Summary. Objective: This paper is aimed at identifying putative morphological changes induced in the rat’s tracheal mucosa by chronic hyperbaric oxygen (HBO) treatment or chronic intermittent hypoxia (CIH).

Study Design: Tracheal samples were obtained from three groups of 11, 12 and 13 adult Wistar rats. The first group was submitted to 20 sessions of 100 min-long HBO treatment; the second group was submitted to eucapnic CIH for 35 days; and the third group was not submitted to any CIH or HBO therapy.

Methods: Four proximal tracheal rings were collected after sacrifice and neck dissection of the animals. The samples were processed for both light microscopy and morphometric analysis. Inflammatory leukocyte infiltration was evaluated by a semi-quantitative method. Unpaired t test and Bernoulli distribution were applied to evaluate statistical differences in the data collected from the three groups.

Results: Both CIH and HBO promote an increase in the thickness of the epithelium and of the basement membrane of the rat tracheal mucosa, as well as an increment in the number of infiltrating leukocytes, when compared with results seen in the untreated group. In the HBO group there was a significant lack of seromucous glands, as opposed to the results obtained in the CIH group.

Conclusions: Chronic HBO and CIH exposure causes only minor changes in the architecture of the tracheal mucosa of the rat. The respiratory tract of the rat showed a mild inflammatory response when subject to variations of pressure and oxygen content. Apparently these effects do not constitute a critical issue on prescribing HBO treatments and in the management of sleep apnea patients.

Key words: Hyperbaric Therapy, Chronic intermittent hypoxia, Trachea, Epithelium, Leukocytes, Rat, Inflammation

Introduction

Chronic intermittent hypoxia (CIH) occurs in highly prevalent diseases, namely in sleep apnea, a condition with repetitive short term episodes of hypoxia followed by compensatory hyperoxia. Chronic hyperoxia through prolonged treatments in hyperbaric chambers is used in diseases with underlying ischemia/hypoxia. Both can be considered models of hypoxic/hyperoxic conditions that cause histopathological changes in the airway, although the differences between the pathophysiological mechanisms of both conditions are not clear. A significant impairment of airway morphology may impose restrictions to the therapeutic use of chronic hyperbaric oxygen (HBO), anticipating the need for early sleep apnea treatment.

CIH alters immune response to allergen with collagen deposition and matrix degradation, in proximal and distal airways, leading to airflow limitation in rats, (Broytman et al., 2014). Also, it promotes the
remodeling of the rat’s diaphragm (Shorrtt et al., 2013). It was demonstrated that CIH induces cell proliferation, namely type II alveolocytes, and an increase in alveolar surface area (Reinke et al., 2011) in the rat’s lungs. In addition, treatment of sleep apnea with air positive pressure apparatus is also injurious by reducing the airway surface liquid (White et al., 2014) or stimulating nasal inflammation.

Hyperbaric oxygen therapy (HBO) is the medical treatment that attains the highest amount of oxygen in the plasma. HBO consists of the intermittent inspiration of near 100% high pressure oxygen, inside a treatment chamber, thus enhancing the amount of oxygen dissolved in the plasma of the patient (Narozny et al., 2002). HBO is an elective procedure in the treatment of emergency situations such as decompression illness or carbon monoxide poisoning (Wattel and Mathieu, 2006).

In addition, HBO has been approved by The Undersea and Hyperbaric Medical Society for the treatment of a number of disorders such as idiopathic sudden sensorineural hearing loss (Bennett et al., 2012; Cvorovic et al., 2013) gas gangrene, acute traumatic ischemia, prolonged failure of wound healing, air or gas emboli, severe blood loss, intracranial abscess, necrotizing soft tissue infections, osteomyelitis, osteoradionecrosis, skin grafts or flaps and thermal burns (McDermott et al., 1992; Bouachour et al., 1996; Bartlett 1997; Moon and Sheffield, 1997; Neovius, 1997; Jain, 1999; Kirby and Deschler, 1999). The therapeutic outcomes of HBO are related to the direct physical effects of oxygen on blood and tissues, and also to the physiological and biochemical effects of cumulative hyperoxigenation (Neumeister et al., 2004; Sahni et al., 2004). However, the underlying mechanism is poorly understood.

Most of the studies on HBO-induced changes in the respiratory tract report lung alterations which are believed to be triggered by oxidative stress and the inflammatory effects of breathing 100% oxygen. HBO treatment in rats appears to be effective in the healing of tracheal anastomosis when following irradiation and after tracheal resection and end-to-end anastomosis. This is revealed by a decrease in fibrosis and alveolar congestion, when compared with controls (Celik et al., 2010) A study with costal cartilage surgery in rabbits demonstrated that HBO also provides increased healing in the cartilage beds and allows for the formation of new cartilage, when comparing the same procedure without HBO exposure (Celik et al., 2015).

Intermittent exposure to atmospheric air is used as a protective strategy against HBO toxicity. In fact, it has been documented that continuous HBO exposure may lead to the activation of inflammatory mediators, to an increase in lung protein nitration, and to the activation of inducible NOS (iNOS) mRNA. Inflammatory responses were not observed after intermittent exposures to the same cumulative oxygen time duration (Chavko et al., 2008). Regarding the nasal mucosa, it has been reported that HBO treatment alters mucociliary transport (Narozny et al., 2002), probably as a result of the high oxygenation of blood plasma, the enhancement of the metabolism of ciliated cells, and the decrease in substance P, which is seen in cluster headache patients (Di Sabato, 1996). We have recently reported that chronic HBO treatment of humans is related to mild granulocyte infiltration and to an increase in the thickness of the basal membrane of the nasal mucosa (Vera-Cruz et al., 2008).

Considering the importance of the trachea in air conduction, thermoregulation and secretions clearance, and the lack of data concerning the effects of extreme variations of oxygen on its morphology, we performed the present study with the purpose of comparing the microanatomy of the tracheal mucosa, in a homogeneous population of Wistar rats exposed to validated models of CIH or HBO.

The results of this study will contribute to clarify whether the putative damage caused by CIH is due to the lack of oxygen or to compensatory hyperventilation. Furthermore, the use of the trachea, in comparison to the nasal mucosa, has the advantage of avoiding the mechanical injury in upper airway that occurs in obstructive sleep apnea with or without continuous positive airway pressure (CPAP) treatment.

Materials and methods

Animals

Experiments were performed in twenty-three male adult Wistar rats (Rattus norvegicus L.), aged between 6 weeks and 2 months, weighing 150-175 g, obtained from the NOVA Medical School animal facility. The animals were housed in polycarbonate cages with wire lids (Tecniplast, Buguggiate, Varese, Italy), 1 or two per cage, under 12 h light/dark cycles (8 am - 8 pm), at a room temperature 22±2.0°C and relative humidity 60±10%. Rats were maintained on standard laboratory diet (SDS diets RM1) and reverse osmosis water, given ad libitum.

Applicable institutional and governmental regulations concerning ethical use of animals were followed, according to the NIH Principles of Laboratory Animal Care (NIH Publication 85-23, revised 1985) and the European Union Directive for Protection of Vertebrates Used for Experimental and Other Scientific Ends (86/609/CEE). All experimental procedures were previously approved by the Institutional Ethics Committee of the Instituto de Ciências da Saúde Egas Moniz (05Nov2012) and NOVA Medical School and also by the Portuguese authority for animal care and use in research (Direção-Geral de Alimentação e Veterinária - DGV).

Experimental procedure

Rats were divided into 3 experimental groups: group 1 (HBO - animals that were submitted to chronic HBO...
treatments; n=11); group 2 (CIH - animals submitted to chronic intermittent hypoxia; n=13) and group 3 (CONT-control animals; n=12).

The HBO treatment took place in a small hyperbaric chamber, designed for small animals, with capacity for four standard cages. The chamber was built in the Portuguese Navy central shipyard and certified for air or oxygen pressurization up to 4 atmospheres.

Group 1 (HBO) underwent 20 sessions of HBO therapy at 2.5 ATA (1 atmosphere absolute - ATA) which lasted 75 minutes each. They were submitted to a daily session, at the same time, for 20 days. Taking into account the time needed for compression (10 minutes) and decompression (15 minutes), rats spent around 100 minutes inside the chamber in each HBO session. The pressure was obtained through 100% humidified oxygen.

To establish chronic intermittent hypoxia protocol group 2-CIH cages were kept in a eucapnic atmosphere, inside medium A-chambers (Biospherix Ltd, NY, USA), in an atmosphere controlled by an OxyCycler AT series (Biospherix Ltd, NY, USA), using electronically regulated solenoid switches in a three-channel gas mixer, which gradually lowered oxygen in the chamber from 21% to 5%. Rats were exposed during their sleep period to 5.6 CIH cycles/h, 10.5 h/day, for 35 days. The O₂ and CO₂ were purchased as regular gas bottles (Gasin, Portugal), while N₂ was generated from the air by pressure swing adsorption technology using a high output nitrogen generator (Nitrogen 15 Plus, PSA Technology, Sysadvance, Maia, Portugal). CO₂ was continuously monitored and the animals were maintained in normocapnic condition.

Finally, the control group (group 3 - CONT) was never exposed to hyperbaric, hypoxia or enhanced oxygen conditions.

At the end of experiments, rats were anesthetized by intraperitoneal injection with sodium pentobarbital (40 mg/Kg) and the necks were dissected through a longitudinal incision, immediately after the last HBO or CIH session. The 4 most proximal tracheal rings were carefully dissected, keeping the orientation of the anterior-posterior axis. The animals were then euthanized with an intracardiac overdose of sodium pentobarbital, and the death was confirmed by cervical dislocation.

**Light microscopy**

The rat tracheal rings were fixed in buffered 10% formaldehyde, decalcified with 10% nitric acid, dehydrated using increasing concentrations of ethanol, and then embedded in paraffin. Serial 3 μm-thick sections were obtained from each tissue block; paraffin sections were stained with hematoxylin-eosin (H&E) and periodic acid-Schiff (PAS) stain (Bancroft and Gamble, 2002). The epithelium and the basement membrane thickness were evaluated using a calibrated eyepiece at the original magnification of x400. Standard morphometric methods were used to obtain light microscopy measurements (Collan, 1984). Three points of perpendicularly cut epithelium were measured for epithelial and basement membrane thickness in each H&E slide, in order to minimize tangential section artifacts. The mean values of each variable were used for all specimens.

The epithelium and the chorion were assessed for the presence of inflammatory cell infiltrate (lymphocytes and polymorphonuclear [PMN] leukocytes); the presence of submucosal seromucinous glands was recorded. The inflammatory cell infiltrate (lymphocytic and PMN) was classified as mild (+; scattered inflammatory cells in the epithelium or chorion, with less than 5 leukocytes/high power field [HPF] 400x); moderate (++; inflammatory cell infiltrate in the epithelium or chorion, with 5-20 cells/ HPF); or intense (+++; dense inflammatory cell infiltrate in the epithelium or chorion, with 21 or more cells/ HPF). These three categories were used to simplify the statistical analysis. Such a grouping has also been successfully employed in other organs where inflammatory infiltration and its consequences have been studied (Dixon et al., 1996).

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**Fig. 1.** Light microscopy micrograph of rat tracheal rings showing increased thickness of the basement membrane of the HBO group (a) compared with the CIH group (b) or control group (c). H&E, x 400
Statistical analysis

All data are presented as mean ± SD. Comparison between the three groups of animals was performed using the Microsoft Excel® program. Histologic and morphometric differences between control and HBO treated rats were tested with the unpaired t test. The Bernoulli distribution was used to evaluate the statistical significance of the presence of inflammation. Statistical significance for all tests was set at the level of p<0.05.

Results

We found no prominent morphological differences in the general architecture of the tracheal mucosa of control rats when compared to rats exposed to HBO or CIH. Epithelia of the respiratory type was observed on the tracheal rings of all rats. Quantitative comparison of the thickness of the epithelial layer and of the basement membrane revealed that there was a significant increase (p<0.05) in both the group of rats submitted to the chronic HBO treatment and to the CIH exposure. Comparing these two groups, we found that the difference in the epithelium is not relevant, whereas the difference in the basement membrane is statistically significant (Table 1 and Fig. 1).

The 3 groups of samples were also screened for the presence of infiltrating leukocytes in the epithelium and in the chorion of the tracheal mucosa. This evaluation was done using a semiquantitative method that indicated that the rats submitted to chronic HBO presented a significantly higher (p<0.05) number of infiltrating

Table 1. Epithelium and basement membrane thickness (μm).

<table>
<thead>
<tr>
<th>Animal</th>
<th>Epithelium</th>
<th>Basement membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HBO exposed group</td>
<td>CIH exposed group</td>
</tr>
<tr>
<td>Animal 1</td>
<td>0.02375</td>
<td>0.02</td>
</tr>
<tr>
<td>Animal 2</td>
<td>0.025</td>
<td>0.0125</td>
</tr>
<tr>
<td>Animal 3</td>
<td>0.025</td>
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</tr>
<tr>
<td>Animal 4</td>
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<td>0.0125</td>
</tr>
<tr>
<td>Animal 5</td>
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<td>0.005</td>
</tr>
<tr>
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<td>0.03</td>
</tr>
<tr>
<td>Animal 7</td>
<td>0.0175</td>
<td>0.005</td>
</tr>
<tr>
<td>Animal 8</td>
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</tr>
<tr>
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<tr>
<td>Animal 12</td>
<td>0.0125</td>
<td>0.0225</td>
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<tr>
<td>Animal 13</td>
<td>0.03</td>
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Fig. 2. Light microscopy micrograph of rat tracheal rings demonstrating that the number of infiltrating lymphocytes in the chorion of the chronic HBO group (a) was significantly higher than the CIH group (b) or control group (c), sometimes even forming large reactive-looking aggregates. H&E, x 100

Fig. 3. Light microscopy micrograph of rat tracheal rings exhibiting the comparison between the scattered seromucinous glands that could be seen in the HBO group (a) and the normal density seen in the CIH group (b) and control group (c). H&E, x 100
leukocytes in the tracheal chorion than the rats from the other 2 groups (Fig. 2). Both polymorphonuclear and mononuclear cells contributed to the inflammatory infiltrate detected in the tracheal mucosa, though there was a clear predominance of lymphocytes. In contrast, data regarding epithelial infiltration were not significantly different between any of the 3 groups of animals.

As expected, most of the glands observed are seromucinous. We found a statistically significant lack of glands (p<0.05) in the HBO group, but not in the CIH one. (Fig. 3).

Discussion

We have investigated the effects of CIH exposure and HBO treatment on the structure of the rat tracheal mucosa comparing the morphology of samples of the four most proximal tracheal rings of rats submitted to either hypoxia, hyperoxia or no oxygen variations. This was done using light microscopy.

Exposing rats to CIH for 35 days caused a statistically significant increase in the thickness of the epithelium and of the basement membrane. There was an increased number of lymphocytes infiltrating the chorion. This was not, however, statistically significant.

Inflammation is often related to CIH as the origin of, for instance, cardiovascular diseases, when seen together with oxidative stress and sympathetic activation (Dumitrascu et al., 2013). It was found that exposure to CIH for 21 days increased the expression pro-inflammatory cytokines like TNF-α and IL-1b in the rat’s carotid body (Del Rio et al., 2012). On the other hand, a 7 day exposure to CIH revealed a decreased inflammatory status after measurement of Ltb, Spp1, Ccl5, MMP-9, fibronectin, HIF-2α and eNOS levels (Ramirez et al., 2012). Data concerning inflammation in the airway is not largely available rendering our results as a small contribution to understanding the response of the airway exposed to CIH.

We found that the number of seromucous glands in the tracheal mucosa of controls rats did not differ from the one on rats exposed to CIH. In contrast, when comparing the results achieved in the aforementioned groups with results from the HBO treatment group, we found a statistically significant (P<0.05) lack of glands. The loss of glands after HBO was observed in other tissues, such as mammary solid tumors, associated with a down-regulation of glandular secretory proteins (parotid secretory protein (Psp); common salivary protein 1 (Csp1); prolactin induced gene (Pip); cystein-rich secretory protein (crisp-1) and proline rich proteoglycan 2 (Prpg2) (Raa et al., 2007). The results achieved are of utmost importance when dealing with HBO lung toxicity or when HBO is applied to improve healing after tracheal anastomosis or tracheal reconstruction.

Chronic HBO treatment was also found to cause mild inflammation of the tracheal mucosa. This was expressed by moderate lymphocyte infiltration of the chorion and by an increase in the thickness of the tracheal epithelial layer and of the basement membrane. The nasal mucosa of rats treated with HBO was found to be affected in the same way as their tracheal mucosa (Vera-Cruz et al., 2008).

Injury caused by prolonged exposure to hyperoxia related to inflammation is well established in the lungs. Several groups are working to find better treatments because the respiratory implications of hyperoxia are very serious (Vosdoganes et al., 2013; Bao et al., 2014; Lv et al., 2014).

Beyond hyperoxia, the increased pressure that occurs in the HBO treatment can be a mechanical aggressor. As an example, the chronic application of CPAP on the rat nose was able to induce a mild inflammatory reaction mediated by macrophage inflammatory protein-2 (MIP-2) that results in neutrophil extravasation (Almendros et al., 2008). This rodent chemokine is homologous to human interleukin 8 (II-8), which is a potent neutrophil chemo-attractant. Taking into account these data, it is pertinent to consider that the inflammatory effects that we detected are likely to derive from the combined action of 100% O₂ and enhanced atmospheric pressure.

In our study we approached the same organ (trachea) in extremely different chronic conditions regarding oxygen exposure. As we expected the results were similar for hypoxia and hyperoxia, taking into consideration the morphologic changes revealing the presence of inflammation.

Further studies are necessary to verify what happens in lungs when exposed to HBO or CIH, so that HBO prescribing decisions or the urgency to treat a sleep apnea can be balanced for potential benefits and disadvantages (namely, airway inflammation) for each individual patient.

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References


Rat tracheal mucosa changes after hypoxia or hyperoxia


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