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Effect of Hyperbaric Oxygen on the Anticancer Effect of Artemisinin on Molt-4 Human Leukemia Cells

YUSUKE OHGAMI[1*], CATHERINE A. ELSTAD[1], EUNHEE CHUNG[1*],
DONALD Y. SHIRACHI[3], RAYMOND M. QUOCK[1] and HENRY C. LAI[2]

[1]*Department of Pharmaceutical Sciences, College of Pharmacy, Washington State University, Pullman, WA 99164, U.S.A.;*
[2]*Department of Bioengineering, University of Washington, Seattle, WA 98195, U.S.A.;*
[3]*Chico Hyperbaric Center, Chico, CA 95926, U.S.A.*

Abstract. *Background: Artemisinin selectively kills cancer cells which have more intracellular free iron than do normal cells. Hyperbaric oxygen (HBO_2) may be beneficial in the treatment of cancer. The hypothesis of this study was that HBO_2 enhances anticancer activity of artemisinin. Materials and Methods: After pretreatment with 12 μM holotransferrin, Molt-4 human leukemia cells were cultured in 10 μM artemisinin and exposed for 90 min to one of three different conditions: control, room air control, and HBO_2. Cell growth was determined for 48 h after exposure. Results: Differences in growth were noted after 6 h of incubation. After 48 h of incubation, growth of cells treated with artemisinin alone or HBO_2 alone was 85% of that of cells grown under artemisinin-free control conditions. Combined artemisinin and HBO_2 treatment resulted in an additional 22% decrease in growth. Conclusion: Combined HBO_2 and artemisinin exposure may be an effective anticancer chemotherapeutic strategy.*

Artemisinin (qinghaosu) is a sesquiterpene lactone that was initially isolated from the wormwood plant *Artemisia annua L.* (qinghao) in 1971 (1). In addition to its antimalarial activity, artemisinin has more recently been reported to exert a cytotoxic effect on cancer cells (2,3). This cytotoxic effect results from reactive oxygen species that are formed when two oxygen atoms linked together in an endoperoxide bridge in the artemisinin molecule react with free iron atoms. Cancer cells require and take up large amounts of free iron in order to proliferate (4). Hence, they are more susceptible than normal mammalian cells to the cytotoxic effect of these free oxygen radicals.

Hyperbaric oxygen (HBO_2) therapy is a complementary therapy that involves intermittent delivery of 100% oxygen at elevated atmospheric pressures for limited periods of time (60-90 min). Under hyperbaric conditions, the hemoglobin becomes saturated with oxygen and further hyperoxygenation of the blood occurs by oxygen dissolving within the plasma. In humans, breathing room air (21% O_2) at 1.0 atm, alveolar pO_2 (pAO_2) is approximately 102 mmHg, and breathing 100% oxygen increases the pAO_2 to 673 mmHg. During HBO_2 therapy, the pAO_2 increases rapidly as the pressure in the hyperbaric chamber increases. At 2.0 atm, the pAO_2 rises to 1433 mmHg; at 2.5 atm, it is approximately 1813 mmHg, a 17-fold increase as compared to breathing air at 1.0 atm (5). There is evidence that administration of HBO_2 can enhance the delivery of oxygen to hypoxic tumor cells and increase their susceptibility to the cytotoxic effects of radiation and chemotherapy (6-9).

Since oxygen free radicals are formed from oxygen, it was hypothesized in this study that the anticancer activity of artemisinin can be enhanced by an increase in oxygen tension. To test this hypothesis, a human leukemia cell line (Molt-4-lymphoblastoid cells) that has been shown to be selectively killed by artemisinin when compared to normal human lymphocytes was used (2, 3). Experiments were designed to assess cell death in different groups treated with artemisinin and/or hyperbaric oxygen with appropriate controls.

Materials and Methods

Cell culture. Molt-4 cells were maintained in RPMI-1640 with 10% FBS. For experiments, cells (3.2×10^5 cells/ml) were pretreated with 12 μM human holotransferrin at 37˚C and 5% CO_2/95% room air for 1 h. Holotransferrin served as a source of iron for the cells (2). Artemisinin was dissolved in dimethylsulfoxide (DMSO). RPMI-

**Present address:* Department of Scientific Criminal Investigation, Chungnam National University, 200 Gung-Dong Yuseong-Gu Daejon 305-764, South Korea

Correspondence to: Catherine A. Elstad, Department of Pharmaceutical Sciences, College of Pharmacy, Washington State University, Pullman, WA 99164, U.S.A. e-mail: elstad@wsu.edu

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1640 with 10% FBS was added to the dissolved artemisinin to attain final concentrations of 10 μM artemisinin and 1% DMSO. Artemisinin (10 μM) and DMSO vehicle were added to cells cultures which were subsequently exposed for 90 min to one of three different conditions: control (5% CO_2/95% room air at 37˚C and normal atm); room air (room air at room temperature (23˚C) and normal atm); and HBO_2 (100% O_2 at room temperature and 3.5 atm). In addition to these groups, there were three additional groups of cells that were not pretreated with artemisinin prior to exposure to control, room air and HBO_2 conditions as described; all of these groups contained 1% DMSO vehicle. HBO_2 exposure took place in a B-11 research hyperbaric chamber (Reimers Systems, Inc., Lorton, VA, USA). Following 90-min exposure to these conditions, cells were transferred to an incubator with 5% CO_2/95% room air at 37˚C throughout the rest of the experiment. Cell growth and viability were determined at 1, 2, 4, 6, 12, 24 and 48 h. Cell numbers were counted using a hemacytometer, and viability was determined using trypan blue.

Reagents and drugs. The following reagents were used in this study: fetal bovine serum (FBS), human holotransferrin and dimethyl sulfoxide (DMSO) (Sigma Aldrich/Research Biochemicals Inc., Natick, MA, USA) and RPMI-1640 (Gibco, Grand Island, NY, USA). Artemisinin was a gift from Holley Pharmaceuticals (Fullerton, CA, USA).

Statistical analysis. The mean number of Molt-4 cells in different treatment groups was compared using a two-way analysis of variance (2-way ANOVA). When a significant main treatment effect was found, *post-hoc* analysis was performed using the Bonferroni's multiple comparison test (Graphpad Prism; Graphpad Software, San Diego, CA, USA).

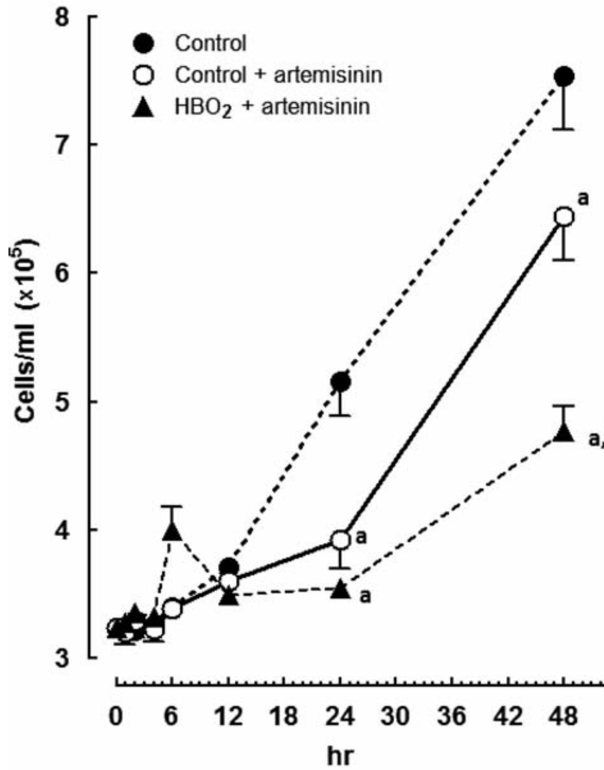

Figure 1. *Growth of Molt-4 leukemia cells treated with Artemisinin or Artemisinin+HBO_2. Cell numbers ($\times 10^5$/ml) represent the mean (±SEM) of 8 experiments. Significant differences (p<0.05): a, significantly different from Control group; and b, significantly different from Control+Artemisinin group.*

Results

Cell growth and viability were determined at 1, 2, 4, 6, 12, 24 and 48 h [Treatment: $F(5,336)=16.21$, $p<0.0001$; Time: $F(7,336)=334.7$, $p<0.0001$; Interaction: $F(35,336)=9.981$, $p<0.0001$]. Significant differences in growth among treatments were noted after six h of incubation. After 48 h of incubation, the growth of cells treated with artemisinin alone or exposed to HBO_2 alone was 85% of the growth of those cells grown under artemisinin-free control conditions. Combined artemisinin treatment and HBO_2 exposure caused an additional 22% decrease in growth (Table I). Figure 1 highlights selected data from Table I which show that an increase in oxygen tension does indeed enhance the anticancer activity of artemisinin.

Discussion

The anticancer effect of artemisinin with the involvement of intracellular iron was first demonstrated in cell culture against Molt-4 lymphoblastoid leukemia cells (10). Two hundred μM dihydroartemisinin (an analog of artemisinin) co-incubated with holotransferrin killed all the Molt-4 cells within 8 h. It was 100 times less toxic to human lymphocytes in culture under the same conditions. Holotransferrin is iron-loaded transferrin and increases the potency of the cytotoxic effect of artemisinin by delivering iron into cancer cells (2, 3).

The anticancer activity of artemisinin has been confirmed in a number of studies. Dihydroartemisinin and holotransferrin kills 98% of human breast cancer cells *in vitro*; cancer cells undergo apoptosis and necrosis, while normal breast cells are unaffected by artemisinin (10). Artemisinin is also effective in producing apoptosis in human liver cancer cells (11, 12), breast cancer cells (13), lung carcinoma cells (14), oral cancer cells (15) and other human tumor cell lines (16).

Several mechanisms have been proposed to explain the anticancer activity of artemisinin. In addition to production of cytotoxic reactive oxygen species, artemisinin has also been reported to suppress TNF-α-induced production of proinflammatory cytokines (17), inhibit hypoxia-inducible factor 1α activation (18), directly damage DNA (19), reduce levels of estrogen receptor-alpha (20), induce apoptosis (21, 22), inhibit angiogenesis (23, 24), and suppress metastasis (25).

The current results show that as individual treatments, artemisinin and HBO_2 were each effective in significantly

Table I. *Growth of Molt-4 leukemia cells treated with artemisinin, hyperbaric oxygen (HBO_2), or artemisinin+HBO_2 conditions. Cell numbers ($\times10^5$/ml) represent the mean (±SEM) of 8 experiments. Significant differences ($p<0.05$): [a]significantly different from Control group; [b]significantly different from Control+Artemisinin group; [c]significantly different from Room Air group; [d]significantly different from Room Air + Artemisinin group; and [e]significantly different from HBO_2 group.*

Time (h)	Treatment					
	Control§	Control + Artemisinin	Room Air§	Room Air + Artemisinin	HBO_2§	HBO_2 + Artemisinin
0	3.24±0.07	3.24±0.07	3.24±0.07	3.24±0.07	3.24±0.07	3.24±0.07
1	3.21±0.06	3.21±0.10	3.23±0.05	3.30±0.14	3.24±0.05	3.28±0.04
2	3.22±0.07	3.29±0.13	3.33±0.03	3.24±0.05	3.39±0.07	3.36±0.03
4	3.26±0.06	3.23±0.10	3.30±0.03	3.26±0.06	3.29±0.04	3.32±0.03
6	3.40±0.05	3.39±0.05	3.46±0.06	3.65±0.21	3.87±0.18	4.00±0.18[a,b]
12	3.71±0.07	3.60±0.04	3.66±0.08	3.64±0.08	3.60±0.07	3.49±0.06
24	5.16±0.27	3.92±0.22[a]	5.42±0.13[b]	3.82±0.09[a,c]	4.26±0.13[a,c]	3.55±0.07[a,c,e]
48	7.54±0.42	6.44±0.34[a]	7.57±0.36[b]	5.69±0.14[a,b,c]	6.49±0.33[a,c,d]	4.77±0.19[a,b,c,d,e]

§Cells not exposed to artemisinin include control (5% CO_2/95% room air at 37˚C and normal ATA); room air (room air at room temperature and normal ATA); and HBO_2 (100% O_2 at room temperature and 3.5 ATA) groups. All data are given as ±the standard error of the mean.

decreasing the growth of Molt-4 human leukemia cells. Interestingly, after 24 and 48 h of incubation, artemisinin and HBO_2 had similar effects; growth of Molt-4 cells exposed to either artemisinin alone or HBO_2 alone was 85% of the growth of controls. When used in combination, artemisinin and HBO_2 caused an additional 22% decrease in growth.

In summary, these results confirm the anticancer activity of artemisinin and HBO_2 as single agents. More importantly, these findings suggest the possibility that combined artemisinin and HBO_2 exposure might be developed into a chemotherapeutic strategy for cancer treatment, with effectiveness increased over that of artemisinin or HBO_2 alone.

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