Combined Effect of Hyperbaric Oxygen and N-Acetylcysteine on Fibroblast Proliferation

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Objective: To examine the combined effect of hyperbaric oxygen and N-acetylcysteine, a well-studied antioxidant, on fibroblast proliferation and production of 3 specific growth factors: basic fibroblast growth factor, vascular endothelial growth factor, and transforming growth factor β1.

Design: In vitro study.

Subjects: None.

Interventions: Human dermal fibroblasts were propagated in serum-free medium and subjected to daily 90-minute 2-atm hyperbaric oxygen treatments with varying concentrations of N-acetylcysteine for 7 consecutive days. Cell proliferation and growth factor assays were performed on days 0, 1, 3, 5, and 7.

Results: Population doubling time decreased significantly with 40 µmol/L of N-acetylcysteine supplementation of 2-atm hyperbaric oxygen treatment. Higher levels of N-acetylcysteine increased population doubling time.

Conclusions: Supplementation of hyperbaric oxygen therapy with 40 µmol/L of N-acetylcysteine appears to increase fibroblast proliferation without producing an unfavorable growth factor profile for normal healing. This suggests that this level of N-acetylcysteine may foster an ideal redox environment for fibroblast proliferation in a hyperbaric oxygen environment.


Hyperbaric oxygen (HBO) has been recognized for decades as an appropriate treatment modality for more than a dozen clinical conditions ranging from decompression sickness and arterial gas embolism to the treatment of thermal burns. Raising oxygen content in cells via 100% oxygen administration at greater than normal atmospheric levels has also been indicated as a treatment for problem wounds. Although the mechanisms remain unclear, several studies have shown HBO therapy to offset poor wound healing associated with tissue hypoxia. In addition to promoting an environment less hospitable to anaerobes, the presence of oxygen is known to speed the process of wound healing, whether from being required for the production of collagen matrix and subsequent angiogenesis, from the presence and beneficial effects of reactive oxygen species (ROS), or from yet undetermined means.

Relatively few studies have examined the effect of HBO on cultured cells in vitro. Dimitrijevich et al studied the effect of HBO on human skin cells in culture and human dermal and skin equivalents and found an increase in fibroblast proliferation, collagen production, and keratinocyte differentiation at pressures up to 3 atm for daily 90-minute treatments during 10 days. Roberts and Harding showed that HBO exposure increased production of glycosaminoglycans in cultured fibroblasts but that fibroblast proliferation actually decreased 7%. Recently, our laboratory reported that HBO treatment up to 2.0 atm enhances proliferation and autocrine growth factor production of normal human fibroblasts grown in a serum-free culture environment.

The administration of HBO has also been shown to have negative effects, due perhaps to the toxic effects of hypoxia from ROS, or from the mechanically induced effects of high atmospheric pressure. Tompach et al showed maximal proliferation of fibroblasts at 2.4 atm of HBO, with declined cellular activity at 4 atm. Dimitrijevich et al showed increased growth at 1 and 2.5 atm of HBO, but no benefit at 3 atm. Our laboratory showed no benefit beyond or below 2 atm of HBO. This indicates that there is a delicate balance between having enough and too much oxygen and/or atmospheric pressure when promoting fibroblast growth.
Recent publications suggest that finding the balance requires an understanding that low concentrations of ROS may play a beneficial role(s) in wound healing, contrasting the common view that all ROS are damaging to cells. Oxidizing species such as free radicals and hydrogen peroxide may serve as cellular messengers mediating complex redox-sensitive processes such as extracellular matrix formation, cytokine action, angiogenesis, and cell motility. However, high levels of ROS and hydrogen peroxide (3% is used as a clinical disinfectant) still prove toxic to cells.

Glutathione is a cysteine-containing tripeptide whose redox properties are essential for the viability and function of virtually all cells, ranging from intracellular defense against oxidative stress to regulation of signal transduction and systemic defense against drug toxicity. In periods of oxidative stress, such as the presence of overwhelming ROS, glutathione levels in the cell decrease. In addition, studies have shown that glutathione levels decrease to 60% to 70% of normal after wound insult. N-acetylcysteine (NAC) is a prodrug that supplies bioavailable cysteine for glutathione replenishment and prevents oxidative damage.

The purpose of this study was to examine the combined effect of HBO with NAC, a well-studied antioxidant, on fibroblast growth and production of 3 specific growth factors: basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and transforming growth factor β1 (TGF-β1). Experiments were conducted with a serum-free model to allow measurement of these growth factors without the confounding variables associated with serum-containing media.

FIBROBLAST PRIMARY CULTURES

A fibroblast cell line was established from skin obtained from the upper eyelid of a 68-year-old man. Approval to use operative specimens that would otherwise be discarded was obtained in advance from the human subjects committee at Stanford University, Stanford, Calif. With the use of sterile technique in a sterile laminar flow hood, operative specimens were washed free of blood in Dulbecco phosphate-buffered saline (PBS) (Gibco, Grand Island, NY). Epidermal fat and tissues were removed, and the remaining dermis was minced into 1-mm³ fragments. The specimens were washed again in PBS and then transferred to scored 75-cm² tissue culture flasks (BD BioCoat Collagen Type I; BD Biosciences, San Jose, Calif) with 10 mL of primary culture medium (Dulbecco Modified Eagle Medium containing 1% l-glutamine, 10% fetal bovine serum, and 1% penicillin-streptomycin-amphotericin) (Gibco). The flasks were then stored in a humidified incubator at 37°C with a 5% carbon dioxide atmosphere.

After 24 hours, the medium was changed with 10 mL of primary culture medium, and it was changed every 2 to 4 days until fibroblasts were visualized under a light microscope to be growing from explanted tissue. After significant fibroblast cell growth, explant tissue was removed and adhered cells were subcultured into additional 75-cm² flasks by washing with PBS, exposing cells to 5 mL of 0.05% trypsin (Gibco) in PBS for 5 minutes, neutralizing trypsin with 10 mL of primary culture medium, and then distributing to new flasks. Primary culture medium was changed every 4 days until plates reached confluence.

CELL PLATING

IN SERUM-FREE MEDIUM

Confluent cells from the first passage were washed 3 times with 10 mL of PBS and released from the flask wall with the use of 5 mL of 0.05% trypsin for 5 minutes at 37°C. The trypsin was then inactivated with soybean trypsin inhibitor (Gibco) in a 1:1 ratio. Cells were then centrifuged, suspended in a serum-free medium with 1-glutamine (UltraCULTURE; Cambrex, Walkersville, Md), and counted in duplicate by means of a hemocytometer. Cell viability was confirmed with trypan blue dye exclusion. Fibroblasts were then diluted in the serum-free culture medium or medium plus NAC (Sigma-Aldrich Co, St Louis, Mo) at densities appropriate for each assay. For the water-soluble tetrazolium salt (WST-1) assay determining cell proliferation and growth curve generation, fibroblasts were seeded at 1 × 10⁵ cells per 100 µL of the serum-free culture medium in 96-well plates (Falcon; BD Biosciences) for each concentration studied: 5 mmol/L, 1 mmol/L, 200 µmol/L, and 40 µmol/L of NAC. For enzyme-linked immunosorbent assays (ELISAs) measuring growth factor production, cells were seeded at 6 × 10⁵ cells per 500 µL in 24-well plates. Cells remained in the same initial suspension (control or experimental with NAC) through the course of the 7-day experiment.

HBO ADMINISTRATION

Fibroblast cultures within the plates were transferred to and sealed in an HBO chamber (BioTime, Berkeley, Calif) and exposed to 2 atm of 100% oxygen at room temperature daily for 90 minutes for 7 consecutive days. Fibroblast cultures within the plates were transferred to and sealed in an HBO chamber (BioTime, Berkeley, Calif) and exposed to 2 atm of 100% oxygen at room temperature daily for 90 minutes for 7 consecutive days. Oxygen was then inactivated with soybean trypsin inhibitor serum, and the remaining dermis was minced into 1-mm³ fragments. The specimens were washed again in PBS and then transferred to scored 75-cm² tissue culture flasks (BD BioCoat Collagen Type I; BD Biosciences, San Jose, Calif) with 10 mL of primary culture medium (Dulbecco Modified Eagle Medium containing 1% l-glutamine, 10% fetal bovine serum, and 1% penicillin-streptomycin-amphotericin) (Gibco). The flasks were then stored in a humidified incubator at 37°C with a 5% carbon dioxide atmosphere. After 24 hours, the medium was changed with 10 mL of primary culture medium, and it was changed every 2 to 4 days until fibroblasts were visualized under a light microscope to be growing from explanted tissue. After significant fibroblast cell growth, explant tissue was removed and adhered cells were subcultured into additional 75-cm² flasks by washing with PBS, exposing cells to 5 mL of 0.05% trypsin (Gibco) in PBS for 5 minutes, neutralizing trypsin with 10 mL of primary culture medium, and then distributing to new flasks. Primary culture medium was changed every 4 days until plates reached confluence.

METHODS

CELL COUNTING AND GROWTH CURVE GENERATION

Cell counts were obtained with the WST-1 assay (Takara Mi-rus Bio Inc, Madison, Wis) at 1, 3, 5, and 7 days after initiation of HBO treatment to obtain data to plot a growth curve. A day 0 baseline was also collected from cells 6 hours after transfer to the culture wells to confirm initial population size. Data for each day were collected from triplicate wells. The WST-1 assay is a colorimetric assay that tests proliferation and viability of cells based on the ability of viable cell mitochondrial dehydrogenases to cleave a tetrazolium salt (WST-1) substrate. Assays were read with an automated plate reader (ELx800; Bio-Tek Instruments Inc, Winooski, Vt). Optical densities were analyzed with KC4 software (Bio-Tek Instruments Inc). Cell counts were determined by comparison with a standard curve generated from known cell quantities. Growth curves were plotted from acquired data, and population doubling times (PDTs) were measured.

GROWTH FACTOR ASSAYS

Expression of bFGF, VEGF, and TGF-β1 at various times after treatment was measured by means of ELISA kits (R&D Systems, Minneapolis, Minn) from supernatants collected before that day’s HBO treatment at days 1, 3, 5, and 7. Samples des-
Ignated day 0 were collected 6 hours after the cells were introduced to the wells to establish pretreatment baseline levels of cytokines. Assays were read with an automated plate reader; optical densities were analyzed with KC4 software. Standard curves generated from known kit-provided concentrations of growth factors were determined with each ELISA.

STATISTICAL ANALYSIS

Differences in cell proliferation and population counts via WST-1 assay were compared by 2-tailed paired t test. The PDTs were also compared with the paired t test. Quantitative analysis of growth factor production via ELISA was analyzed by comparing pooled growth factor production in each well and then comparing this with the use of the paired 2-sample t test for means. Differences in data were considered significantly different at P<.05.

RESULTS

Fibroblast cultures were maintained in the serum-free culture medium successfully for the duration of the experiment. Cell proliferation was measured by means of WST-1 data (Figure 2). All cultures doubled in cell count between days 0 and 3, and ranged from 3 to 6 times the original count by day 7. Cells exposed to 2 atm of HBO and 5 mmol/L of NAC demonstrated erratic growth (not shown), with the population first growing, then shrinking, and then increasing again in size. At day 1, counts of cells exposed to HBO only and to HBO plus 40 µmol/L of NAC appeared lower than those of the non-HBO control and the conditions with higher (200-µmol/L to 1-mmol/L) NAC concentrations, with significant differences in comparing HBO plus 40 µmol/L of NAC with HBO plus 200 µmol/L of NAC (P = .02) and 1 mmol/L of NAC (P = .01). At day 7, HBO plus 40 µmol/L of NAC exhibited higher cell counts than HBO plus 200 µmol/L of NAC and HBO plus 1 mmol/L of NAC, although differences were not significant (P = .06 for both).

The WST-1 data were further analyzed by constructing logarithmic best-fit curves to determine PDTs (Table). The population exposed to HBO plus 40 µmol/L of NAC exhibited a significantly (P = .005) shorter PDT than the normal control. In addition, the population exposed to HBO plus 1 mmol/L of NAC had a significantly longer PDT than the HBO-only control.

Growth factor assays showed a gradual decline in total bFGF production between days 0 and 7 (Figure 3). Aside from the comparison of the normal control vs HBO plus 5 mmol/L of NAC at day 0 (where bFGF production was significantly lower in the HBO plus 5 mmol/L of NAC population), there were no statistical differences between conditions within days of analysis.

Total TGF-β1 production modestly increased for all conditions between days 0 and 7 (Figure 4). At day 7, TGF-β1 production in the 2-atm HBO plus 5-mmol/L NAC condition was significantly greater than the HBO-only control. Similarly, VEGF production increased during the course of the experiment (Figure 5). Overall production in the 2-atm HBO plus 5-mmol/L NAC population was significantly lower than in all other conditions (P<.05 for all comparisons) for days 1, 3, 5, and 7. At day 7, the amount of NAC introduced to cells treated with HBO appears negatively correlated with VEGF production, although statistical significance was not established.

Figure 1. The hyperbaric oxygen chamber used for this study (BioTime, Berkeley, Calif). Fibroblasts were exposed to daily 90-minute hyperbaric oxygen treatments at 2 atm. Normal control cells were exposed to ambient room pressure and oxygen content during treatments.

Figure 2. Mean growth curves of fibroblasts exposed to daily 2-atm 90-minute hyperbaric oxygen (HBO) treatments. The HBO-only control involved daily HBO treatments in normal media while the other experimental conditions used media containing 5-mmol/L (not shown), 1-mmol/L, 200-µmol/L, and 40-µmol/L of N-acetylcysteine (NAC). The HBO plus 40-µmol/L NAC counts were statistically significantly different from the HBO plus 200-µmol/L NAC and HBO plus 1-mmol/L NAC at days 1 and 7.
Although the range of clinical uses for HBO therapy is broad as defined by the Undersea and Hyperbaric Medical Society, the validation of HBO use as a modulator for wound healing has been hindered by a lack of understanding of its direct cellular effects. Previous in vitro studies have proposed that the mechanism of HBO action on wounds is complex and involves intricate interplay of oxidative species and cellular pathways.

The insult of a wound establishes hypoxia through inaccessibility of local vasculature and the energetic demands of regenerating tissue. Understanding that oxygen delivery is critical to healing as a nutrient and mediator of angiogenesis, cell motility, and extracellular matrix formation suggests that increasing the partial pressure of supplied oxygen facilitates wound repair. It has been shown that, in human fibroblasts, oxygenation is required for hydroxylation processes involved in collagen release. In addition, hyperoxia has been shown to enhance collagen and glycosaminoglycan synthesis, enhance bactericidal activity, and speed overall wound healing. Yet, the toxic effects of ROS or the mechanically induced effects of high atmospheric pressure establish an upper boundary for effective treatment, as HBO administration above 2 to 3 atm has been shown to inhibit fibroblast and keratinocyte differentiation and proliferation.

Recently, some wound-healing studies have suggested that, in contrast to the traditional understanding that they are inherently damaging to cells, the presence of low levels of ROS may actually be essential in the

<table>
<thead>
<tr>
<th>Condition</th>
<th>Population Doubling Time, h (Mean ± SD)</th>
<th>Comparison With Normal Control</th>
<th>Comparison With HBO-Only Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>63.6 ± 6.9</td>
<td>NA</td>
<td>.50</td>
</tr>
<tr>
<td>2 atm HBO-only control</td>
<td>119.4 ± 67.3</td>
<td>.50</td>
<td>NA</td>
</tr>
<tr>
<td>2 atm HBO + 1 mmol/L of NAC</td>
<td>224.1 ± 56.4</td>
<td>.13</td>
<td>.96</td>
</tr>
<tr>
<td>2 atm HBO + 200 µmol/L of NAC</td>
<td>123.6 ± 43.8</td>
<td>.12</td>
<td>.36</td>
</tr>
<tr>
<td>2 atm HBO + 40 µmol/L of NAC</td>
<td>36.6 ± 7.8</td>
<td>.005</td>
<td>.047</td>
</tr>
</tbody>
</table>

Abbreviations: HBO, hyperbaric oxygen; NA, not applicable; NAC, N-acetylcysteine.
wound-healing response. Gordillo and Sen\textsuperscript{15,16} postulate that each cell in the wound microenvironment enzymatically contributes to the development of ROS that conduct redox-sensitive messages between cells regulating cytokine action, angiogenesis, cell motility, and extracellular matrix formation. Still, higher levels of ROS are thought to complicate wound healing and remodeling of tissue. We believe that the combined administration of HBO and NAC, a precursor to the redox-orchestrating protein glutathione, to cultured fibroblasts may contribute to the understanding of redox-regulated wound-site biology and assist in elucidation of the optimal wound-healing environment.

In our experiment, the supplementation by 40 µmol/L of NAC of normal fibroblasts treated daily with 2 atm of HBO appears to significantly stimulate proliferation (decrease PDT). This suggests that this level of NAC may foster an ideal redox environment for fibroblast growth. Higher concentrations of NAC appear to inhibit population doubling, suggesting that these levels either become toxic to cells or overcompensate for beneficial ROS. Interestingly, the cells derived from this patient did not show significantly improved PDTs when exposed to only 2 atm of HBO as compared with the normal control. This somewhat contrasts a previous study performed in our laboratory with similar eyelid dermal fibroblasts treated with 2 atm of HBO.\textsuperscript{13} In this study, neither pretreatment (day 0) nor posttreatment (days 1–7) levels were statistically significantly different from the control. Interestingly, the previous study showed bFGF production being statistically significantly higher in 1- to 2.5-atm HBO–treated cells at 1 day after initiation of therapy when compared with the normal control. The hypothesis that the HBO therapy induced the increased bFGF production could not be fully supported because pretreatment bFGF production of the same cells was not measured. The disparity in data may be due to a number of variables, from varying genetic patient profiles to differences due to cell line development (this study used first-passage cells closer to the native tissue state, while the previous study used second-passage cells).

Transforming growth factor β1 is a cytokine key in the initiation and termination of tissue repair, stimulating production of extracellular matrix proteins such as collagen, proteoglycan, and fibronectin as well as enhancing mitogenesis and inhibiting protease production.\textsuperscript{20,21} Production of this growth factor is increased in the fetal state and keloid formation.\textsuperscript{20,30} In this study, TGF-β1 expression increased during the course of the study, reflecting an expected wound-healing response by the treated fibroblasts. No statistically significant differences were seen between the control cells, HBO-treated cells, and HBO plus NAC–treated cells. The one exception was for the cells treated with 5 mmol/L of NAC, which showed elevated TGF-β1 production at day 7 when compared with the HBO-only control. This increased TGF-β1 production may be a reflection of increased response to insult triggered by the large concentration of NAC (the same high concentration of NAC is associated with causing the erratic decline and growth of treated cells, as well as significantly decreased VEGF production, discussed below).

Vascular endothelial growth factor is an angiogenesis stimulator, the increased production of which has been linked to intermittent hyperoxia in the setting of wound healing. Marx et al\textsuperscript{31} showed that increased vascularity was induced with daily 2.4-atm HBO treatment, but not with 1-atm treatments. Sheikh et al\textsuperscript{32} showed in a rat wound model that VEGF levels were significantly increased with exposure to intermittent 2-atm HBO treatment. In this study, VEGF production similarly rose among experimental groups and controls with no significant difference between conditions. The one exception was the 2-atm HBO plus 5-mmol/L NAC–treated group, which showed a significantly lower VEGF expression compared with the other groups at days 1, 3, 5, and 7. This suggests that a 5-mmol/L concentration of NAC impairs VEGF production and thus inhibits angiogenesis and the wound-healing response, either through an imbalance in the cell redox state or through a yet-to-be-determined mechanism.

Consideration of both cell proliferation data and ELISA-generated growth factor production data suggests that antioxidant agents such as NAC may optimize the redox environment of fibroblasts undergoing HBO therapy to enhance the wound-healing response. The 40-µmol/L supplementation with NAC of fibroblasts treated daily with 2 atm of HBO was shown in this study to significantly increase proliferation via reduction in PDT. In addition, ELISA analysis of the production of autocrine growth factors in treated fibroblasts suggests that the combined effect of 2-atm HBO treatment with moderate (<5-mmol/L) NAC supplementation does not produce unfavorable growth factor profiles for normal healing. The negative effects seen with 5-mmol/L NAC may be the result of an imbalance in the oxidation-reduction environment in the cell, causing a more reducing environment incompatible with a normal wound-healing response. Indeed, these data support the suggestions by Gordillo and Sen\textsuperscript{15,16} that a delicate balance may truly exist between too few and too many ROS in the wound-healing response.

This study adds to the growing evidence that the tissue redox environment is important in the wound-healing response and that strategies to manipulate such environments are likely to be of considerable significance in clinical care. In current and future experiments, our laboratory intends to further evaluate the balance of ROS and antioxidants in the wound environment and to elucidate the mechanisms involved in the hope of developing new treatment modalities.