Effects of hyperbaric oxygen therapy on experimental burn wound healing in rats: A randomized controlled study.

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A body of data supports the efficacy of hyperbaric oxygen (HBO2) therapy in the treatment of thermal burns, but the role of HBO2 in the treatment of burn injury remains a subject of controversy. The aim of this study was to evaluate possible positive effects of HBO2 on the experimental burn wound healing. Deep second degree burns were produced on the depilated backs of 70 male Wistar rats using a validated burn protocol. The animals were assigned randomly to one of two groups: 35 to the control group, which was treated with silver sulphadiazine and placebo gas, and 35 to the experimental group, which was treated with silver sulphadiazine and HBO2. The main outcome measure was wound healing, characterized by formation of post-burn edema, neoangiogenesis, number of regeneratory active follicles, necrosis staging, margination of leukocytes, and time of epithelization. A significant reduction of the post-burn edema after treatment with HBO2 (p=0.009) was found. HBO2 had a beneficial effect on neoangiogenesis (p=0.009). The number of preserved regeneratory active follicles was significantly higher (p=0.009) and epithelial regeneration was more rapid in the experimental group (p=0.048). There were no significant differences for margination of leukocytes (p=0.55) or necrosis staging (p=1.00). These data further support earlier conclusions that HBO2 is beneficial in the healing of burn wounds.

INTRODUCTION

The management of burns is in a continuous state of evolution, but the first principle is to achieve healing as quickly as possible with a minimum of scarring. Initial treatment of burns aims to minimize edema, preserve marginally viable tissue, protect the microvasculature, enhance host defense, and promote wound closure. Adjunctive HBO2 has been shown to enhance the treatment of severe thermal injury (1-10). When used as an adjunct in a comprehensive program of care for severe burns, HBO2 can significantly decrease mortality (11), lessen the need for surgery (12,13), and reduce length of hospital stay (14). The postulated mechanisms of a beneficial effect of HBO2 on burn wounds are decreased edema formation due to hyperoxic vasoconstriction (15), increased collagen formation (16), and improved phagocytic killing of bacteria (17). However, this treatment is still not uniformly accepted (18,19). The randomized controlled study described here was undertaken to further explore the role and possible mechanisms of action of HBO2 in the treatment of second degree burn wounds.
MATERIALS AND METHODS

The study involved 70 male Wistar rats with a mean weight of 250±50 g. They were assigned randomly to one of two groups, experimental or control, 35 in each. The experimental protocol was approved by the Ethical Committee for Biomedical Researches, School of Medicine, University of Split, Croatia. The burn model of Mikus et al. was used (20) to induce deep partial skin thickness burns on the back of animals by controlled burning (1.5x1.5 cm or about 20% of total body area) under anesthesia induced with intraperitoneal administration of ketamine (100 mg/kg body wt). The anesthetized animals were exposed to direct flame for 5 to 7 sec through a 1.5x1.5 cm window in an asbestos network. This procedure resulted in no mortality. All the animals' burns were treated with 1% silver sulfadiazine cream every eight hours, which is an accepted topical remedy (21).

Two hours after burn, the experimental group was placed in the hyperbaric chamber and exposed to 100% oxygen at 2.5 bars (253.25 kPa) for 60 minutes. That is the average pressure used in earlier studies of experimental burn wounds (22). The procedure was the same every day and the experiment lasted for 21 days. The rats assigned to the control group were treated in the hyperbaric chamber pressurized by an artificial gas mixture produced in the Laboratory for Gas Analyses of the Department of Undersea and Hyperbaric Medicine of the Naval Medicine Institute, Split, Croatia, comprising of 8.4% oxygen and 91.6% nitrogen, which at 2.5 bars (253.25 kPa), produces normoxic conditions. Thus, the inspired gas mixture was the only variable that differed between the groups.

The concentrations of oxygen and carbon dioxide were measured at the beginning and at the end of each session. The pathohistological outcome measures included edema, blood vessel formation, number of preserved hair follicles, margination of leukocytes, stage of necrosis, and number of rats with signs of reepithelization. After subcutaneous anesthesia with lidocaine to enable painless skin biopsy, skin samples were taken on days 1,2,3,5,7,15, and 21. For each sampling, five rats from each group were randomly chosen and euthanased afterwards in a painless manner. Normal (unburned) tissue was not sampled in either group since the results of a pilot study we conducted prior to this experiment showed no influence of the treatment protocol on normal skin. Also, we found no influence of subcutaneous anesthesia with lidocaine on normal skin histology (23).

Standard tissue slices were prepared and stained with hematoxylin-eosin. Edema was determined by measuring the distance from the muscular layer to the surface of burned area on the 1st to the 5th day after burn. Angiogenesis was determined by counting the number of blood vessels on the 1st to the 21st day after burn. The number of preserved hair follicles was counted on the margins of the burned area on the 1st to the 7th day after burn. Necrosis was scored using a modified Suzuki scale (24) as follows: Grade 1: necrosis within the epidermal layer; Grade 2: necrosis up to the deepest layer of hair follicles; Grade 3: necrosis exceeding the deepest layer of hair follicles, and Grade 4: necrosis exceeding the muscular layer. Margination of leukocytes was scored as follows: Grade 1: no marginated leukocytes; Grade 2: scant margination (1-10 leukocytes marginated); Grade 3: abundant margination (11-20 leukocytes marginated), and Grade 4: very massive margination (21 and more leukocytes marginated to the endothelium wall). Margination of leukocytes was measured on the 1st to the 15th day. Development of epithelization was expressed as the number of animals with complete (or begun) epithelization on the 15th and the 21st day. All microscopic measurements were carried out by two independent blind observers.
(pathologists). Three fields were always used to determine the number of blood vessels and other pathologic outcomes. The data were analysed by Kruskall-Wallis, Mann-Whitney, and Fisher's exact probability test. Differences were considered to be significant for $p \leq 0.05$.

RESULTS

1. **Edema Formation.** The Kruskal-Wallis testing revealed a statistically significant change in size of edema formation (Table 1) between days in the control group ($x^2=14.4; df=3; p=0.002$) and the experimental group ($x^2=9.6; df=3; p=0.022$). In the control group there was a noticeable increase in extent of edema over the period of observation. Comparing the edema between the groups on the same day showed no statistically significant differences on the first ($p=0.60$), second ($p=0.35$), or third day ($p=0.47$), but only on the fifth day of observation ($p=0.009$).

2. **Neoangiogenesis.** The Kruskal-Wallis testing showed a statistically significant difference in the number of blood vessels (Table 2) between days in the control ($x^2=27.00; df=6; p<0.001$) and the experimental group ($x^2=26.5; df=6; p<0.001$). Statistically significant differences between the groups in the number of blood vessels were noticed on the second ($p=0.047$), on the third ($p=0.009$), on the fifth ($p=0.009$), and on the seventh day ($p=0.009$) after the burns.

3. **Regenerative Active Follicles.** The Kruskal-Wallis testing showed no significant difference in the number of preserved follicles (Figure 1) in the control group among days ($x^2=9.4; df=4; p=0.059$). In the experimental group, there was a significant difference in the number of preserved follicles ($x^2=13.5; df=4; p=0.009$). In the experimental group, there were notably more preserved follicles on all the days of observation and examination ($1st: p=0.016, 2nd: p=0.009, 3rd: p=0.009, 5th: p=0.009, and 7th: p=0.009$).

4. **Necrosis Staging.** The Mann-Whitney test showed no statistically significant difference in necrosis staging between the groups (Table 3).

5. **Margination of Leukocytes.** No significant difference was noted for this parameter by Mann-Whitney test (Table 4).

6. **Epithelization.** Full epithelization did not occur in a single animal. Whenever noticeable epithelization was found, it was marked as positive. In the experimental group, signs of epithelization were present in 9 out of 10 animals, and in the control group in 1 out of 10 animals ($p=0.048$).

In the experimental group, the mean concentration of oxygen at the beginning of the session was 99.4±0.2%, and at the end 99.2±0.3%. The mean concentration of carbon dioxide at the start was 0.06±0.02%, and at the end 0.08±0.03%. In the control group, the mean concentration of oxygen at the beginning was 8.3±0.2%, and at the end 8.5±0.2%. The mean concentration of carbon dioxide at the start was 0.07±0.02%, and at the end 0.06±0.03%.
Table 1. Results and statistical analysis of size of edema formation in the control and in the experimental group by the days of observation. Legend: *Mann-Whitney test; **Kruskal-Wallis test; SD: standard deviation.

<table>
<thead>
<tr>
<th>Control group</th>
<th>Experimental group</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Days</strong></td>
<td><strong>Median (range) [µm]</strong></td>
<td><strong>Mean value ± SD [µm]</strong></td>
</tr>
<tr>
<td>1.</td>
<td>1072 (712-1419)</td>
<td>1099.80±284.26</td>
</tr>
<tr>
<td>2.</td>
<td>956 (669-1462)</td>
<td>1000.40±313.45</td>
</tr>
<tr>
<td>3.</td>
<td>1726 (1621-2356)</td>
<td>1915.40±343.17</td>
</tr>
<tr>
<td>5.</td>
<td>1820 (1552-2759)</td>
<td>1932.00±487.29</td>
</tr>
</tbody>
</table>

Table 2. The results and statistical analysis of the number of blood vessels in the control and in the experimental group by the days of observation. Legend: *Mann-Whitney test; **Kruskal-Wallis test; SD: standard deviation.

<table>
<thead>
<tr>
<th>Control group</th>
<th>Experimental group</th>
<th>p*</th>
<th>p**</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Days</strong></td>
<td><strong>Median (range) [µm]</strong></td>
<td><strong>Mean value ± SD [µm]</strong></td>
<td><strong>Median (range) [µm]</strong></td>
</tr>
<tr>
<td>1.</td>
<td>22 (8-32)</td>
<td>21.20±8.67</td>
<td>27 (10-47)</td>
</tr>
<tr>
<td>2.</td>
<td>21 (20-33)</td>
<td>24.00±5.61</td>
<td>40 (24-46)</td>
</tr>
<tr>
<td>3.</td>
<td>11 (7-20)</td>
<td>12.40±4.93</td>
<td>40 (28-43)</td>
</tr>
<tr>
<td>5.</td>
<td>20 (13-24)</td>
<td>18.40±4.72</td>
<td>40 (29-68)</td>
</tr>
<tr>
<td>7.</td>
<td>14 (10-19)</td>
<td>14.60±3.85</td>
<td>148 (112-186)</td>
</tr>
<tr>
<td>15.</td>
<td>159 (129-186)</td>
<td>156.00±26.05</td>
<td>135 (128-203)</td>
</tr>
<tr>
<td>21.</td>
<td>97 (88-122)</td>
<td>101.80±12.97</td>
<td>99 (73-213)</td>
</tr>
</tbody>
</table>

Fig. 1. The dynamics of changes in the number of regeneratory active follicles in the control and the experimental group by the days of observation.
Table 3. The results of statistical analysis of necrosis staging in the control and in the experimental group by the days of observation. Legend: Mann-Whitney test; SD: standard deviation.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control group</th>
<th>Experimental group</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (range)</td>
<td>Mean value ± SD</td>
<td>Median (range)</td>
</tr>
<tr>
<td>1.</td>
<td>2 (2-2)</td>
<td>2.00±0.00</td>
<td>2 (2-2)</td>
</tr>
<tr>
<td>2.</td>
<td>3 (2-4)</td>
<td>3.20±0.84</td>
<td>2 (2-4)</td>
</tr>
<tr>
<td>3.</td>
<td>4 (4-4)</td>
<td>4.00±0.00</td>
<td>4 (2-4)</td>
</tr>
<tr>
<td>5.</td>
<td>4 (4-4)</td>
<td>4.00±0.00</td>
<td>4 (4-4)</td>
</tr>
<tr>
<td>7.</td>
<td>4 (4-4)</td>
<td>4.00±0.00</td>
<td>4 (4-4)</td>
</tr>
</tbody>
</table>

Table 4. The results of statistical analysis of leukocyte margination in the control and in the experimental group by the days of observation. Legend: *Mann-Whitney test; SD: standard deviation.

<table>
<thead>
<tr>
<th>Day</th>
<th>Control group</th>
<th>Experimental group</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (range)</td>
<td>Mean value ± SD</td>
<td>Median (range)</td>
</tr>
<tr>
<td>1.</td>
<td>3 (2-3)</td>
<td>2.60±0.55</td>
<td>2 (2-3)</td>
</tr>
<tr>
<td>2.</td>
<td>3 (2-3)</td>
<td>2.60±0.55</td>
<td>3 (2-3)</td>
</tr>
<tr>
<td>3.</td>
<td>1 (0-3)</td>
<td>1.40±1.14</td>
<td>1 (0-2)</td>
</tr>
<tr>
<td>5.</td>
<td>3 (3-3)</td>
<td>3.00±0.00</td>
<td>3 (2-3)</td>
</tr>
<tr>
<td>7.</td>
<td>0 (0-1)</td>
<td>0.40±0.55</td>
<td>0 (0-1)</td>
</tr>
<tr>
<td>15.</td>
<td>3 (2-3)</td>
<td>2.60±0.55</td>
<td>2 (1-3)</td>
</tr>
</tbody>
</table>

DISCUSSION

This study yielded a statistically significant reduction in edema formation in the experimental group when compared with controls (p=0.022). Several experimental studies support the efficacy of HBO₂ in significantly reducing edema in burns. Nylander and co-workers showed in a validated animal model that HBO₂ reduced the generalized edema associated with burn injury (25). Kaiser and colleagues also showed a significant reduction of subcutaneous edema in burned animals treated with HBO₂ (26). They reported progression of the burn wound in controls, while wound size decreased in the hyperbaric-treated animals. Hammarlund and co-workers showed that HBO₂ reduced edema formation (27). Several other authors demonstrated reduction of edema formation in animal models (7,28-30). The reduction of edema in the treatment of burns may be related not only to reduced blood flow and capillary perfusion but also to preservation of aerobic metabolism (7). This may play a major role in maintaining the integrity of the microvasculature. Reduced edema formation is probably the result of maintaining microvascular integrity and providing the oxygen necessary to sustain cellular viability. This is the usual rationale for HBO₂ in thermal burns (7). An intact microvasculature is a critical factor in the ability to provide cellular and humoral elements to the site of burn injury. Any microvascular improvement, whether it be preservation of intact capillaries or control of interstitial edema, should influence burn outcome favorably.

Formation of new blood vessels in this study was significantly higher in the experimental group, confirming the positive effect of HBO₂ on blood vessel formation (p<0.001). Saunders and colleagues showed preservation of microcirculation in animals treated with HBO₂ vs controls (31). Angiogenesis represents a limiting factor for epithelization, since the primary source of
epithelial cells needed for epithelization is vascular (32,33).

One of the most intriguing descriptors in the analysis of burn wound healing is the number of regeneratory active follicles in the deeper skin layers. The speed of healing of dermal burns depends on the number of viable hair follicles and other appendages (34). Oxygen-protective effects in preserving vital follicles is the main reason for faster wound epithelization. It remains unclear if this is a result of direct effect of oxygen on epithelial cells or a result of angioprotective effect of oxygen. In the present study, the number of regeneratory active follicles was significantly higher in the experimental relative to the control group (p=0.009). We found no data about effect of HBO$_2$ on burn wound healing that were derived from numbering preserved follicles as a wound healing descriptor.

Margination of leukocytes, as a parameter of estimation of inflammatory reaction, showed no significant difference between the groups, so we concluded that HBO$_2$ in this study had no effect on inflammatory reaction. However, Thom and co-workers demonstrated that HBO$_2$ at 2.8 or 3.0 bars inhibited beta2-integrin-dependent neutrophil adherence (35). The difference between results could be due to lower oxygen pressure used in our study, and the fact that rat neutrophils differ significantly from human neutrophils, so comparing these study results should be done with care (36-39).

In analysing necrosis staging, there was no difference between the two groups. HBO$_2$, in a randomized controlled trial designed like this one, had no effect on necrosis staging.

Epithelization is probably the most important event in the healing of deep second degree burn because it signifies the end of the initial stage of healing. There are several possibilities for how HBO$_2$ may affect the epithelization of the second degree burn wound: it can aid in minimizing the deleterious influences following burn injury and thus leave more viable tissue to resurface the wound; it may increase the mitotic rate of subepithelial cells or it may speed up the migration of the epithelial cells and thus allow faster coverage (28). Korn and co-workers showed that second degree burn wounds heal faster when treated with HBO$_2$ (28). Studies of epithelial tissue indicate that it can survive without oxygen, but cells cannot divide or migrate (40). Sufficient oxygen in tissue to enable epithelial cells to migrate and divide is essential to wound healing (28). Epithelization depends on the total cell population that survives the initial and subsequent injury, and their mitosis and migration. HBO$_2$ appears to affect this process by allowing less wound dessication and destruction and increasing oxygenation of hypoxic, thermally damaged cells that might not survive otherwise (41). In this study we showed faster epithelization in the experimental group vs control, which supports earlier conclusions that adequate oxygen level is mandatory for wound healing (4,41-43).

Not all reports of HBO$_2$ therapy in burns are favorable. Perrins and colleagues reported no effect of HBO$_2$ in a pig scald model (18). Niccole and co-authors reported no advantage in wound healing achieved by HBO$_2$ when the modality was compared to topical antibiotics (19). They proposed that HBO$_2$ alone acted merely as a mild antiseptic.

In choosing our experimental design for this study, we considered several points, including the potential advantages and disadvantages of different protocols. There is no standard protocol for HBO$_2$ in human burn wounds. As suggested by the Hyperbaric Oxygen Committee of the Undersea and Hyperbaric Medical Society, treatment should be provided on a regimen of 90 minutes at 2.0 to 2.4 bars (44). Standard HBO$_2$ sessions at the Naval Medical Institute in Split, Croatia, are 60 minutes at 2.2 bars. Because of that and because of the need to account for differences
in oxygen toxicity between species, we decided to use 2.5 bars for 60 minutes, which is the average oxygen pressure used in previous burn experiments (22). The two-hour delay from burn to HBO\(_2\) was chosen for practical reasons, although such a short delay might be unrealistic in clinical practice. We did not follow the animals far enough for complete burn wound healing because healing could not have occurred in the control group. These rats had no remaining hair follicles that could have supported epithelization and only late scarring after necrosis could have been expected. This is why we considered the beginning of epithelization to be a valid measure of the outcome in this model.

The use of HBO\(_2\) as an adjunct merits further consideration in the comprehensive management of thermal burns. Some of the questions which remain unanswered are as follows: What other treatment modalities could enhance the effect of HBO\(_2\) on burn wound healing? Is there sufficient objective evidence that the usage of HBO\(_2\), as adjunctive therapy to standard burn wound management, really aids in healing? Is HBO\(_2\) efficient, ethically legitimate, cost effective, and without harmful consequences?

In this study, HBO\(_2\) exerted a positive, beneficial effect on the burn wound healing. Such an effect was probably caused by reducing edema and preserving the microcirculation as much as maintaining the viability of dermal elements (such as follicles) which are obligatory for faster epithelization of the burn wound.

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