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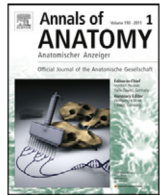

Research article

Bronchopulmonary dysplasia in a double-hit mouse model induced by intrauterine hypoxia and postnatal hyperoxia: Closer to clinical features?

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SUMMARY

Despite increased survival of very preterm newborns, bronchopulmonary dysplasia (BPD) remains a major threat, as it affects long-term pulmonary function and neurodevelopmental outcome. Recent research focused on mechanisms of lung repair. Animal models of BPD in term rodents use postnatal hyperoxia in order to mimic features observed in very preterm human neonates: reduced alveolarization and impaired septal architecture without profound inflammatory changes. In contrast, BPD in very preterm human neonates involves prenatal hits e.g. infections and growth restriction plus postnatal ventilation. BPD induced in rodents by postnatal hyperoxia also exhibits reduced alveolarization however without septal pathology but with marked inflammation.

We therefore aimed to establish an animal model combining prenatal growth restriction (FiO_2 0.1 for 4 days) with postnatal hyperoxia (FiO_2 0.7 for 2 weeks). In double-hit mice the development was retarded: body weight and length, lung and brain weight were significantly reduced by day P14 compared with normoxic controls. Histomorphometric analysis revealed reduced alveolarization and increased septal thickness without pronounced inflammatory lesions. A down-regulation of *SftpB* and *SftpC* genes was observed in double-hit animals compared with controls. Thus, we established a new model of BPD using pre- and postnatal hits.

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1. Introduction

Bronchopulmonary dysplasia (BPD) remains a major threat of very preterm birth (<32 weeks gestational age) and is currently observed at a rate of up to 20% among these children (Gortner et al., 2011) and is a relevant factor for neonatal mortality (Stichtenoth et al., 2012). As main clinical risk factors have been identified the degree of prematurity, pre- and postnatal infections, postnatal asphyxia, intrauterine growth restriction (IUGR) and male gender. These data were obtained from a European multicenter trial enrolling about 5000 preterm neonates (MOSAIC-Study) (Gortner et al., 2011). As very preterm neonates account for about 1.5% of all births in Europe (Field et al., 2009), the disorder still represents a burden for affected individuals and society. Therapy of BPD still is controversial in neonatology. An increased survival without BPD has been demonstrated following the introduction of surfactant replacement therapy, more than 20 years ago (Seger and Soll, 2009). Thus, intratracheal surfactant administration became standard in neonatal intensive care. However, further pharmacological prophylaxis and therapy of BPD are still a matter of controversy.

Postnatal corticosteroid therapy has been identified to reduce the number of babies with BPD, however, it was associated with an increased number of infants surviving with cerebral handicaps (Wood et al., 2005). Thus, this pharmacological approach is reserved only to life-threatening courses of BPD (Doyle et al., 2010). Further drug therapies, proven to marginally reduce the incidence of BPD, include postnatal administration of caffeine and vitamin A (Thomas and Speer, 2008).

Short-term effects of BPD during early childhood include an increased risk of re-hospitalization, mainly secondary to life-threatening acute respiratory infections (Greenough, 2008), and at long-term an increased risk for asthma and persistent airway hyperresponsiveness until the early adult age (Greenough, 2008; Vrijlandt et al., 2006). These findings were attributed to an impaired

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Table 1
TaqMan® Gene Expression Assays used for quantitative RT-PCR.

Gene	Symbol	Taqman assay ID
Surfactant-associated protein C	*SftpC*	Mm00488144_m1
Surfactant-associated protein B	*SftpB*	Mm00455681_m1
Elastin	*Eln*	Mm00514670_m1
Mammalian target of rapamycin	*Mtor*	Mm0044968_m1
Hypoxia inducible factor 1, alpha	*Hif1a*	Mm00468869_m1
Insulin-like growth factor 1	*Igf1*	Mm00439560_m1
Vascular endothelial growth factor	*Vegf*	Mm01281449_m1
Transforming growth factor, beta 1	*Tgfb1*	Mm01178820_m1
Transforming growth factor, beta 2	*Tgfb2*	Mm00436955_m1
Transforming growth factor, beta 3	*Tgfb3*	Mm00436960_m1
Retinoic acid receptor, alpha	*Rara*	Mm01296312_m1
Retinoic acid receptor, beta	*Rarb*	Mm01319677_m1
Retinoic acid receptor, gamma	*Rarg*	Mm00441091_m1
Retinol binding protein 1	*Rbp1*	Mm00441119_m1
Cellular retinoic acid binding protein 1	*Crabp1*	Mm00442776_m1
Actin, beta[a]	*ActB*	Mm00607939_s1

[a] Endogenous control.

lung repair and development after the neonatal insult. The most critical impact on long-term outcome however represents the association of BPD with an adverse neurodevelopmental prognosis (Bassler et al., 2009; Mello et al., 2009). A recently published study from the NICHD network proved an association of BPD with spastic diplegia (Van Marter et al., 2011).

The present data underline the need for experimental data in order to provide further insight into mechanisms of lung repair. As an adequate experimental model of BPD in small animals for evaluation of innovative therapies is still lacking, the goal of the present study was to develop a perinatal double-hit model of BPD in neonatal mice, which is close to clinical features in humans. It was aimed to administer a pre- and postnatal noxious insult which mimics a typical clinical setting in very preterm neonates.

2. Materials and methods

2.1. Animal model

Date-mated pregnant mice (C57BL/6 from Charles River, Sulzfeld, Germany) were housed in individual cages being fed at libitum. The animals were randomly assigned to a normoxia group as controls (4 litters, 32 pups) or prenatal hypoxia and postnatal hyperoxia as an experimental double-hit group (15 litters, 116 pups). Not all of the animals were included in all the analyses. For auxological measurements, at least 9 animals per group were used (see Table 2). For molecular biology at least 10 animals per group were used and at least 5 animals per group were included in the stereological analysis. Experimental procedure is shown in Fig. 1. Hypoxia and hyperoxia were created in a ventilated chamber by applying nitrogen or oxygen respectively into the chamber. FiO_2 was regulated by an autoregulatory unit (model 110, Biospherix, Ltd. Redfield NY, USA). The animals' condition was visually checked twice a day.

One litter ($n = 9$) of the animals exposed to prenatal normoxia was exposed to prenatal hyperoxia.

The experimental protocol was approved by the local board for animal welfare (Landesamt für Gesundheit und Verbraucherschutz, Abteilung Lebensmittel und Veterinärwesen, Saarbrücken, Germany, AZ: H-1 2.4.2.2).

2.2. Auxological measurements

We obtained auxological data from the mice with respect to body weight at P1, P7 and P14 by means of a precision scale (see Table 2). In addition at P14, the body length and fronto-occipital head length were measured using a digital caliper. The dams were weighed at day E14 and at days P1, P7 and P14.

2.3. Lung tissue preparation

For further evaluation of lung development, lungs were excised and kept both for molecular analyses and for histological investigation. Therefore, the pups were sacrificed at P14 by injecting 400 mg pentoparbital per kg body weight intraperitoneally. The lungs were immediately removed, shock frozen in liquid nitrogen and stored at −80 °C for molecular analysis or fixed for morphological evaluation (0.1 M cacodylate buffer, 1.0% paraformaldehyde, 1.0% glutaraldehyde, pH 7.3–7.4). The fixation solution was instilled into the lung by introducing a fine catheter (0.6 mm × 19 mm, BD Vasculon™ Plus) into the trachea and letting the buffer flow into the lung with a pressure of approximately 20 cm of water. To guarantee that the pressure was the same for all the lungs, the catheter with the lung was connected to a junction line (1.1 mm × 2.2 mm, Riplast, Neunkirchen-Saar, Germany) and suspended from a syringe filled with fixation solution. Thus, the pressure was only determined by the height of the water column and was the same for all preparations. This kind of lung inflation is a common technique used to apply constant pressure while fixing different samples (Vasilescu et al., 2012; Knust et al., 2009). It may result in different distension depending on the stiffness of the organ, but as the pressure remains constant the different distension should always be the same in all the organs with comparable stiffness. Thus, if immaturity influences the stiffness of the lung in one group, there should be a different distension which should be comparable for all the samples in the group. The lungs were then transferred into fixation solution and stored at 4 °C.

2.4. Brain tissue preparation

The mice were decapitated and brains dissected and embedded in tissue freezing medium (Leica, Nussloch, Germany) and frozen in the liquid nitrogen gas phase. Histology was performed on frontal cryosections of 10 μm thickness, which were mounted on poly-L-lysine coated slides and air dried at 40 °C for 30 min (Meier et al., 2006). Histology on mouse brain cryosections according to Kluver–Barrera was performed as described previously (Meier et al., 2006).

2.5. Sampling for stereological evaluation

The sampling and stereological methods are in accordance with the guidelines for quantitative assessment of lung structure (Hsia et al., 2010).

The right single lung was embedded in 2% aqueous agar-agar. Afterwards, the organ was cut from apical to caudal into parallel slices with a thickness of 2 mm by means of a tissue slicer. The position of the first cut was chosen at random between 0 and 3 mm (Fehrenbach and Ochs, 1998). Normally four to six slices were obtained per single lung. The organ slices were put in the correct order with the apical section looking up. Starting with a random number, slices were alternatingly taken for light and for electron microscopy. Tissue blocks for electron microscopy were received as follows. A transparent point grid was superimposed at random orientation over the collection of remaining slices. Parts of the lung slices touched by a point were excised and cut into small tissue blocks with a size of 1–2 mm^3. This systematic uniform random sampling method guarantees to obtain specimens which were representative of the whole organ (Fehrenbach and Ochs, 1998).

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