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Review

Cyclosporine and hyperoxia-induced lung damage in neonatal rats☆

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ABSTRACT

Cyclosporine effects on hyperoxia-induced histopathological and functional changes in the rat adult lung are controversial and the newborn lung has not been studied. Thus, we evaluated the effects of cyclosporine in young rats after 60% hyperoxia exposure postnatally. Experimental categories included: (1) room air for the first 5 postnatal weeks with daily subcutaneous injections of saline from postnatal day (PN)15 to PN35; (2) room air with daily injections of cyclosporine from PN15 to PN35; (3) 60% oxygen from PN0 to PN14 and then daily saline injections during the following three weeks; (4) 60% oxygen from PN0 to PN14 followed by cyclosporine treatment from PN15 to PN35. Hyperoxia significantly reduced the number of secondary crests and microvessel density, and it increased the mean alveolar size and septa thickness. Cyclosporine treatment did not significantly modify the hyperoxia-induced changes. Conversely, in normoxia, cyclosporine reduced microvessel density and the number of secondary crests. In conclusion, cyclosporine did not modify alveolar and microvascular parameters in hyperoxia exposure, although it caused some changes in normoxia.

1. Introduction

Cyclosporine is an immunosuppressive drug used in the treatment for organ transplantation and autoimmune diseases. It acts by blocking activation and proliferation of T lymphocyte and by inhibiting their cytokine production. Cyclosporine may also indirectly affect B-cell immune response through inhibition of T helper lymphocytes (Osadchy and Koren, 2011). Cyclosporine is excreted into human milk and women in treatment with cyclosporine are usually advised not to breastfeed (American Academy of Pediatrics Committee on Drugs, 2001). The literature describes cases of breast-feeding without adverse effects and with undetectable cyclosporine concentrations (Nyberg et al., 1998; Munoz-Flores-Thiagarajan et al., 2001), but therapeutic concentrations have also been observed in infant blood (Moretti et al., 2003). Cyclosporine shows controversial effects on the adult lung, but the effects in the first postnatal period are largely unknown. Moreover, cyclosporine has been reported to show some therapeutic effects on lung hyperoxic damage in adult experimental animals (Matthew et al., 1999, 2003; Pagano et al., 2004), but there are no reports addressing its possible role in experimental models of bronchopulmonary dysplasia (BPD). BPD is the most common chronic lung disease of prematurity. The classic severe form of BPD is strongly related with oxygen toxicity and mechanical injury, whereas the milder forms which are seen nowadays, mainly in small premature infants surviving after prolonged mechanical ventilation, relate more to immaturity, perinatal infection and inflammation, persistent ductus arteriosus, and disrupted alveolar and capillary development (Bancalari et al., 2003). BPD mainly results in impaired alveolar growth and dysmorphic vascular architecture (Thebaud and Abman, 2007). An experimental model of BPD involves exposure to hyperoxia of rats and mice in the early postnatal period. Hyperoxia disrupts postnatal alveolar development, leading to smaller numbers of enlarged and simplified alveoli, thicker septa, increased numbers of alveolar macrophages, and changes in microvascular development (Dauger et al., 2003; Balasubramaniam et al., 2007). Hyperoxia may also cause injury to alveolar type II cells and impair surfactant phospholipids synthesis and decrease surfactant proteins and mRNA (Minoo et al., 1992). The mechanisms of action of cyclosporine could, theoretically, modify some of these pulmonary changes. Thus, the aim of the present work was to assess the effects of postnatal cyclosporine exposure in young rats and, specifically, in a rat model of BPD, to ascertain whether cyclosporine exposure worsens hyperoxia-induced changes or whether it may show some therapeutic effects.

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2. Methods

2.1. Materials

Female wild-type Sprague-Dawley rats (Harlan, Udine, Italy) and their offspring were housed and handled in accordance with the guidelines of the Helsinki Declaration and the recommendations of the Italian public health authorities. The study had approval from the Ethics Committee of the University of Padua for experiments on animals. The study was conducted on male or female rat pups kept together with their nursing mother in clear polished acrylic chambers, where oxygen and CO_2 were continuously monitored (BioSpherix, OxyCycler model A84XOV, Redfield, NY). The animals were maintained under standardized conditions of light (alternate 12-hour cycles of light and dark, starting with light on at 08.00 h) at room temperature of 22 °C and humidity of 50%. After term gestation, the pups were randomly distributed between the following four experimental groups: (1) neonatal rats ($n = 7$) raised in room air for the first 5 postnatal weeks with subcutaneous daily injection of saline (NaCl 0.9%) from postnatal day (PN)15 to PN35 (21%+ Saline); (2) neonatal rats ($n = 6$) raised in room air with daily subcutaneous injection of 15 mg/kg cyclosporine from PN15 to PN35 (21%+ Cyclosporine); (3) neonatal rats ($n = 6$) raised in 60% oxygen from PN0 to PN14 and then given daily injection of saline during the following three weeks (60%+ Saline); (4) neonatal rats ($n = 5$) raised in 60% oxygen from PN0 to PN14 followed by daily injection of cyclosporine from PN15 to PN35 (60%+ Cyclosporine). The nursing dams were rotated every one or two days to prevent any negative effects of hyperoxia on nursing. The pups' body weight was measured daily. On postnatal day 35, the animals were euthanized with an overdose of tiletamine-zolazepam (Zoletil®) and xylazine (Rompun®). The lungs were then removed *in toto* and fixed in buffered formalin, serially dehydrated in rising concentrations of ethanol, and embedded in paraffin.

2.2. Morphometric analysis

For each rat, two 4-μm sections of the lungs were stained with hematoxylin and eosin, two sections were stained with azan-Mallory and two with Weigert–van Gieson staining. All assessments were conducted with a Leica DM 4000B microscope (Leica, Solms, Germany) integrated with a camera (Leica DFC 280). Morphometric analyses were performed according to Grisafi et al. (2012). Briefly, photomicrographs were obtained on a field of 568 μm × 422 μm with a LeicaDM4000B microscope (Leica, Solms, Germany) integrated with a camera (Leica DFC 280). Lung morphometric analyses were performed by two independent researchers blinded to the treatment strategy, using ImageJ, a public domain Java image processing program created by Wayne Rasband at the Research Services Branch, National Institute of Mental Health, Bethesda, MD (http://rsb.info.nih.gov/ij). In particular in each section, from a specific plug-in which leads to the evaluation of the skeletonized air spaces into each high powered field (hpf), the mean alveolar size was evaluated by considering the alveolar minimum and maximum diameter and excluding the areas of large airways or vessels from analysis. A cell counter was applied for assessing the secondary crests number/hpf.

2.3. Immunohistochemistry

Microvessel density was calculated according to Porzionato et al. (2004) and Grisafi et al. (2012) in two 4-μm-thick sections immunostained with anti-CD31 antibody. Lung sections were hydrated gradually and were incubated in 0.03% hydrogen peroxide in deionized H_2O, to eliminate endogenous peroxidase activity and to enhance antibody penetration in the tissue. Antigen unmasking was performed with 10 mM sodium citrate buffer, pH 6.0, at 96 °C for 30 min. Sections were incubated for 30 min in blocking serum (0.04% bovine serum albumin (A2153, Sigma–Aldrich, Milan, Italy) and 0.5% normal goat serum (X0907, Dako Corporation, Carpinteria, CA, USA) to eliminate unspecific binding. Sections were then incubated for 1 h at room temperature with a mouse monoclonal antibody against CD31 (Dako, Milan, Italy) diluted 1:50 in PBS. Primary antibody binding was revealed by incubation with anti-rabbit/mouse serum diluted 1:100 in blocking serum for 30 min at room temperature (DAKO® EnVision + TM Peroxidase, Rabbit/Mouse, Dako Corporation, Glostrup, Denmark) and developed in 3,3′-diaminobenzidine for 3 min at room temperature. Lastly, sections were counterstained with hematoxylin. Sections incubated without primary antibodies showed no immunoreactivity, confirming the specificity of the immunostaining. For each case, we examined ten fields per section at a Leica DM 4000B microscope (Leica, Solms, Germany) integrated with a camera (Leica DFC 280). The number of CD31-positive vessels (<100 μm in size) was counted per hpf. The mean values of microvessel density were calculated for each case and for the entire experimental groups.

2.4. Statistical analysis

Results are expressed as mean values ± SD. Statistical analysis was performed with one-way ANOVA and Tukey's multiple comparison test. A $P < 0.05$ was considered statistically significant. Statistical calculations were conducted with Prism 3.0.3 (GraphPad Software Inc., San Diego, CA).

3. Results

No statistically significant differences in body weight were found between the different experimental groups. Histopathological examination of the lung sections showed an impaired alveolar development in the two hyperoxic groups in comparison with the control animals grown in room air. The distal airspaces were fewer in number and enlarged, with reduced septation. Patchy areas of marked interstitial thickening were also appreciable, with increased content of collagen and elastin at azan-Mallory and Weigert–van Gieson stainings. Cyclosporine treatment in hyperoxia-exposed animals did not significantly modify the above-mentioned hyperoxia-induced changes in alveolarization. Conversely, in normoxic groups, cyclosporine treatment induced a slight reduction in septation and an increase in the thickness of the alveolar septa. Azan-Mallory and Weigert–van Gieson stainings also showed a slightly increased content of collagen and elastin in the alveolar septa of normoxic rats treated with cyclosporine (Figs. 1 and 2).

The results of the lung morphometric analysis are shown in Figs. 3 and 4. The mean number of secondary crests per high-powered field was higher in the control rats (21%+ Saline: 12.7 ± 0.8) than in all the other experimental groups (21%+ Cyclosporine: 9.5 ± 0.9, $P < 0.05$; 60%+ Saline: 8.3 ± 0.8, $P < 0.01$; and 60%+ Cyclosporine: 6.3 ± 0.7, $P < 0.001$). The mean alveolar surface in the rats exposed to hyperoxia and saline (60%+ Saline: 1837.0 ± 96.6 μm^2) was higher than in both normoxic groups (21%+ Saline: 958.2 ± 64.7 μm^2, $P < 0.01$; 21%+ Cyclosporine: 1107.0 ± 224.3 μm^2, $P < 0.05$). Conversely, cyclosporine treatment was found not to significantly modify the mean alveolar surface after hyperoxia exposure (60%+ Cyclosporine: 1399.0 ± 310.5 μm^2, $P > 0.05$). The pulmonary microvessel density was also higher in the control rats (21%+ Saline: 22.9 ± 1.3) than in all the other experimental groups (21%+ Cyclosporine: 18.5 ± 1.0, $P < 0.05$; 60%+ Saline: 16.8 ± 0.9, $P < 0.01$; 60%+ Cyclosporine: 17.6 ± 1.2, $P < 0.05$).