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Hyperbaric Oxygenation on Lung Tissue´s Morphology

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ABSTRACT

Introduction: Hyperbaric oxygen therapy (HBO) involves the inhalation of intermittent 100% oxygen under an atmospheric pressure (ATA) greater than 1 atmosphere absolute in a pressurized chamber. Despite all the benefits of HBO, there is little data in the literature regarding its repercussions on lung morphology, especially in experimental models with healthy animals. We aimed to evaluate the safety of the intervention through by evaluating the morphological parameters of the lung tissue from animals subjected to HBO.

Materials and Methods: 24 rats were assigned to Control (control, n = 12) or HBO (treated with HBO, n = 12). HBO consisted of breathing 100% oxygen at 2.5 ATA, 60 min/day, 5 days/week, for 5 weeks. After euthanasia, the lungs were processed and submitted to histopathological and histomorphometric analyses.

Results: no significant differences were found regarding histopathological analysis or morphometric parameters such as the average thickness of the alveolar wall, alveoli count, alveoli mean size and alveolar wall area.

Conclusion: our results showed that HBO did not significantly alter the tissue density of small airways, alveolar wall thickness, or airspaces number and dimensions. This clinically based protocol was considered safe for the lung tissue's morphology in healthy conditions.

Keywords: hyperbaric oxygenation; Experimental model; Respiratory system; Histomorphometry.

Introduction

Hyperbaric oxygen therapy (HBO) corresponds to the intermittent inhalation of 100% oxygen inside a chamber pressurized above one atmosphere absolute (1 ATA) to increase the levels of dissolved oxygen in the plasma[1] and, consequently, in tissues[2]. Currently, HBO is recommended as standard care in the management of diverse clinical conditions, including decompression sickness, carbon monoxide poisoning, diabetic wounds, delayed radiation injury, necrotizing fasciitis, gas gangrene and refractory osteomyelitis[3]. It is known that oxygen diffusion capacity in HBO may increase by up to four times greater than in normobaric air breathing, thus benefiting previously hypoxic regions, such as in injuries. In these regions, re-oxygenation can activate fibroblasts and stimulate the formation of new blood capillaries to facilitate tissue repair[4]. In this context, HBO was also associated with reduction of edema[5], and improvement of immune function, and is also related to a decrease in the regulation of inflammatory cytokines[6]. Since hyperbaric oxygen is firstly assimilated in the lung tissue, studies have aimed to understand the effects of HBO on the respiratory system[7,8]. In general, standard HBO protocols are considered safe in relation to lung function. Patients with heterogenous indications for HBO presented increase of the peripheral oxygen saturation and decrease of the pulse rate, without altering other functional parameters such as blood pressure, and pulmonary volumes and capacities[8]. Yamanel and coworkers demonstrated that HBO was able to reduce inflammation and injury in the septic rat lung[9]. Similarly, HBO was able to reduce apoptosis in lung tissue, as well as the pulmonary secretion of inflammatory cytokines and formation of oxidative products in lipopolysaccharide-induced acute lung injury[10].Despite all these benefits in disease setting, data regarding HBO repercussions on the morphology of the healthy lung is still scarce. Based solely on subjective pathological scoring, You *et al*[11] and Oruç *et al*[12] both reported lung tissue damage in animals receiving HBO. However, the excessive pressures (3 ATA for 1.5 h/day)[12] and the long exposition periods per session (2–8 h with 2.5 ATA)[11] used in those experiments does not fit the therapeutic regimens used for the most HBO indications, which typically ranges from 1.5–2.5 ATA for 1–1.5h[13,14,15]. In view of this inconclusive evidence, the present work aimed to evaluate the safety of HBO for the lung tissue through histomorphometry as well as histopathological evaluation in healthy rats given an usual, clinically-based HBO regimen of 2.5 ATA, 1 h a day, for 5 weeks.

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Materials and Methods

Animals and ethics: Twenty-four male rats (Rattus norvegicus "Wistar"), weighing between 220 and 300 g, aged 60 days, were obtained from the animal facilities of the Bioscience Center of the Federal University of Rio Grande do Norte (Natal, Rio Grande do Norte, Brazil) and randomly assigned to the groups Control (control, n = 12) or HBO (treated with hyperbaric oxygen, n = 12). Animals were kept in polypropylene boxes (four animals per box) in a temperature-controlled room (24 °C) on a 12-hour light-dark cycle, with chow and water *ad libitum*. Body weight, food intake and water intake were weekly monitored throughout the experimental period. This work was approved by the Commission of Ethics in the Use of Animals (CEUA, protocol number 125.054/2018) and are in strict accordance with the Guide for the Care and Use of Laboratory Animals published by US National Institute of Health. All efforts were made to minimize animal discomfort, including gentle handling and daily cleaning of the hyperbaric chamber and boxes.

HBO treatment protocol and tissue harvesting: All rats were acclimated to the environment of a hyperbaric chamber (Ecobar 400, Ecotec Equipment and Systems, Mogi das Cruzes, Brazil) for five consecutive days (1 h/day), breathing normobaric room air. After 48 h, animals from HBO group received daily treatment with 100% oxygen at 2.5 ATA, 60 min/day, 5 days/week, for 5 weeks, as described by our group previously[16]. After 48 h of the end of the experimental period, all animals were overnight-fasted, anesthetized with isoflurane (2–3%), and weighed. Euthanasia was performed by cardiac exsanguination. Lungs were harvested and kept in a fixative buffer with formalin (10%). After 24 h, samples of lungs were dehydrated and embedded in paraffin. Five μm sections were made and slides were stained with hematoxylin and eosin (HE).

Morphological analyses: Five fields from each lung sample were captured on a light microscope (Leica DM750) with 10x and 40x objectives coupled to an ICC50 HD camera. For automated morphometric analysis, the airspaces (alveoli and sacs) in each image were counted, their individual areas were measured, and the alveolar tissue was analyzed in terms of its area fraction (alveolar tissue area/total area of lung section) and septa thickness using macros in the ImageJ software (NIH, Bethesda, USA; https://imagej.net/Fiji). Histopathological evaluation was based on the observation of three parameters chosen in the lung tissue: 1) inflammation; 2) congestion and 3) hemorrhage. The final lung damage score was calculated based on an average of each parameter A scale of 0 (absent) to 3 (intense) was used for pathological grading of each image with 10x and 40x objective lenses[17].

Statistical analysis:

Data were analyzed and plotted using GraphPad Prism software version 7.0 (GraphPad Software, San Diego, USA). Groups were compared using unpaired t test. Descriptive data were expressed as mean ± standard error of the mean (SEM). Significance was set at $p < 0.05$.

Results

Table 1 shows the results of food consumption, water intake, and body weight of the animals. A significant reduction of 19.5% in the food intake ($p < 0.05$) was observed in HBO when compared to the Control. The other variables did not demonstrate any significant differences between both groups. The general architecture of the lung tissue is shown in Figure 1. In Control, alveoli can be observed in their normal appearance with a morphology characterized by irregularly arranged, spongiform contours and septa with a delicate thickness (Figure 1A). Absence of inflammatory infiltrate (Figure 1C) and congested vessels (Figure 1A) can also be noted in the healthy lung tissue. When analyzing the HBO group (Figures 1B, 1D and 1F), the presence of some congested vessels (Figure 1B), a discrete perivascular inflammatory infiltrate (figure 1D) and hemorrhage points (figure 1F) can be observed. Lung damage or injury was calculated through the mean of three parameters: vascular congestion, inflammation, and hemorrhage, being represented separately and together (Table 2). A slight increase in congested vessels was observed in HBO when compared to Control, although not statistically significant. A discrete increase in congested vessels was noted in the HBO when compared to Control, although not statistically significant. Moreover, there were no significant changes regarding these parameters when viewing the two groups. Table 2 demonstrates that, although there is a tendency in the appearance of some histopathological findings and morphological changes in lung tissue with the use of HBO, these were not significant when compared to Control. When observing the parenchyma of animals

Table 1. Food Intake, water intake and weight.

	FI (g)		WI (ml)		W (g)	
	Week 1	**Week 5**	**Week 1**	**Week 5**	**Week 1**	**Week 5**
Control	85.10 ± 1.13	91.30 ± 3.25	128.70 ± 16.54	147.55 ± 7.70	250.25 ± 19.46	333.75 ± 51.79
HBO	70.06 ± 3.42*	73.15 ± 2.61*	130.6 ± 11.31	111.7 ± 15.31	233.85 ± 17.17	315.57 ± 51.90

Food Intake, water intake and weight. FI, Food intake, WI, Water intake, W, Weight. Data expressed as mean ± SEM. *Significant difference versus control group.

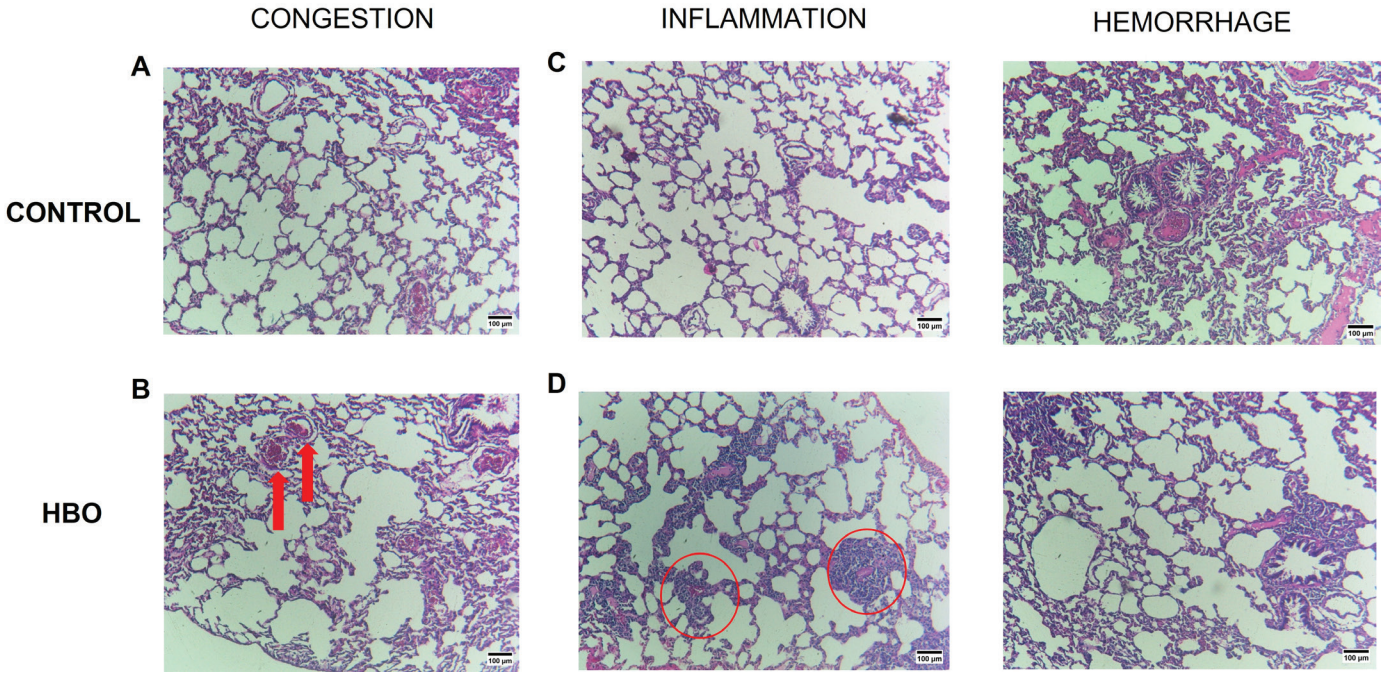

Figure 1. Representative images of lung tissue morphology from control animals (Fig. 1A, 1C and 1E) and animals subjected to HBO (Fig. 1B, 1D and 1F) are presented. In Control, pulmonary ducts, sacs and alveoli with their delicate septa are evident, interspersed with bronchioles and blood vessels without morphological changes (Figures 1A, 1C and 1E). The appearance of some congested vessels (Figure 1B) indicated by the red arrow and the presence of some inflammatory foci (Figure 1D) marked by the red circle were observed in animals in the HBO group. Images captured at 10x magnification. Scale bar = 100 µm.

Table 2. Analysis of morphological parameters.

	Congestion	Hemorrhage	Inflammation	Lung damage
Control	1 ±0.5	0.11 ± 0.33	1.14 ± 0.69	0.75 ± 0.55
HBO	1.11 ± 0.60	0.2 ± 0.42	0.85 ± 0.69	0.72 ± 0.46

Analysis of morphological parameters. Congestion, hemorrhage, inflammation and lung damage.

submitted to HBO, normal lung morphology was maintained, which is evident in Figure 1F. The fraction of alveolar tissue in relation to the total lung section area (or respiratory area %) was calculated in the control group and HBO, as represented (Table 3). The average size of the alveoli, the area of the alveolar wall, as well as the average thickness of the alveolar wall were quantified in groups. No significant differences were observed between the groups for any variable.

Discussion

There is still controversy about what oxygen at high pressures causes in the lungs of healthy living organisms. In the present study, HBO did not promote significant morphological changes in the lung tissue of healthy rats. However, it is known that HBO generates changes in molecular levels, especially in injured organs. For instance, HBO decreased the intercellular adhesion molecule (ICAM)-1 expression in an *in vitro* endothelial cell injury study and induced the production of endothelial nitric oxide synthase (eNOS)[18]. In another work, it was shown that HBO significantly reversed hypoxemia and reduced lung injury assessed at 5 and 24 hours in Sprague-Dawley rats in 24 hours[19]. This was an analysis of an in vitro model of I/R injury in endothelial cells, in which HBO effectively downregulated ICAM-1 expression. Like other studies, these results indicate that HBO has a role in the lungs of animals, but in an already affected context and signal the need for more studies on the respiratory system using hyperbaric oxygen. In this context, there is still debate regarding the potential adverse effects of HBO on lung tissue for those who use it to treat non-pulmonary conditions. This work addressed this question in the subjective histopathological and objective morphometric aspects, seeking greater elucidation by exposing the healthy rat lung to a clinically based HBO regimen. In the present study, objective morphometric analysis of the alveolar tissue and airspaces showed no significant differences in respiratory area fraction between the

Table 3. Morphometric analysis parameters.

	Control	HBO
Alveoli Count	12.25 ± 3.32	13.00 ± 4.45
Alveoli mean size (µm²)	3772.41 ± 2674.14	2986.04 ± 1476.04
Alveolar wall area (%)	30.36 ± 4.07	33.45 ± 7.89
Alveolar wall mean thickness (µm)	19.12 ± 5.86	19.57 ± 6.35

Morphometric analysis parameters. Alveolar count, alveolar mean size, alveolar area fraction, and average alveolar wall thickness.

groups, suggesting that the HBO protocol consisting of 1-h HBO daily sessions for 5 weeks using 2.5 ATA does not damage the gas exchange-specialized structures in rat lung. Moreover, as this treatment protocol did not increase the size of the individual airspaces nor reduced its number, we also infer that destruction of septa was not present in the HBO group. In diabetic wounds, HBO is implemented to stimulate collagen synthesis and healing[6]. Given this, the hypothesis could be raised that HBO would also induce the same effect in the alveolar interstitium resulting in fibrosis and impaired hematosis. This would be especially for diabetic patients as hyperglycemia itself causes collagen deposition and septal thickening, as seen in other studies[20,21]. Although collagen was not assessed directly herein, we assume that HBO did not trigger profibrotic signaling as the thickness of alveolar walls was preserved, and thereby the gas diffusion distances. Conversely to our data, it was previously stated the presence of severe pulmonary edema followed by death in rats subjected to 4 ATA HBO for more than 3h[22]. Histopathological signs of lung injury were also reported by You *et al*, associated with the increase of the matrix metalloproteinase 9 and caspase 3 protein expressions, and of oxidative stress when exposing rats to 2.5 ATA HBO for 2–8 h in a single session[11]. As in other studies, there was no relevance of antioxidant signaling compensation, which occurs during the longer period of treatment[23,24,25]. In rabbits submitted to an HBO protocol of 3 ATA for 1.5 h/day for 7 and 28 days, pulmonary changes involved vessel congestion, leukocyte infiltration, interstitial edema, and alveolar dilation[12]. Of note, the adopted pressures in HBO care are usually 2–2.5 ATA and session duration vary between 1 and 1.5 h[13,14]. However, by setting such long exposure times and/or high pressures for HBO, those studies have clearly focused on the induction of lung toxicity rather than closely mimicking clinical protocols. As stressed by Oter *et al* and supported by a recent meta-analysis[15], clinicians should pressurize patients as low as possible to make HBO safer since oxidative response is pressure-dependent[13]. The data on the topic come from studies that induced pulmonary toxicity using HBO parameters above what is currently considered clinically safe[13,14,15]. Indeed, not only oxidative stress but also the incidence of clinical adverse events was proven minimal as lower the treatment pressure and the shorter its course relative to the approved therapeutic limits (3 ATA maximum and 2 h duration)[13,15]. To minimize edema, oxidative stress, and other adverse events (e.g., ear barotrauma), using optimum time intervals and rates of compression is also recommended for reducing any sudden changes[22,26]. Therefore, the amount of pressure manipulated, and treatment time are critical parameters, as they determine the risk of HBO toxicity, and must be studied and readjusted before use. In parallel, it is necessary to regularly monitor possible adverse effects[13]. To date, it is still unknown what the lung tissue looks like in patients who receive HBO to treat any condition (non-pulmonary) for which HBO is first line, such as burn injury, limb amputation/crushing, a diabetic foot wound, anemia, etc. In addition to the local benefits resulted from the treatment, side effects on the lung should be considered by the clinicians since HBO acts systemically.

Conclusion

Our results demonstrated that using a treatment regimen that abides current HBO evidence and practice was not able to promote significant alterations on the lung tissue of rats, especially in the tissue density of small airways, alveolar wall thickness, or airspaces number and dimension. So, the present study demonstrated that a clinically based HBO protocol was a safe procedure for the lungs of healthy rats at a morphological level.

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