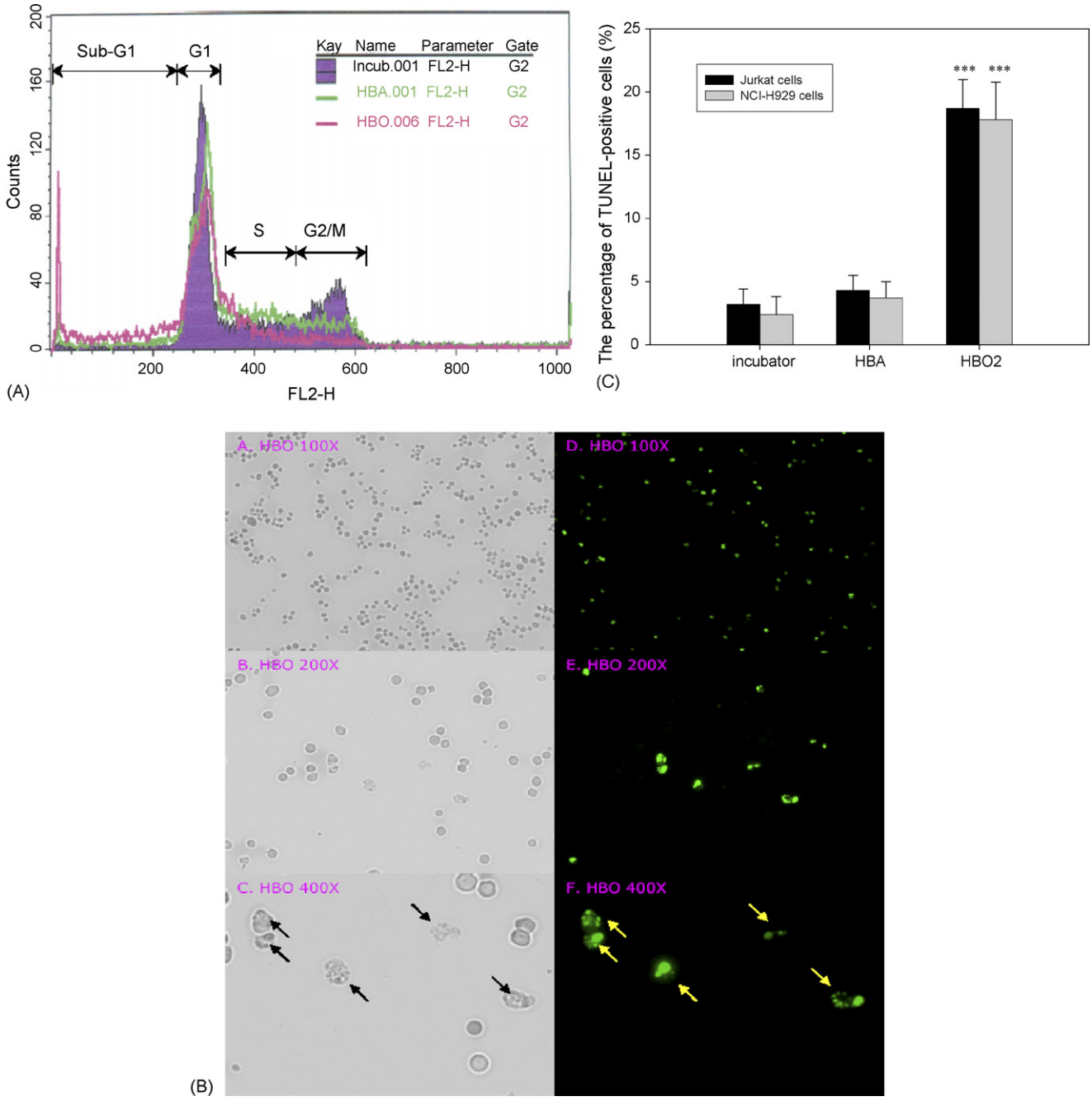

Fig. 2. (A) Effects of HBO_2 exposure on cell cycle in Jurkat cells. Cell cycle was examined at 8 h after HBO_2 or HBA treatment. DNA degradation was determined by staining intracellular DNA with PI, followed by flow cytometry analysis. (B) Effects of HBO_2 exposure on nucleus fragmentation in Jurkat cells by TUNEL assay (A: 100×; B: 200×; C: 400× under light microscope; D: 100×; E: 200×; F: 400× under fluorescence microscope). Apoptotic ratio was determined by measuring numbers of TUNEL positive cells in 300 total cells. (C) The percentage of TUNEL-positive cells of Jurkat and NCI-H929 cells in groups of cells was measured. $^{***}P<0.001$ when comparing HBO_2-exposed cells to control cells.

integrity. Collapse of $\Delta\Psi_m$ was detected by using $DiOC_6$[3], a mitochondrion-selective potentiometric probe. The $\Delta\Psi_m$ of Jurkat and NCI-H929 cells were examined 8 h after HBO_2 treatment. The gradient change in the cell lines after HBO_2 treatment was significant compared with control and HBA groups ($P<0.001$) (Fig. 4).

3.5. HBO_2 induced caspase-3 and caspase-7 activation, leading to the cleavage of PARP on Jurkat and NCI-H929 cells

Since members of the caspase family are crucial mediators of apoptosis, protein expression of caspase family and

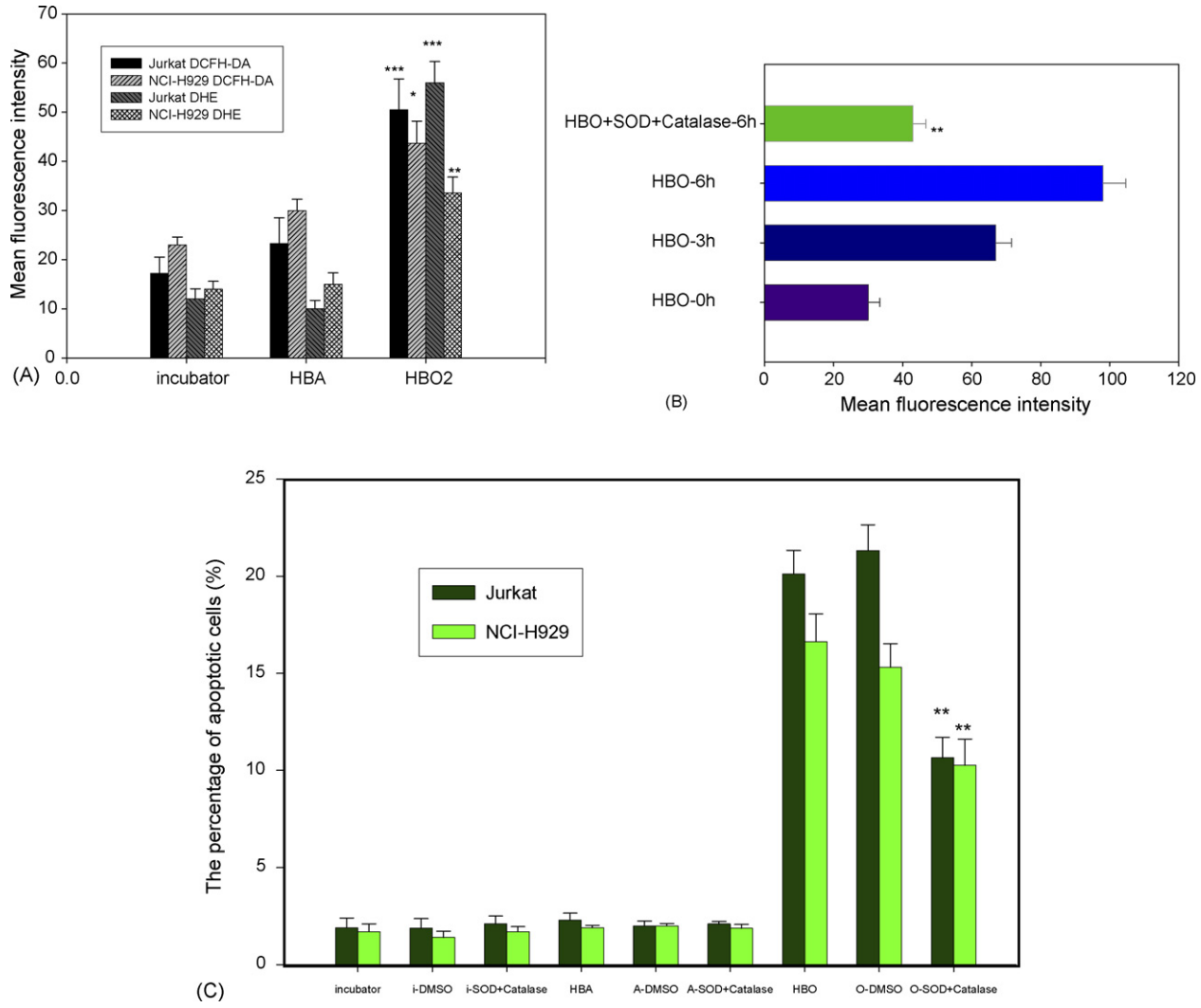

Fig. 3. Intracellular hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet -}$) production in Jurkat and NCI-H929 cells after HBO_2 exposure. Cells were measured for intracellular H_2O_2 and $O_2^{\bullet -}$ immediately after HBO_2 or HBA treatment. (A) The production of H_2O_2 by 2′, 7′-dichlorodihydrofluorescin diacetate (DCFH-DA) fluorescence probe and the generation of superoxide by dihydroethidium (DHE) fluorescence intensity was increased in HBO_2-treated cells. $^{***}P < 0.001$ was emphasized when comparing Jurkat HBO_2-exposed cells to incubator cells. While $^{*}P < 0.05$ was indicated when comparing NCI-H929 HBO_2-exposed cells to incubator cells. (B) The increment of intracellular superoxide was inhibited by SOD and catalase as measured by DHE fluorescence intensity in Jurkat cells. $^{**}P < 0.01$ when comparing (SOD + catalase)-treated Jurkat cells to untreated cells after 3.5 ATA 6 h of HBO_2 exposure. (C) Antioxidants inhibited HBO_2-induced apoptosis in both Jurkat and NCI-H929 cells. $^{**}P < 0.01$ when comparing (SOD + catalase)-treated cells to untreated cells after HBO_2 exposure. To control for the effect of DMSO-inducing cell apoptosis, cells were diluted in DMSO and detected for the percentage of apoptosis by Annexin V/PI staining. I-DMSO, A-DMSO and O-DMSO were represented as cells with DMSO in either incubator, HBA or HBO_2 treatment.

PARP in Jurkat and NCI-H929 cells was assessed at 6 h after HBO_2 or HBA exposure. After HBO_2 treatment, the cleaved caspase-3 (17 and 19 kDa) was shown in Jurkat and NCI-H929 cells. The cleaved caspase-7 (20 kDa) was also demonstrated after HBO_2 exposure and led to the cleavage of PARP in Jurkat and NCI-H929 cells (Fig. 5).

3.6. HBO_2 increased phosphorylation of p38 MAPK in both Jurkat and NCI-H929 cells

To determine if there is an association of p38 MAPK phosphorylation and HBO_2-induced apoptosis of Jurkat and NCI-H929 cells, the expression of mitogen-activated protein kinases (MAPKs) was assessed at 3 h after exposure to HBO_2 or HBA treatment. After HBO_2 treatment, the phosphorylation of p38 MAPKs was increased in both Jurkat and NCI-H929 cells compared with control and HBA-treated cells, while phosphorylation of ERK1/2 was decreased only in Jurkat cells compared with controls (Fig. 6A and B). There was no significant difference in the phosphorylation of JNK among the three conditions for both cell lines (Fig. 6B). Moreover, HBO_2 treatment did not significantly increase p38 MAPK phosphorylation in A549 and MCF-7 cell lines (Fig. 6C).

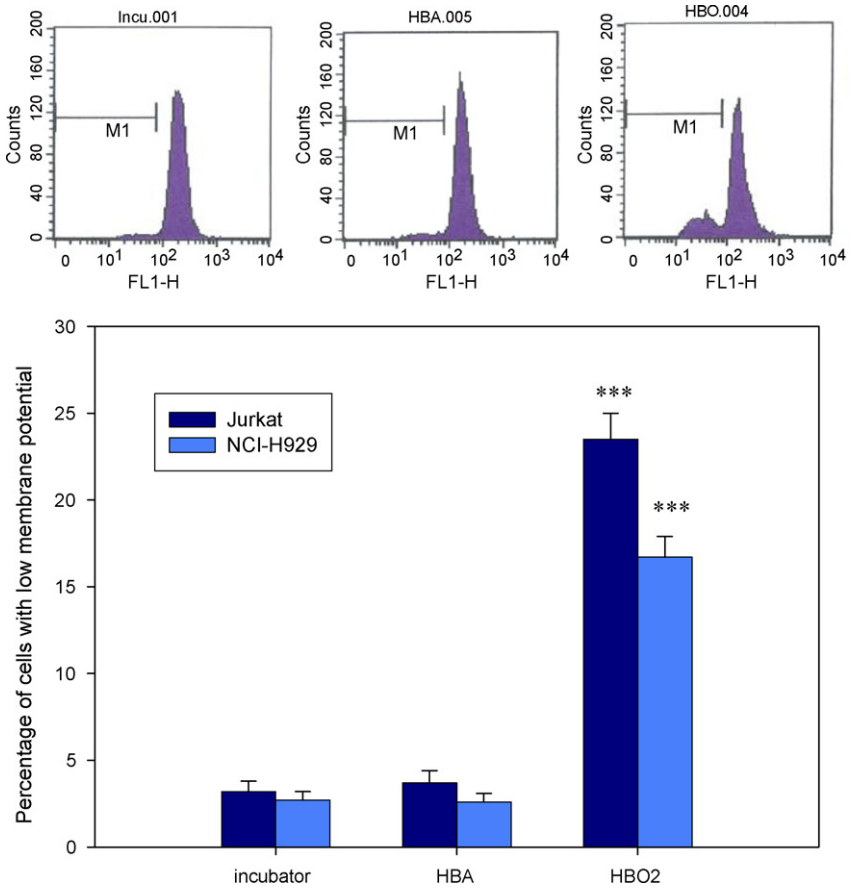

Fig. 4. Collapse of mitochondrial membrane potential in Jurkat cells after HBO_2 exposure. Percentage of Jurkat and NCI-H929 cells with low membrane potential in control, HBA-exposed, and HBO_2-exposed cells. $^{***}P < 0.001$ when comparing HBO_2-exposed cells to control cells.

4. Discussion

Our results argue that cells of hematopoietic origin have a lower threshold to oxidative stress induced by hyperbaric oxygen treatment and HBO_2-induced apoptosis of hematopoietic derived cancer cells may be through the intracellular accumulation of H_2O_2 and $O_2^{\bullet -}$ as well as the involvement of phosphorylation of p38 MAPK. The comparison of incubator, HBA and HBO_2-treated cells suggest that oxygen and pressure are both important in inducing hematopoietic cancer cells to undergo apoptosis.

Kalns and associates reported the effects of hyperbaric oxygen on the growth of two prostate cancer cell lines in cell culture [15,16]. In this study, both cell lines had their growth suppressed by 8.1% and 2.7%, respectively, relative to normobaric controls, after a 90-min exposure to 3.0 ATA of 100% oxygen. Ganguly et al. [17] had explored that HBO_2 enhances spontaneous hematopoietic HL-60 cell apoptosis in a time- and caspase-dependent manner. To control for the effects of hyperoxia, HL-60 cells were exposed to 95% O_2 and 5% CO_2 at 1 ATA and simple hyperoxia alone had only minimal effect on apoptosis in HL-60 cells. HBO_2 also enhanced stimulus-induced apoptosis in hematopoietic Jurkat cells stimulated with anti-Fas antibody underwent 44% more apoptosis when exposed to HBO_2. However, our unpublished data did not show different levels of Fas and FasL antigen expression in Jurkat and NCI-H929 cells after HBO_2 exposure. It is possible that mitochondria-mediated pathway other than receptor-mediated pathway might be crucial in HBO_2-induced apoptosis.

Mitochondria play an important role in the regulation of cell death. Mitochondria have the ability to promote apoptosis through release of cytochrome *c*, which together with Apaf-1 and ATP forms a complex with pro-caspase 9, leading to activation of caspase 9 and the caspase cascade. In addition, anti-apoptotic members of the Bcl-2 family of proteins, such as Bcl-2 and Bcl-XL, are located in the outer mitochondrial membrane and act to promote cell survival [18,19]. It has been suggested that Bax can form a transmembrane pore across the outer mitochondrial membrane, leading to loss of membrane potential and efflux of cytochrome *c* and apoptosis inducing factor (AIF). Bcl-2 and Bcl-XL act to prevent this pore formation. Budinger et al. [20] suggested that a higher concentration of oxygen would induce an interaction among Bax, Bad, and Bcl-XL, further leading cells to apoptosis. However, other studies also demonstrated that Bax, Bad, Bcl-2, and Bcl-XL were not involved in oxygen-induced apoptosis [21]. Jurkat cells are considered

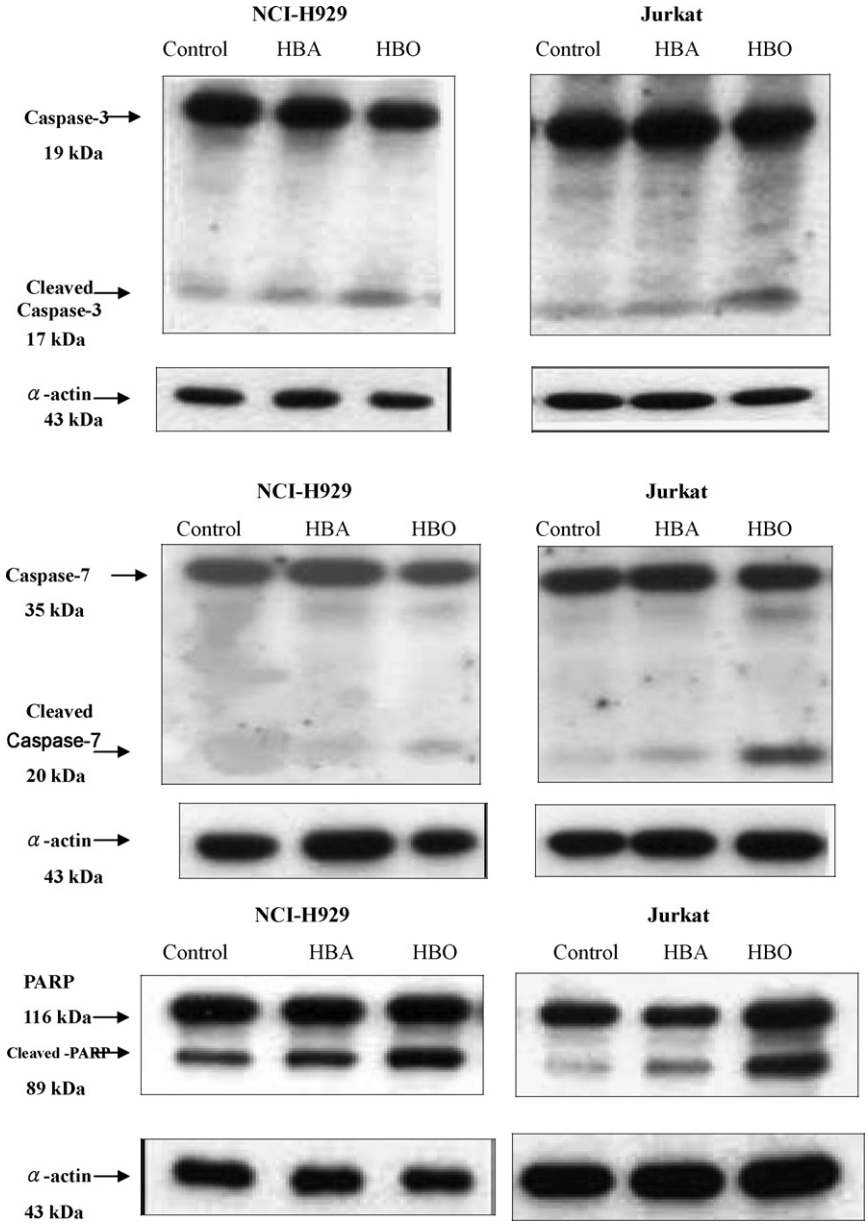

Fig. 5. HBO_2 exposure led to cleavage of caspase-3, caspase-7, and PARP in both Jurkat and NCI-H929 cells.

as type II cells that usually have poor ability to assemble the death-inducing signaling complex (DISC) complex for ligand-signaling and thus, a predominant mode of cell death seen in these cells is mitochondrially-mediated [22]. In our studies, in spite of collapse of mitochondrial transmembrane potential after HBO_2 exposure, there were no significant differences in the expressions of Bcl-2 and Bak after HBO_2 treatment (data not shown). This may further support the argument that activation of p38 most likely stemmed from the activation of receptor-mediated apoptosis in our study.

The mitogen-activated protein kinase (MAPK) superfamily, including the extracellular signal-related protein kinases (ERKs), the c-Jun N-terminal kinases (JNK), and the p38 kinases, is involved in stress-induced apoptosis; activation of JNK or p38 kinase and inhibition of phosphorylation of ERK can promote apoptosis [23]. The results of western blot analysis in our model revealed that both inhibition of ERK phosphorylation and activation of p38 kinase were involved in HBO_2-induced Jurkat cell death. However, there was no significant difference in JNK phosphorylation after HBO_2 exposure. Cell death is thought to be either apoptotic, with shrinking phenotypes and activated caspases, or necrotic, with swelling organelles. Exposure to hyperoxia (95% O_2) for 3 days induced cell necrosis and triggered a sustained activation of p38 MAPK and JNK in MLE-12 cells [24]. In a study by Kannan et al. [25], both ERK1/2 phosphorylation and p38 activity were increased after 2 days of exposure

to hyperoxia 95% O_2 in A549 cells. Overexpression of 8-oxoguanine DNA glycosylase (hOgg1) reduced hyperoxic toxicity in A549 cells. However, the phosphorylation of p38 MAPK was not significantly increased in A549 and MCF-7 cells after HBO_2 treatment in our study. The cell type, the partial pressure of oxygen, as well as the exposure time for oxygen treatment might play important roles in HBO_2-induced cell apoptosis.

In animal models of neurotoxicity, ischemia, or nephrotoxicity, hyperbaric oxygen treatment seems to offer cell protection and seems to be anti-apoptotic [26–28]. Similar observations have been made in case of human oral

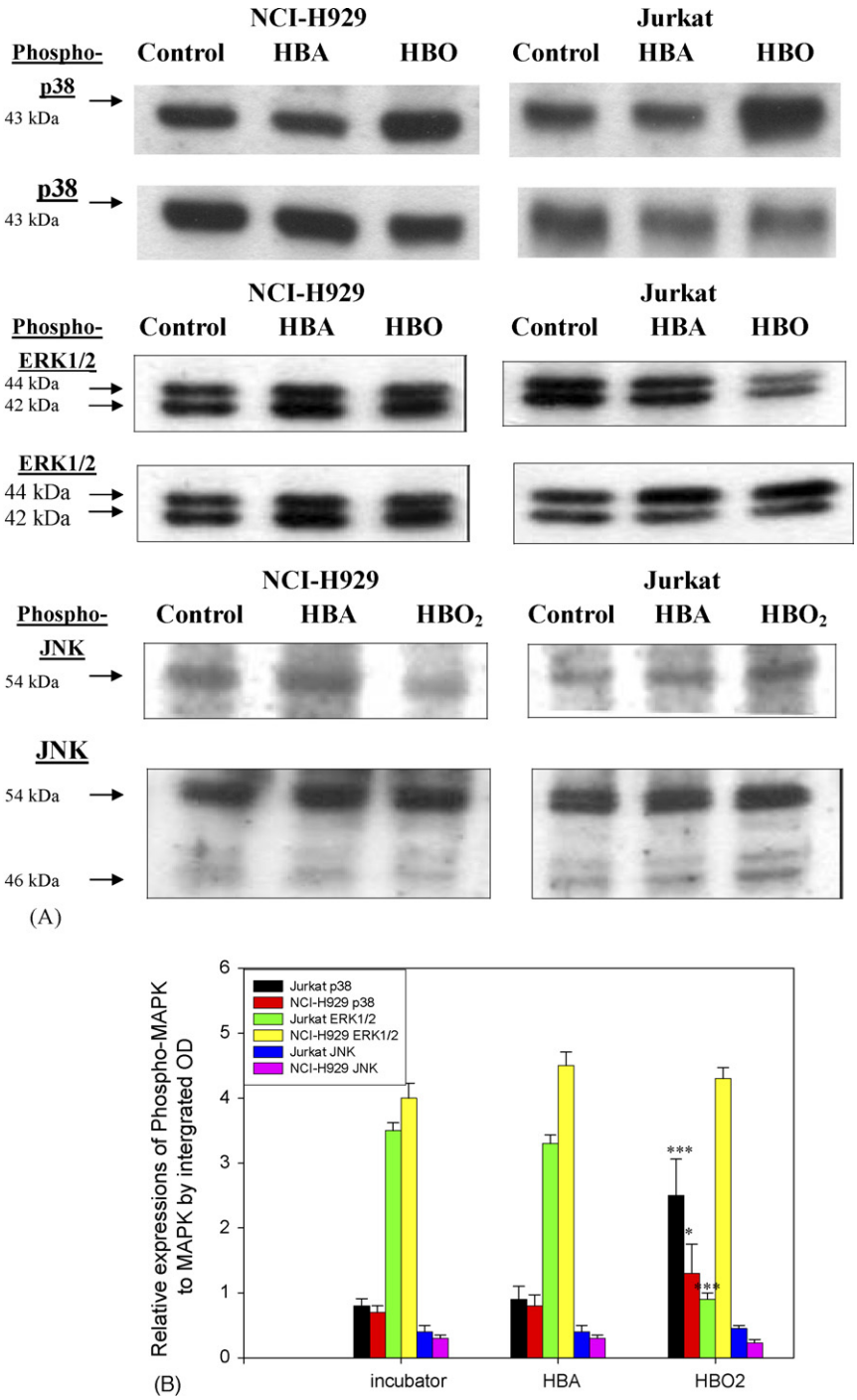

Fig. 6. (A) The expressions of MAPKs (p38, ERK1/2 and JNK) after HBO_2 exposure on Jurkat and NCI-H929 cells in western blotting analysis. (B) Relative expressions of phospho-(p38, ERK1/2 or JNK)/actin to (p38, ERK1/2 or JNK)/actin in Jurkat and NCI-H929 cells were compared after one D-scan analysis. $^{***}P < 0.001$ was emphasized when comparing HBO_2-exposed Jurkat cells to control cells. $^{*}P < 0.05$ when comparing HBO_2-exposed NCI-H929 cells to control cells. (C) Relative expressions of phospho-p38/actin to p38/actin in A549 and MCF-7 cells were compared after one D-scan analysis.

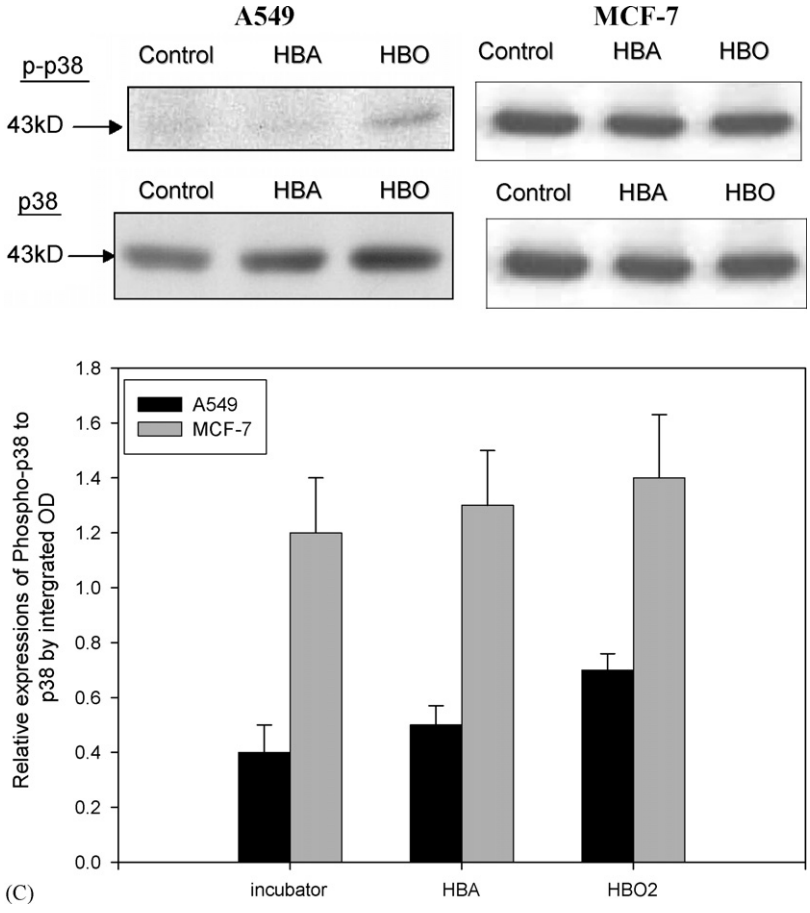

Fig. 6. (*Continued*).

cancer cells and mammary epithelial cells [29,30]. These findings are in line with our results with non-hematopoietic cell lines. Although, HBO_2 did not induce apoptosis in A549 and MCF-7 cells, hyperbaric oxygen inhibited benign and malignant human mammary epithelial cell proliferation but did not promote apoptosis in MCF-7 cells [30]. These observations pose a question if hyperbaric oxygen may predominantly exert apoptotic effects on hematopoietic cells, while inhibiting proliferation on non-hematopoietic cells. Such an observation may have significant implications both in cancer and tissue degenerative disorders. These studies might also provide more strategies about how we apply HBO_2 therapy to tumor malignancy. Future studies will focus on establishing tumor-transferred SCID mice in order to investigate the potential role of HBO_2 treatment in cancer therapy.

Acknowledgments

This study was supported in part by a grant-in-aid from the National Science Council (NSC 93-2314-B-016-026) and CY Foundation for Advancement of Education, Sciences and Medicine.

Contributions. Our PhD student, Yen-Chen Chen was responsible for the conception, design, flow cytometry analysis and data collection, interpretation, assembling of data and drafting the article. Dr. Shao-Yuan Chen and Dr. Huey-Kang Sytwu contributed to the concept and design of this research and critical revision of the article for final approval of the article. Mr. Pey-Shen Ho, Mrs. Chia-Huei Lin and Miss. Ya-Yuan Cheng were responsible for cell culture, flow cytometry and western blotting technical assistance. We want to thank Dr. Jehng-Kang Wang for his substance supply in western blotting analysis.

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