Hyperbaric Oxygen Therapy for Reduction of Secondary Brain Damage in Head Injury: An Animal Model of Brain Contusion

EILAM PALZUR,1 EUGENE VLODAVSKY,2 HANI MULLA,1 RAN ARIELI,3 MOSHE FEINSOD,1 and JEAN F. SOUSTIEL1

ABSTRACT

Cerebral contusions are one of the most frequent traumatic lesions and the most common indication for secondary surgical decompression. The purpose of this study was to investigate the physiology of perilesional secondary brain damage and evaluate the value of hyperbaric oxygen therapy (HBOT) in the treatment of these lesions. Five groups of five Sprague-Dawley rats each were submitted to dynamic cortical deformation (DCD) induced by negative pressure applied to the cortex. Cerebral lesions produced by DCD at the vacuum site proved to be reproducible. The study protocol entailed the following: (1) DCD alone, (2) DCD and HBOT, (3) DCD and post-operative hypoxia and HBOT, (4) DCD, post-operative hypoxia and HBOT, and (5) DCD and normobaric hyperoxia. Animals were sacrificed after 4 days. Histological sections showed localized gross tissue loss in the cortex at injury site, along with hemorrhage. In all cases, the severity of secondary brain damage was assessed by counting the number of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and caspase 3–positive cells in successive perilesional layers, each 0.5 mm thick. Perilesional TUNEL positive cells suggested the involvement of apoptosis in group 1 (12.24% of positive cells in layer 1). These findings were significantly enhanced by post-operative hypoxia (31.75%, p < 0.001). HBOT significantly reduced the severity and extent of secondary brain damage expressed by the number of TUNEL positive cells in each layer and the volume of the lesion (4.7% and 9% of TUNEL positive cells in layer 1 in groups 2 and 4 respectively, p < 0.0001 and p < 0.003). Normobaric hyperoxia also proved to be beneficial although in a lesser extent. This study demonstrates that the vacuum model of brain injury is a reproducible model of cerebral contusion. The current findings also suggest that HBOT may limit the growth of cerebral contusions and justify further experimental studies.

Key words: apoptosis; cerebral contusion; head injury; hyperbaric oxygen; secondary brain damage

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INTRODUCTION

Cerebral contusions are one of the most common clinical findings following traumatic brain injury (TBI) and are responsible for significant mortality (Teasdale and Mathew, 1997). Cerebral contusions most often occur on the basal aspect of frontal and temporal lobes and are commonly related to differential motion of the brain over the skull base during dynamic loads to the head (Gennarelli 1993). Despite multiple forensic and radiological studies characterizing their epidemiological and pathological aspects, cerebral contusions remain a serious threat due to their unique evolving clinical course. Relatively little is known about the mechanisms underlying growth of cerebral contusions. Pathologically cerebral contusions are characterized as a central area of hemorrhagic necrosis surrounded by a perilesional region often referred to as the traumatic penumbra in which both excitotoxic and ischemic events eventually lead to delayed neuronal death. As such, cerebral contusions offer a suitable lesion for the investigation of the adverse consequences of secondary traumatic brain damage. Pathologically, cerebral contusions share several similarities with ischemic stroke, characterized as well by central necrosis and perilesional penumbra (Leker and Shohami, 2002). Ischemic as well as post traumatic penumbra are characterized as potentially viable tissue exposed to secondary deleterious events wherein energy failure is prominent. In this regard, increasing attention has been recently drawn to the beneficial effect of hyperbaric oxygen therapy (HBOT) for the reduction of delayed neuronal death (Neubauer et al., 1994). However, conflicting results regarding the benefits of hyperbaric oxygen have also been reported (Nighoghossian and Trouillas, 1997). In spite of a few, supportive laboratory (Calvert et al., 2002; Wada et al., 2000) and clinical studies (Neubauer et al., 1994; Rockswold et al., 2001) the overall protective influence of HBOT remains controversial. In the present study, we attempted to evaluate the potential value of HBOT as a treatment for reduction of secondary traumatic brain damage in a rat model of focal cerebral contusion.

MATERIALS AND METHODS

Model of Traumatic Brain Injury

The technique of cortical dynamic deformation (DCD) is based on that thoroughly described by Shreiber et al. (Shreiber et al., 1999). Sprague-Dawley rats weighing 370–430 g were anesthetized by intraperitoneal injection of chloral hydrate 40% (1 mg/kg). During surgery, core temperature was maintained by surface heating. A scalp incision was performed, and a 5-mm-diameter burr hole was drilled in the left parietal region. Under magnification, the dura was torn and a hollow screw was connected to the burr hole sealed with bone wax (Fig. 1). A negative pressure of 400 mbar (0.605 ATA) was applied to the cortical surface for 10 sec by a digitally controlled vacuum pump through the screw. The skin was then sutured and the animal allowed to resume normal activity for 3 days. The experimental procedure was approved by the Animal Care Committee of the Israel Ministry of Defence, and the rats were handled in accordance with internationally accepted humane standards.

Twenty-five rats were divided into five groups as follows: (1) DCD alone, (2) DCD and HBOT, (3) DCD-hypoxemia, (4) DCD-hypoxemia and HBOT, and (5) DCD and normobaric hyperoxia.

Hypoxemia

In order to assess the clinical relevance of the model and the impact of posttraumatic hypoxemia on secondary brain damage, prepared animals were exposed to mild hypoxemia. Animals were placed in a closed chamber filled and constantly flushed with an air mixture designed to produce a mild hypoxemia defined by oxygen saturation (SaO₂) ranging between 82% and 86% at atmospheric pressure (Arieli et al., 1994). Exposure to hypoxemia continued for 60 min and was carried out under continuous SaO₂ monitoring (Datex Engstrom Instruments, Finland) and the air mixture by means of mass spectrometer (QP 9000, Morgan Medicals, Rainham, UK).

Hyperbaric Oxygen Therapy (HBOT) and Normobaric Hyperoxia

Treated animals were placed in a 150-L pressure chamber (Roberto Galeazzi, La Spezia, Italy) and received HBOT 3 h after injury and thereafter twice every day for three consecutive days. During each treatment 100% oxygen was delivered at 1 absolute atmosphere (ATA) for normobaric hyperoxia and 2.8 ATA for HBOT during two consecutive sessions of 45 min each. Between the two sessions, a pause of 5 min was made, during which oxygen was replaced by room air in order to prevent oxygen toxicity and its complications. During HBOT, ambient temperature was monitored and maintained at 25°C. Air mixture and humidity were continuously monitored as well.

Pathological Assessment

At the fourth post-operative day, the animals were reanesthetized and transcardially perfused with heparinized saline, 10% sucrose in a buffered saline and 4% buffered
FIG. 1.  TUNEL-positive cells were divided into two groups: type I characterized by densely stained cells retaining their previous nuclear shape (arrow) and type II identified by nuclear staining with chromatine condensation (arrow head). Since type I cells are usually considered to be non-apoptotic cells, only type II were taken into account for analysis.

FIG. 2.  Macroscopic appearance of the focal injury produced by the dynamic cortical deformation model in a coronal section.

FIG. 3.  Caspase-3 staining could be found in few glial cells with pyknotic nuclei though most prominently in macrophages surrounding the necrotic area (arrows).
formaldehyde. Brains were post fixed by immersion into 4% buffered formaldehyde for 72 h and then removed from the skull. Brains were sectioned through the area of the produced lesion and embedded in paraffin. Histological sections of 5 \mu m in thickness were cut through mounted brain cross-sections and stained in hematoxylin and eosin.

**Immunohistochemistry**

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays were used for quantitative evaluation of the post-traumatic penumbra. For this purpose, paraffin sections were stained by in-situ cell death detection kit (Boehringer Mannheim, Mannheim, Germany) according to manufacturer’s protocols. TUNEL-positive cells were then divided into two groups according to previous studies (Smith et al., 2000; O’Dell et al., 2000): type I characterized by densely stained cells retaining their previous nuclear shape and type II identified by nuclear staining with chromatin condensation (Fig. 1). Since type I cells are considered to be non apoptotic cells, only type II were analyzed. Positive cells were counted in five successive perilesional layers each 0.5 mm wide, using an ocular micrometer. For each layer, the number of positive cells was expressed as a percentage of the total positive cells within the same layer as an attempt to normalize the possible variations in cell density in different layers and different areas. Cells were counted in two consecutive sections for each animal. In order to provide further evidence for apoptosis as part of the secondary cell death, paraffin sections were also stained for active Caspase 3 (R&D Systems, Minneapolis, MN) according to manufacturer’s protocols with microwave antigen retrieval (5 min at 95°C). To demonstrate the neuronal, glial or macrophage origin of the cells, consecutive sections were stained for immunohistochemical markers Synaptophysin (Zymed, San Francisco, CA), GFAP, and CD68 (DAKO, Carpinteria, CA).

**Statistical Analysis**

TUNEL-positive cell indices in the different groups were compared separately for each layer using Student’s *t* test and analysis of variance (ANOVA). A *p* value of less than 0.05 was considered be statistically significant.

**RESULTS**

**Structural Changes**

Cortical dynamic deformation model produced a wedge-shaped, highly reproducible lesion involving the parietal cortex and the subcortical white matter (Fig. 2). Grossly, the lesion was characterized by central cavitation encompassed by a zone of hemorrhagic necrosis ranging in diameter from 2.5 \times 1.0 to 4.2 \times 1.7 mm. This zone was well delineated from the surrounding tissue. Microscopically, the necrotic area was surrounded by a rim of macrophages. Fresh hemorrhage was routinely present within and around the necrotic area. Few neurons and glial cells with pyknotic nuclei were usually found adjacent to the area of necrosis. No structural damage, however, could be discerned remote from the injury site beyond 2.5 mm.

**Immunohistochemistry**

TUNEL assay. The number and dispersion of TUNEL-positive cells are summarized in Table 1. Secondary cell death is expressed in each group by the mean TUNEL-positive cell index for each layer. In animals exposed to DCD only, the TUNEL-positive cell index was 12.2% in the first layer, whereas no TUNEL-positive cells could be found beyond 1.5 mm from the injury focus.

Caspase assay. At the fourth post-operative day, Caspase-3 staining could be found in scattered glial cells with pyknotic nuclei prominently in macrophages surrounding the necrotic area (Fig. 3). Accordingly, no quantitative analysis were performed using the TUNEL assay.

**Hypoxia**

Hypoxemia caused a profound lesion enhancement, with a significant increase in the TUNEL-positive cell index in each layer, expanding from 12.2% to 31.8% (*p* < 0.02) with an enlargement of the damaged area to cover five successive layers—that is, 2.5 mm (Table 1).

**Hyperoxia**

HBOT induced a significant decrease in both the radius and severity of brain damage following DCD (Table 1, *p* < 0.002). The lesion surface was significantly reduced from 5.9 ± 2.2 mm² in non-treated animals to 2.3 ± 0.6 mm² (*p* < 0.02) in treated animals. In animals exposed to post-traumatic hypoxemia, reduction of lesion volume and severity by HBOT was even more pronounced than after DCD alone. The TUNEL-positive cell index in the first layer was reduced by 53% in the former group (DCD alone, *p* < 0.002) and 71.7% in the latter (Table 1, *p* < 0.02). In animals treated by normobaric hyperoxia, a similar trend of reduction in the number of TUNEL-positive cells in the different layers could be demonstrated, although to a lesser extent than seen with HBOT (9.3% at 0.5 mm, *p* < 0.001, Table 1).
DISCUSSION

Cerebral contusions are one of the most common clinical findings following traumatic brain injury (TBI) and are responsible for significant mortality (Teasdale and Mathew, 1997). Among those patients harboring CT findings compatible with cerebral contusions on admission, many show progressive clinical deterioration. This negative neurological trend is commonly associated with a significant growth of the contusion on follow-up imaging studies (Kobayashi et al., 1983). Most authors have argued that perilesional ischemia, either induced by reduced cerebral perfusion pressure (Bullock et al., 1992; Roper et al., 1991) or vascular injury (Matthews et al., 1995) is likely to account for contusion growth although the mechanisms underlying evolving contusions remain speculative in nature, hence the need for further investigation.

In an attempt to create an animal model of focal cerebral contusion without associated diffuse brain injury, Shreiber et al. (1999) showed that cerebral lesions produced by a transient non-ablative vacuum pulse applied to the exposed cerebral cortex were structurally similar to those observed in clinical conditions. As such and despite the obvious disparity with the clinical situation, the model described by these authors seems to be a valid laboratory model for studying cerebral contusions and associated evolving damage. In our study, DCD yielded findings similar to that reported by Shreiber et al. (1999). Both macroscopic appearance of hemorrhagic necrosis and microscopic findings of pyknotic neurons and erythrocytic and macrophagic infiltrates were close to that of the clinical situation. A perilesional penumbra could be clearly delineated and differentiated from spared surrounding brain tissue justifying the selection of this model for the investigation of focal traumatic brain injury. The validity of the model was further supported by the profound worsening of pathological findings noted in animals exposed to secondary hypoxia as previously reported in numerous clinical studies (Puka-Sundvall et al., 1997; Calvert et al., 2002).

In order to quantify and investigate secondary cell death, the TUNEL method was used. Indeed, attention has recently been drawn on TUNEL-positive staining of cerebral contusions in both laboratory and clinical settings (Smith et al., 2000; Ng et al., 2000; O’Dell et al., 2000). Since the report of Gavrieli et al. (1992), DNA fragmentation as determined by the TUNEL method has been linked to programmed cell death and increasing evidence has been accumulating on the involvement of the apoptotic process in the delayed post-traumatic neuronal death (Ng et al., 2000; O’Dell et al., 2000; Smith et al. 2000). As such, the present findings further corroborate that apoptosis may contribute to secondary brain damage.

### Table 1. Extent and Severity of Secondary Cell Death in Different Groups

<table>
<thead>
<tr>
<th></th>
<th>DCD</th>
<th>DCD + HBOT</th>
<th>DCD + Hypoxia</th>
<th>DCD + Hypoxia + HBOT</th>
<th>DCD + Normobaric hyperoxia</th>
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<tbody>
<tr>
<td>Radius from the focus of injury</td>
<td>0.5 1 1.5 2 2.5</td>
<td>0 1 1.5 2</td>
<td>0 1 1.5 2</td>
<td>0 1 1.5 2</td>
<td>0 1 1.5 2</td>
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<tr>
<td>Percent of apoptotic cells</td>
<td>12.2 6.4 1.6 0 0</td>
<td>4.7† 0 0 0 0</td>
<td>3 0 † 0 0</td>
<td>0 0 0 0</td>
<td>9 2 † 0.5 0</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>1.2 2.9 1.5 0 0</td>
<td>4.7† 0 0 0 0</td>
<td>3 0 † 0 0</td>
<td>0 0 0 0</td>
<td>7 2.8 1 0</td>
</tr>
<tr>
<td>Radius from the focus of injury</td>
<td>0.5 1 1.5 2 2.5</td>
<td>0 1 1.5 2</td>
<td>0 1 1.5 2</td>
<td>0 1 1.5 2</td>
<td>0 1 1.5 2</td>
</tr>
<tr>
<td>Percent of apoptotic cells</td>
<td>31.8† 26.8‡ 17.5 4.3 0</td>
<td>9 2 † 0.5 0</td>
<td>7 2.8 1 0</td>
<td>0 0 0 0</td>
<td>9 2 † 0.5 0</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>12.6 11.4 9.3 4.3 4.2</td>
<td>7 2.8 1 0</td>
<td>0 0 0 0</td>
<td>0 0 0 0</td>
<td>1.9 1.7 0.7 0</td>
</tr>
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</table>

This table summarizes the percentage of TUNEL type 2 positive cells (apoptotic cells) in the perilesional area for the five groups. This area was divided into five consecutive layers of 0.5 mm in width.

†p<0.001 comparison between DCD and DCD + HBOT at the first layer.
‡p<0.001 comparison between DCD and DCD + hypoxia at the first and second layer.
¶p<0.001 comparison between DCD + hypoxia and DCD + hypoxia + HBOT at the first three layers.
* p<0.001 comparison between DCD and DCD + normobaric oxygen therapy in layer 1 and 2.
in cerebral contusions. TUNEL staining, however, may not be necessarily conclusive of apoptosis as the cause of delayed neuronal death and several studies have provided evidence that positive TUNEL staining can also be associated with necrotic process (Charriaut-Marlangue and Ben-Ari, 1995; Gwag et al., 1997; Zipfel et al., 2000). In this regard, the presence of Caspase-3–positive cytoplasmic inclusions in macrophages in the present model reinforces the involvement of apoptosis in secondary neuron death, assuming that those inclusions are secondary to phagocytosis of dying cells by stained macrophages. Considering the late involvement of Caspase-3 in the apoptotic process, it seems safe to assume that necrosis is not responsible for death in such instance. This finding, however, does not allow a relative quantification of apoptosis vs. necrosis, as the histological assessment was relatively late in respect to the Caspase-activity time course. It has been shown that Caspase-3 activity following brain injury peaks at 6 h after injury and resolves within 72 h (Beer et al., 2000; Keane et al., 2001). In the present study, pathological evaluations were made on the fourth day of injury so that only sparse activity could be revealed, prominently in macrophages.

The effect of hyperbaric oxygen therapy (HBOT) shown in the present study is somewhat ambiguous. Links to necrosis, have been claimed by several authors following both head injury and ischemic stroke (Veltkamp et al., 2000; Rockswold et al., 2001). The energy failure characterizing the ultra-early phase following acute brain injury fits with the potential benefit of HBOT. Cerebral blood flow has been documented to be significantly reduced in the initial post-traumatic period both in animal models (Thomale, 2002; Eriskat et al., 1997) and in humans (Martin 1997). Compromised oxygen delivery may subsequently result in impaired mitochondrial respiration leading in turn to increased lactate production and reduced ATP production (Zauner, 2002; Inao et al., 1988; Goodman et al., 1999; Krishnappa et al., 1999). Energy failure may eventually account for increased membrane permeability, massive calcium entry and finally neuronal death (Faden et al., 1989; Choi, 1988). In hyperbaric conditions, oxygen concentration in the cell vicinity is markedly increased due to improved diffusion gradient (Davis et al., 1973; Hunt et al., 1978). Another possible explanation for anti-necrosis effect of HBOT in head injury is the redistribution of cerebral blood flow. Several authors noted a decrease in cerebral blood flow in hyperbaric conditions and have related this decrease either to vasoconstriction (Demchenko et al., 1998; Kohshi et al., 1991) or nitric oxide inactivation (Demchenko et al., 2000). Since both mechanisms would imply normal autoregulation, relative post traumatic vasoparalysis may result in redistribution of CBF towards injured areas. Oxidative stress with paradoxical antioxidant effect have been also advocated to explain the protective effect of HBOT in acute brain injury. In an animal model of ischemic stroke, Wada et al. demonstrated a protective effect of repeated HBOT exposure and related this improved tolerance to ischemia to increased production of manganese superoxide dismutase (Mn-SOD), most likely due to the initial oxidative stress induced by HBOT (Wada et al., 2000). Over expression of antioxidants such as superoxide dismutase may in turn result in reduced excitatory-induced brain damage. In rat hippocampal slices exposed to hypoxia, Pellegrini-Giampietro et al. showed that glutamate release could be significantly reduced by superoxide dismutase (Pellegrini-Giampietro et al., 1990).

In contrast to the current communication, the protective effect of HBOT has not been attributed to reduced apoptotic activity as suggested by the findings of the present study. In this regard, although apoptosis and necrosis are commonly considered two distinct pathological entities, they are intimately interconnected (Kane et al., 1993). Several insults may trigger both death mechanisms and each mechanism may influence the course of the other. For instance, release of cytochrome c, a compound involved in the intrinsic apoptosis pathway, has been shown to be associated with increased free radical production (Murphy, 1999). Conversely, overexpression of Bcl-2, a gene associated with anti-apoptotic activity, has been found to be responsible for decrease of superoxide production (Cai and Jones, 1998) and to protect from free radical-induced damage (Kane et al., 1993). Interestingly, Wada et al. showed that repeated exposure to HBOT in gerbils resulted in Bcl-2 over expression in addition to the antioxidative effect previously discussed (Wada et al., 2000). As such, the reduced apoptosis in our model may have been linked to a decreased production of free radicals via either improved membrane integrity, reduced calcium entry, improved energy balance or enhanced antioxidant production (Lewen et al., 2000).

Although the mechanisms by which HBOT may be beneficial remain speculative, the results of the present study demonstrate a definite reduction of the extent and severity of secondary brain damage through this approach. Indeed, treated animals revealed lesions less than half the surface area of non-treated animals, with the number of TUNEL positive cells declining to one third of that observed in non-treated animals. Although high pressure HBOT was used in this study, normobaric hypoxia also proved to be beneficial. This observation supports previous studies of normobaric hypoxia in ischemia (Singhal et al., 2002), suggesting that less rigorous HBOT approaches may prove useful in the clinical
setting, making the implementation of HBOT easier in critically ill patients.

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