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Single, double, and triple-hit strategies to establish a long-term premature rabbit model of bronchopulmonary dysplasia

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Abstract

Background Bronchopulmonary dysplasia (BPD) is a chronic lung condition of premature neonates, yet without an established pharmacological treatment. The BPD rabbit model exposed to 95% oxygen has been used in recent years for drug testing. However, the toxicity of the strong hyperoxic hit precludes a longer-term follow-up due to high mortality after the first week of life. This study aimed to extend the preterm rabbit model to postnatal day (PND) 14 to mimic the evolving phase of BPD and enable the investigation of therapeutic interventions at later and more relevant time points.

Methods Preterm rabbit pups delivered on the 28th day of gestation were either exposed to room air or different degrees of hyperoxia (50% and 70% O_2) for 14 days. Single (immediately after birth) or double (at birth and at PND5) intratracheal lipopolysaccharide (LPS) administrations were also tested in combination with 50% O_2. Age-matched rabbits delivered vaginally at term were used as controls. Survival, weight gain, lung function, pulmonary artery micro-ultrasound Doppler analysis, lung histology (alveolarization, lung injury score, and design-based stereology), and longitudinal micro-CT imaging were used to compare the outcomes at PND14.

Results Premature birth itself, without any other BPD hit, was associated with lung function deficits, delayed lung development, and cardiovascular abnormalities. The BPD-like lung phenotype was enhanced by 70% O_2 but not by 50% O_2 hyperoxia. Intratracheal LPS delivered immediately after birth was associated with significantly higher lung injury scores at PND14 and increased tissue damping, a marker of parenchymal air resistance.

Conclusion Several strategies are feasible to extend the preterm rabbit model of BPD to PND14. Preterm birth at the saccular phase itself, even in the absence of other postnatal BPD hits, was associated with lung function deficits, delayed lung development, and cardiovascular abnormalities compared with age-matched term rabbit pups. Enhanced BPD-like phenotypes can be further achieved by continued exposure to moderate hyperoxia (70% O_2) and the intratracheal administration of LPS.

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Keywords Premature birth, Bronchopulmonary dysplasia, BPD, Preterm rabbits, micro-CT, Hyperoxia, LPS exposure, Lung stereology

Background

Bronchopulmonary dysplasia (BPD) is a chronic lung condition of premature neonates with long-term pulmonary sequelae [1, 2]. BPD occurs almost exclusively in extremely premature infants (i.e., born below 28 weeks of gestation) [3, 4] who display underdeveloped lungs and usually require intensive respiratory support and supplemental oxygen to adapt to extrauterine life [5]. Although life-saving, protracted mechanical ventilation and supplemental oxygen have been shown to induce inflammation, which in turn activates complex injury and repair mechanisms that ultimately lead to arrested alveolarization and vascular development [6]. Perinatal inflammation may also stem from antenatal (i.e., chorioamnionitis) and postnatal infections, which further increase the risk of developing BPD [7]. Unfortunately, there is no cure for BPD, and the search for pharmacological interventions remains an unmet clinical need.

Animal models are crucial for the preclinical development of pharmacological treatments. Newborn rodents delivered at term and exposed to postnatal hyperoxia [8–11] and other perinatal inflammatory insults, such as antenatal lipopolysaccharide [LPS]) [12–14], as experimental BPD hits have been widely used for basic explorative research in the context of BPD. However, rodent lungs are fully functional at birth and thus do not mimic the gestation interruption triggered by premature birth [15]. Large animal models like preterm lambs, piglets, and baboons, combining premature birth with other BPD hits like antenatal inflammation, mechanical ventilation, and hyperoxia, have also been used in the late-stage preclinical development of BPD drugs (e.g., postnatal steroids, Vitamin A, rhIGF-1/rhIGFBP3) [16–23]. Large animal models, however, are less cost-effective and bear ethical concerns; therefore, they are less suitable for early exploratory research on BPD therapeutics.

The rabbit model provides a good compromise between small and large BPD models. Premature rabbits delivered at 28 days of gestation (i.e., at the early saccular phase) display mild to moderate respiratory distress and impaired postnatal lung development at postnatal day (PND) 7 compared to age-matched term rabbit pups [24]. Therefore, premature birth alone can be considered as the first experimental BPD hit. Preterm rabbits exposed to an additional hyperoxic hit (95% O_2) for seven days have been used for pharmacological testing in the context of BPD [25–28]; however, this model has a relatively short follow-up compared to rodent models or premature lambs, which can be maintained for several weeks [29–31]. This limitation is due to the strong and irreversible toxicity generated by the seven-day 95% O_2 exposure in preterm rabbits, which reduces the lifespan of the pups [32] and limits the pharmacological assessment of new compounds to finding a partial attenuation of the hyperoxic damage at PND7 [25, 27, 28]. A longer-term preterm rabbit model, up to PND14, would theoretically allow for pharmacological interventions at later and more relevant time points, offering the possibility of treating the early (inflammatory phase) and evolving course (remodeling phase) of experimental BPD. Therefore, this study aimed to investigate the feasibility of establishing a long-term BPD model, leveraging the prematurity feature of preterm rabbits (i.e., the unique common feature to all human BPD cases) and combining it with additional postnatal hits like hyperoxia and the intratracheal delivery of LPS.

Materials and methods

Animal care and delivery

All experimental procedures involving animals were approved by the local animal ethics committee (Animal Welfare Body: Organismo Preposto al Benessere Animale [Italy], file number n°783/2019-PR) and met the standard European regulations on animal research. All Animal experiments were carried out at Chiesi Farmaceutici, an AAALAC accredited facility.

Time-mated New Zealand White rabbits were obtained from Charles River Laboratories (Domaine des Oncins, France) and maintained at 15–21 °C, relative humidity of 55%±15, 12:12 h light/dark cycles, and food and water *ad libitum* until the Caesarian section (C-section) or natural birth occurred.

Premature rabbit pups were delivered at 28 days of gestation (term 31 days). For the C-section, does (3.8 ± 0.3 kg) were sedated with intramuscular (i.m.) medetomidine 2 mg/kg (Domitor®, Orion Pharma, Finland). Ten minutes later, they received 25 mg/kg of ketamine (Imalgene 1000®, Merial, France) and 5 mg/kg of xylazine (Rompun®, Bayer, Germany) i.m. Subsequently, does were euthanized with an overdose of pentothal sodium (50 mg/kg, MSD Animal Health, USA). The abdomen was immediately opened, and the uterus was exposed to extract all pups through hysterectomy. Pups were immediately dried, stimulated, and placed in *ad hoc* incubators (Okolab, Italy), where temperature, oxygen, and humidity were set according to the experimental condition of each group. Preterm rabbits were placed on sterilized soft bedding and remained in the incubator except for the feeding. Housing material was changed daily. Pups were fed twice daily (morning and afternoon)

via a 3.5 Fr tube (Vygon, France) with a milk replacer (Day One®, Protein 30%, Fat 50%; FoxValley, USA) dissolved in water (250 mg/mL). Probiotics (25 mg/mL, Bio-Lapis®; Probiotics International Ltd, UK) were added daily, whereas additional immunoglobulins (15 mg/mL, Colo-Cat®, SanoBest, Netherlands) were only administered in the first two days. The volume of feeds was progressively increased from 80 mL/kg/day on the day of birth to 100 mL/kg/day on PND1, 150 ml/kg/day on PND2, and 200 mL/kg/day from PND3 to the end of the experimental period (PND14). The total volume was divided into two daily feeds. On day 2, vitamin K was administered intramuscularly (0.25 mg/kg, Konakion®; Roche). Pups were stimulated to urinate twice daily before feeding. From PND7 to PND14, pups were let to urinate before their weight was measured in the morning.

Term pups were vaginally delivered and fostered by their mothers. Does were placed in special cages equipped with an external box where they could build their nest.

Experimental groups

Rabbit pups from independent experimental sessions conducted between November 2019 and April 2021 were allocated to one of the six experimental groups (Fig. 1). Premature birth was considered as the first BPD hit, and hyperoxia and LPS-mediated inflammation were added in selected experimental groups as second and third BPD hits.

1) Age-matched term pups were naturally delivered and kept under Normoxia for 11 days (**Term** group, $n = 24$).
2) Preterm pups kept under Normoxia for 14 days (**Nox** group, $n = 90$).
3) Preterm pups kept under Hyperoxia (Hox) 50% O_2 for 14 days (**Hox-50%** group, $n = 65$).
4) Preterm pups kept under Hox 50% O_2 for 14 days that received an intratracheal injection of LPS (Escherichia coli 0111: B4, 5 mg/kg) dissolved in 100 mg/kg of Poractant alfa (Curosurf, Chiesi Farmaceutici, Italy) one hour after birth, using the intratracheal surfactant administration protocol previously described [33] (**Hox-50% + x1LPS** group, $n = 44$).
5) Preterm pups kept under Hox 50% O_2 for 14 days that received two intratracheal injections of LPS dissolved in Poractant alfa (100 mg/kg): the first dose delivered one hour after birth and the second dose at PND5 (**Hox-50% + x2LPS** group, $n = 26$).
6) Preterm pups kept under Hyperoxia (Hox, 70% O_2) for 14 days (**Hox-70%** group, $n = 40$).
7) An attempt was made to wean a few preterm pups from 95% O_2 (Hox-95% + weaning group, $n = 7$), applying a gradual O_2 lowering protocol from 95% at PND6 to 75%, 55%, 35%, and 21% at PND7, PND8, PND9 and PND10-14, respectively. However, mortality at day 14 was high in this group and was not included as part of the general study.

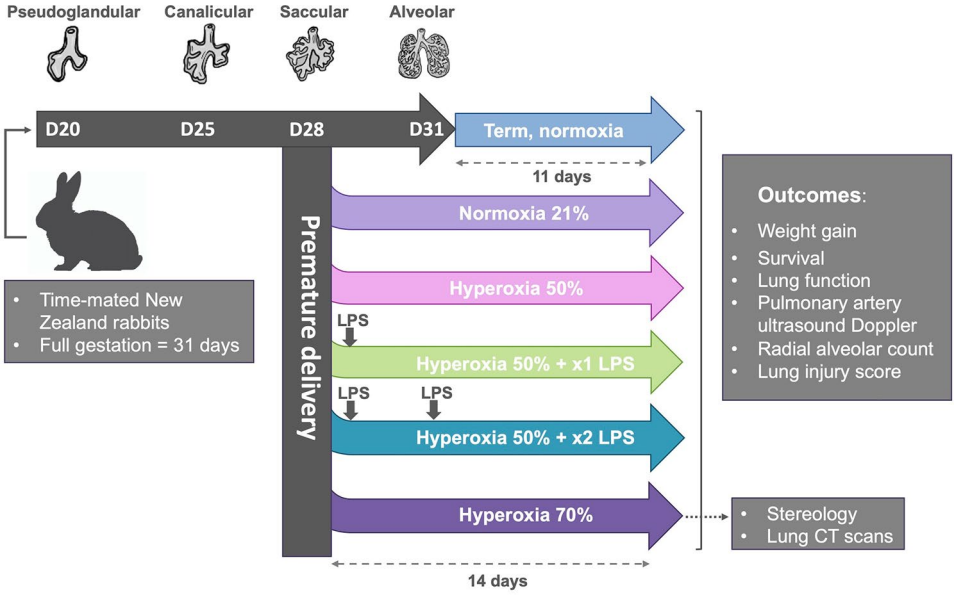

Fig. 1 Scheme of the experimental design. Premature rabbit pups were delivered at 28 days (D28) of gestation, in the saccular phase of lung development, and managed postnatally under Normoxia or different degrees of Hyperoxia (50% or 70% O_2). Two groups of preterm rabbits managed under hyperoxia 50% O_2 also received intratracheal lipopolysaccharide (LPS), either a single exposure immediately after birth or two exposures at birth and at postnatal day five. Age-matched term pups delivered vaginally at day 31 (D31) were included as controls. Outcomes were assessed at postnatal day 14

Lung function measurements

At PND14 (PND11 for age-matched term pups), invasive lung function testing was performed using the forced oscillation technique with the flexiVent™ apparatus equipped with module 2 or 3 (SCIREQ, Canada), depending on the weight of pups (module 2 for pups < 80gr and module 3 for pups > 80gr). Preterm rabbits were anesthetized with ketamine (35 mg/kg) and xylazine (5 mg/kg) i.m. initially. Then, a tracheostomy was performed with a 14-gauge metal needle, and pups were connected to the volume-controlled mechanical ventilation of the flexiVent™ (tidal volume [V_T] 10 mL/kg, breath rate [BR] 120 breaths/min, positive end-expiratory pressure [PEEP] of 3 cmH_2O). Inspiratory capacity, lung tissue damping (tissue resistance, G), lung tissue elastance (H), and static compliance (C_{st}) were determined. G and H were measured using the Primewave-8 forced oscillation, whereas C_{st} was determined using the pressure-volume curve. All measurements were performed until three consistent measurements were obtained, with a coefficient of determination > 0.95 as the limit to accept the measurement. The average of three measurements was calculated and used in the analyses.

Pulmonary artery micro-ultrasound doppler

At PND14 (PND11 for age-matched term pups), pups were anesthetized with isoflurane and placed supine on the Vevo Imaging station (Visualsonics, Canada). The pulse-wave Doppler sample was positioned as previously described [34]. Two-dimensional images of the pulmonary artery were obtained from a parasternal short-axis view at the level of the aortic valve using the MX-550D transducer (40 MHz, Broadband Frequency 22 MHz – 55 MHz, Vevo 3100). The pulmonary artery acceleration time (PAT)/ ejection time (PET) ratio was obtained from offline measurements (Vevo Lab software package V3.0). The average of three cardiac cycles was used for the analyses.

Histological analyses

Lungs of a subset of pups at PND14 (PND11 for age-matched term pups) were used for histological analysis. The numbers of animals per group were the following: Term age-matched, $n = 11$; Preterm Nox, $n = 29$; Preterm Hox-50%, $n = 29$; Preterm Hox-50% + 1x LPS, $n = 25$; Preterm Hox-50% + 2x LPS, $n = 24$; Preterm Hox-70%, $n = 34$. The lungs were removed *en bloc*, and a 14-gauge catheter was secured inside the trachea. The lungs were fixed with 10% buffered formalin (Sigma-Aldrich, Germany) for at least 4 h under constant pressure (25 cmH_2O). After that, the lungs were left in formalin for at least 24 h and then dehydrated in graded alcohol solutions, xylene clarified, and paraffin-embedded. Dorsal serial sections 5 µm thick were obtained at approximately the same height for each animal using a rotary microtome and stained with Hematoxylin and Eosin (H&E), according to the manufacturer's specifications (Histo-Line Laboratories). Histological slides were acquired as whole slide images by digital slide scanner (Nanozoomer S-60, Hamamatsu, Japan). The radial alveolar count (RAC) was performed by drawing a perpendicular line from the lumen of the terminal bronchiole to the nearest connective tissue septum or pleural margin, and the number of saccules or alveoli crossed by this line was counted [35, 36]. Ten counts were performed per lobe (20 measurements in total). The acute lung injury score (ALI) was performed according to the method proposed by the American Thoracic Society [37]. The method is based on five histological findings (i.e., neutrophils in the alveolar space, neutrophils in the interstitial space, hyaline membranes, proteinaceous debris in the airspace, and alveolar septal thickening). At least 20 random high-power fields (400× total magnification), ten per lobe, were independently scored in a blinded fashion for each condition. The 20 fields, ten per lobe, were sampled by generating successive random displacements from the current position, in both the x and y directions, taking care that the minimum distance between each field was at least one high power field in length and to obtain a uniform sampling of the tissue. Furthermore, fields with at least 50% of the area occupied by lung alveoli were accepted for analysis, while fields that consisted predominately of the lumen of large airways or vessels were rejected. To generate the ALI score, the sum of each of the five independent variables was weighted and then normalized to the number of fields evaluated [37]. The resulting ALI score was a continuous value between zero and one. The average between the right lung and left lung was calculated for each parameter and used in the analysis. Both RAC measurement and ALI scores were performed by a single researcher expert in the field. To minimize intra-observer variability, the observer intermittently used reference control cases.

Design-based lung stereology

A subset of pups from the Nox, Hox-70% O_2, and Term groups were assessed using design-based lung stereology. Lungs were inflation fixed at a pressure of 25 cmH_2O with a fixative containing 1.5% glutaraldehyde and 1.5% paraformaldehyde in 0.15 M HEPES buffer. Prior to embedding in Technovit resin, lung volume measurements were assessed by volume displacement (based on Archimedes' principle) [38], and lung tissue was sampled using systematic uniform random sampling [39]. This was done by cutting the lung tissue into 2 mm thick slices, and every other slice, with a random start, was embedded in Technovit resin as described previously [40]. From the term born rabbits, only every third slice was sampled, as the lungs were larger. Resin-embedded lung tissue was

cut into 1.5 µm thick sections and stained with toluidine blue. Histological slides with tissue sections were then scanned and digitalized with a microscopic slide scanner (AxioScan.Z1. Zeiss, Germany) at a 20x magnification.

Stereological analysis was done with the NewCast™ software (Visiopharm®, Denmark) on digitalized slides. To estimate parenchymal, non-parenchymal, and ductal airspace volumes, automated random subsampling was performed at a 5x magnification and with a sampling fraction of 5–12%. A point grid consisting of 36 points with a subsampling fraction of 4 was used to estimate the respective volume densities, as described previously [41]. Respective total volumes were assessed by multiplying the densities with the lung volume.

To estimate alveolar airspace, septal volume, septal thickness, and septal surface area, an automated random subsampling was performed at a 20x magnification and with a sampling fraction of 0.5-1%. A test line grid with 12 lines and a length per point of 9.37 µm was superimposed over the images, and points and intersections were counted. Respective volume and surface densities were estimated as described previously [41], and total volume and surface areas were obtained by multiplying the densities with the parenchymal lung volume.

Micro-CT lung imaging

Longitudinal micro-CT lung imaging was performed in a subset of pups from the Pre-Nox, Pre-Hox 70% O_2, and age-matched term groups ($n = 6$ rabbit pups in each group). The same pups underwent micro-CT imaging at PND7 and PND13. Before imaging, rabbit pups received dexmedetomidine (Dexdormitor, Zoetis, Inc.) intraperitoneally (0.25 mg/kg) and were supplied with oxygen until 100% saturation was reached to reduce the risk of hypoxemia during image acquisition. Pups were then positioned supine inside the CT scan and anesthetized with 1% isoflurane in oxygen using a facemask. The homemade facemask was designed to fit snugly on the muzzle of the rabbit pup without obstructing its mouth or nose and minimizing the dead space to avoid carbon dioxide rebreathing [42].

Lung imaging was performed with Quantum GX Micro-CT (PerkinElmer). Images were acquired in free-breathing rabbit pups with the following parameters: X-ray tube voltage 90 KV, X-ray tube current 88 µA over a total angle of 360° for a total scan time of 4 min. A respiratory gating technique, consisting of a region of interest (ROI) positioned around the diaphragm, monitored the breathing pattern during the acquisition. The retrospectively gated acquisition was in the 'high speed' mode and generated two three-dimensional datasets with 90-µm isotropic reconstructed voxel size corresponding to the two main phases of the breathing cycle (end-inspiration and end-expiration) [43]. The same field of view and resolution were maintained between different time points. For each animal, both respiratory phases were post-processed. Briefly, each image was converted from a grayscale to a Hounsfield Unit (HU), setting −1,000 HU as the density of air and 0 HU as the density of water. The lung was semi-automatically segmented to assess the volume (mm^3) and the Mean Lung Attenuation (MLA, HU) in order to quantify the volume of "air" and "tissue" components (Functional Residual Capacity [FRC], which represents the volume of air at the end of expiration). $V_{T,}$ Minute Ventilation (MV), and BR were determined as previously described [42]. The CT scanner is calibrated monthly with standard phantoms for noise, uniformity, low contrast, and resolution [44].

Following micro-CT imaging on PND13, rabbit pups underwent flexiVent™ measurement on PND14, and the two functional outcomes were finally correlated.

Statistical analyses

The number of pups analyzed with each technique is displayed in Table 1. All data are presented as mean ± SD unless otherwise stated. Log-rank test was used to compare the survival between groups. A one-way ANOVA, followed by Tukey's multiple comparisons test, was used for the other comparisons. A $P < 0.05$ was considered statistically significant. A Spearman matrix was generated to evaluate the relationship between flexiVent™ data and micro-CT functional parameters (Spearman's

Table 1 Number of preterm pups analyzed with each technique

Group	Starting sample size (*n*)	Lung Function (*n*)	Micro-ultrasound Doppler (*n*)	Histology, RAC and ALI (*n*)	Lung Stereology (*n*)	Micro-CT (*n*)
Term	24	24	18	11	7	6
Nox	90	54	55	29	8	6
Hox-50%	65	46	44	29	-	-
Hox-50% + x1LPS	44	25	25	25	-	-
Hox-50% + x2LPS	26	14	14	14	-	-
Hox-70%	40	32	32	32	9	6
Hox-95% + weaning	7	-	-	-	-	-

ALI, Acute Lung Injury; CT, Computed Tomography; LPS, Lipopolysaccharide; N, Sample Size; Nox, Normoxia; RAC, Radial Alveolar Count

R corresponding to each correlation is reported in each square of the matrix). Statistical analyses were performed using GraphPad software, version 8.1.

Results

Survival and weight gain

70% O_2 was well tolerated and achieved a significantly higher survival than 50% O_2 and Normoxia conditions (Fig. 2A). Exposure to 95% O_2 for six days produced a strong hyperoxic hit, which precluded oxygen weaning and was associated with a > 80% mortality at PND14. Except for the hyperoxia 95%-weaning group, mortality mainly occurred in the first six days of life. Survival in the age-matched Term group was 100% (not shown in the graph).

Rabbit pups in the Hox-70% group showed a significantly higher weight at PND 14 than animals in the Hox-50% O_2 groups (Fig. 2B). Animals treated with LPS showed the lowest weight gain at PND14, particularly

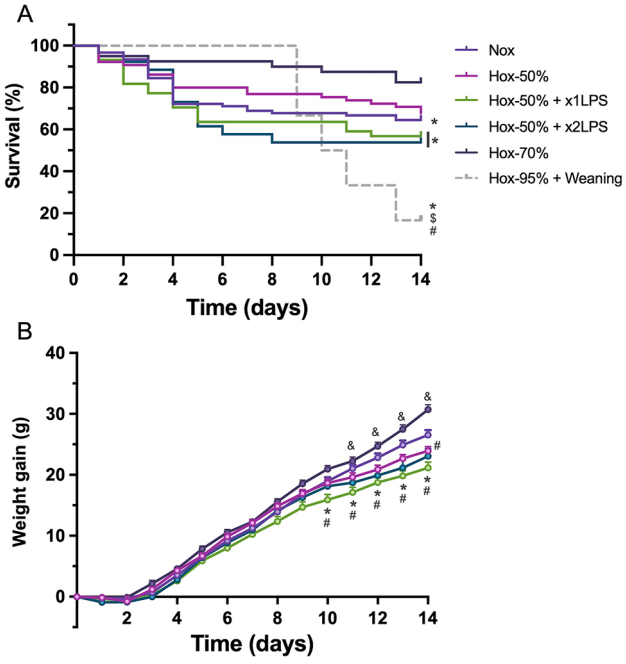

Fig. 2 (**A**) 14-day survival in preterm rabbits pups managed with Normoxia (Nox, $n = 90$), Hyperoxia 50% O_2 (Hox-50%, $n = 65$), Hox-50% and a single lipopolysaccharide (LPS) intratracheal injection immediately after birth (Hox-50% + 1x LPS, $n = 44$), Hox-50% combined with two LPS intratracheal injections, one immediately after birth and another one at day 5 (Hox-50% + 2x LPS, $n = 26$), Hox-70% ($n = 40$), or Hox-95% for six days followed by a weaning protocol consisting of applying a gradual O_2 lowering protocol from 95% at day six to 75%, 55%, 35%, and 21% at days 7, 8, 9 and 10–14, respectively (Hox-95% + weaning, $n = 7$)). (**B**) Weight gain from birth through day 14 (mean ± SEM). Log-rank test for survival and one-way ANOVA for daily comparisons in weight gain. $^{*}P < 0.05$ vs. Hox-70%; $^{\#}P < 0.05$ vs. Nox; $^{\$}P < 0.05$ between Hox-50% and Hox-95%+ weaning; and $^{\&}P < 0.05$ between Hox-70% and Hox-50% and Hox-50% + 2xLPS. Log-rank test was used to compare the survival between groups. One-way ANOVA was used to compare groups at single time points

those receiving a single LPS administration. Daily weight gain could not be determined for term pups because foster mothers reject the pups that leave the fostering chamber in the first days of life.

Lung function measurements

Preterm pups showed significantly lower inspiratory capacity and C_{st} values at PND14 than term pups. Among preterm groups, those exposed to Hyperoxia had lower inspiratory capacity values than preterm pups kept under Normoxia conditions (Fig. 3A). LPS and 70% O_2 exposure were associated with significantly lower C_{st} compared with the Nox group (Fig. 3B). Tissue damping (G), a measure of air resistance in the small airways and alveoli, was significantly increased in the rabbit pups exposed to LPS (Fig. 3C). Lung elastance was significantly higher in all preterm groups compared to age-matched term pups (Fig. 3D).

Pulmonary artery micro-ultrasound doppler

The PAT/PET ratio at PND14 was significantly reduced in all preterm groups compared to term animals, with no differences observed between normoxia, hyperoxia, or LPS-exposed preterm rabbit pups (Fig. 4).

Histological analyses

The RAC was significantly lower in preterm pups compared with age-matched term-born animals (Fig. 5A). Among the preterm groups, the RAC was significantly lower in the Hox-50% O_2 + x2LPS and Hox-70% groups than in the Nox group.

Compared to term animals, the ALI score was significantly higher in preterm pups exposed to Hyperoxia but not in those kept under Normoxia conditions (Fig. 5B). LPS exposure on top of Hox-50% O_2 significantly increased the ALI compared to the Nox group, as evidenced by a marked neutrophilic infiltration (Fig. 5C). The Hox-50% + x1LPS group showed a significantly higher ALI than the Hox-70% group.

Stereological analyses

Preterm pups showed a significantly lower lung volume than term pups, mainly driven by differences in the parenchymal volume (Fig. 6A-C). Similarly, the parenchymal airspace volume was significantly lower in preterm pups, with no difference in ductal volume and a significantly higher alveolar airspace volume in term animals (Fig. 6D-F). The total septal surface was significantly higher in term animals and there were no differences between the Nox and Hox-70% groups (Fig. 6G).

Micro-CT lung imaging

Representative longitudinal axial micro-CT lung images obtained at the end-inspiratory and end-expiratory

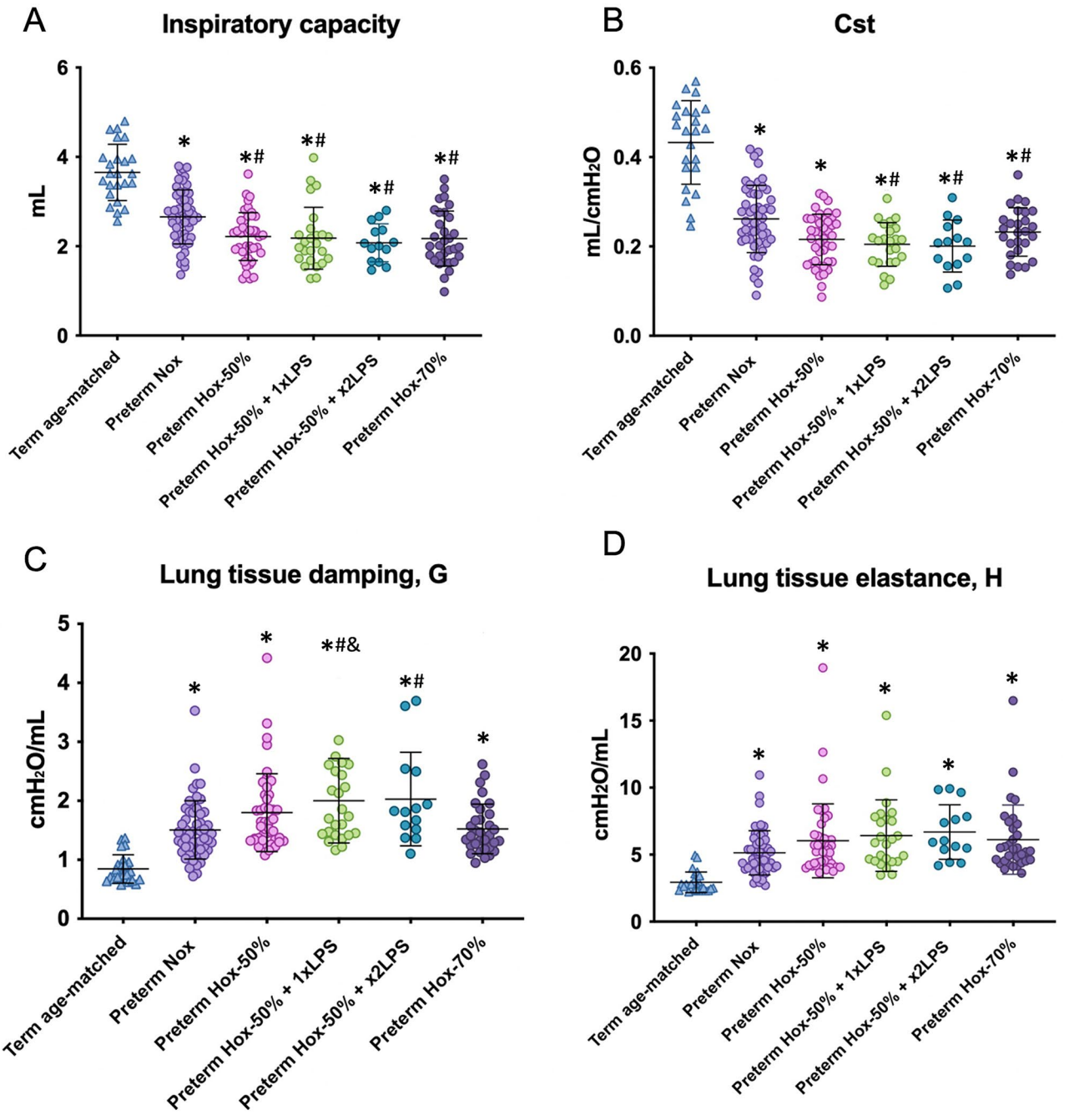

Fig. 3 (**A**) Inspiratory capacity, (**B**) static compliance (C_{st}), (**C**) lung tissue damping (G), and (**D**) lung tissue elastance (H) measured at postnatal day 14 in age-matched term rabbit pups (Term, age-matched, $n = 24$) and in preterm rabbits managed with Normoxia (Preterm Nox, $n = 54$), Hyperoxia 50% O_2 (Preterm Hox-50%, $n = 46$), Preterm Hox-50% combined with a single lipopolysaccharide (LPS) intratracheal injection immediately after birth (Preterm Hox-50% + 1x LPS, $n = 25$), Preterm Hox-50% combined with two LPS intratracheal injections, one immediately after birth and another one at day 5 (Preterm Hox-50% + 2x LPS, $n = 14$), and hyperoxia 70% O_2 (Hox-70%, $n = 32$). $^{*}P < 0.05$ vs. Term age-matched; $^{\#}P < 0.05$ vs. Preterm Nox; $^{\&}P < 0.05$ vs. Hox-70% One-way ANOVA

phases show several differences between the Term and preterm groups at both PND7 and PND13 (Fig. 7A). There were no significant differences between groups in total lung volume at PND7. However, both the volume of aerated lung areas and lung tissue components significantly increased in Term pups from PND7 to PND13, whereas no evident changes occurred in preterm groups (Fig. 7B).

Term animals exhibited a significantly higher FRC at PND13, whereas it was only slightly improved in Pre-Nox pups and declined in the Pre-Hox group (Fig. 7C).

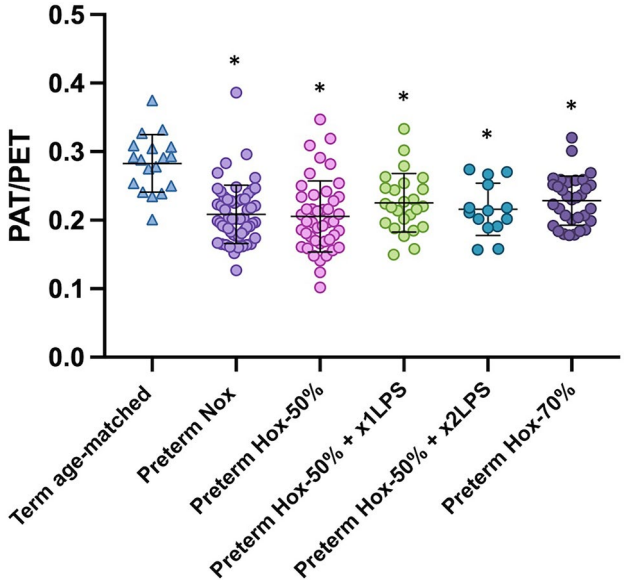

Fig. 4 Pulmonary artery acceleration time (PAT) / pulmonary ejection time (PET) ratio was determined at postnatal day 14 in age-matched term rabbit pups (Term age-matched, $n = 18$) and in preterm rabbits managed with Normoxia (Preterm Nox, $n = 55$), Hyperoxia 50% O_2 (Preterm Hox-50%, $n = 44$), Preterm Hox-50% combined with a single lipopolysaccharide (LPS) intratracheal injection immediately after birth (Preterm Hox-50% + 1x LPS, $n = 25$), Preterm Hox-50% combined with two LPS intratracheal injections, one immediately after birth and another one at day 5 (Preterm Hox-50% + 2x LPS, $n = 14$), and hyperoxia 70% O_2 (Hox-70%, $n = 32$). *$P < 0.05$ vs. Term age-matched. One-way ANOVA

CT-derived tidal volume (V_T) was significantly higher in term rabbit pups compared to preterm groups at PND7. On PND13, V_T in the Pre-Nox groups was significantly higher than in pre-Hox 70% group (Fig. 7D). The BR rate increased from PND7 to PND13 in the Pre-Hox 70% and Term groups, while it remained stable in the Pre-Nox over time and significantly lower compared to Term animals at PND13 (Fig. 7E).

The Minute Ventilation (MV) was significantly higher in Term pups than in preterm groups at both time points (Fig. 7F). However, if MV was normalized by body weight, a significant difference was only detected between Term and pre-Hox 70% groups (Fig. 7G).

Figure 7H shows the Spearman matrix correlation between flexiVentTM and micro-CT-derived parameters from the same pups. IC and C_{st} positively correlated with FRC, V_T, and MV ($R > 0.7$), whereas tissue damping (G) and tissue elastance (H) displayed a negative correlation with FRC, V_T, and MV, thus suggesting that lung tissue resistance may impair air exchange during normal breathing.

Discussion

We investigated the feasibility of extending the preterm rabbit model of BPD from PND7 to PND14, using single, double, and triple hit strategies. We showed that delivering preterm rabbit pups at the saccular phase represents a clear disadvantage by itself, as evidenced by less alveolar development and the inability of preterm pups to catch up regarding lung function with age-matched term animals at PND14 (i.e., first BPD hit). We also combined prematurity with other BPD hits like postnatal hyperoxia and intratracheal LPS administration. Hyperoxia at 70% O_2 improves survival and enhances the BPD-like phenotype. On the other hand, intratracheal LPS administration was feasible in preterm rabbits and enhanced the lung inflammatory phenotype.

The preterm rabbit model has been proposed in recent years to test pharmacological interventions in the context of BPD [15]. Unlike rodent models, preterm rabbits combine structural and functional prematurity and are more cost-effective than large premature models because preterm rabbits do not need the infrastructure of a complete neonatal intensive care unit [15]. We have recently shown that preterm birth by itself, without any other insult, impairs the postnatal development of the rabbit lung, resulting in lung function deficits and diminished alveolarization compared to age-matched term pups at PND7 [24, 45, 46]. Moreover, longitudinal transcriptomic data comparing the gene expression of preterm and age-matched term rabbits' lungs has shown a consistent increase in the number of dysregulated genes from PND1 to PND14 [46, 47], suggesting that the effect of premature birth on the lung molecular pathways persist beyond the first week of life. Our results confirm this observation, showing that premature delivery at the saccular phase is an important hit itself associated with lung function deficits, lower alveolarization, and cardiovascular abnormalities.

Hyperoxia has been the most widely used postnatal hit to mimic BPD in rodent models. Hyperoxia disrupts the homeostatic balance of cellular processes due to exacerbated reactive oxygen species and activates inflammation cascades and cytokines, triggering lung injury/remodeling processes that resemble a BPD-like phenotype [48, 49]. Hyperoxia (> 95% O_2) has also been used in the 28-day gestation preterm rabbit model for drug testing purposes [25, 27, 28]. Although this model is useful to mimic the initial inflammatory phase of BPD, it has some limitations. For instance, the strong hyperoxic hit might limit the response to therapeutic interventions, which, at most, can just attenuate the effects of hyperoxia [25, 27, 28]. In addition, exposure to 95% O_2 for seven days produces acute and apparently irreversible toxicity in preterm rabbits, precluding a longer-term follow-up. Mortality after 95% O_2 exposure has been reported to be higher than 85% at PND11 [32]. Here, we aimed to extend the preterm rabbit model to PND14 to mimic the evolving phase of BPD and investigate therapeutic interventions at later and more relevant time points. PND14

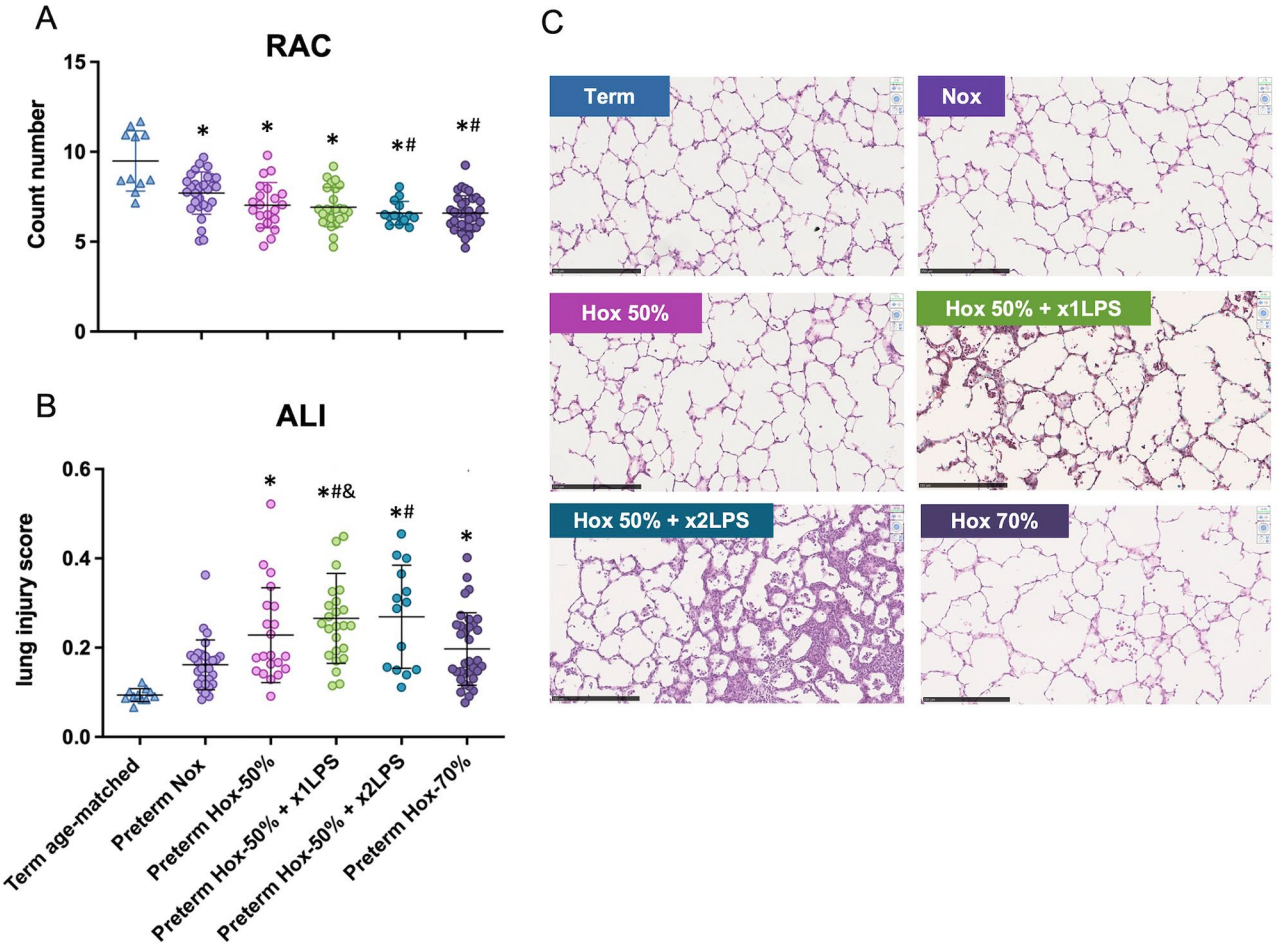

Fig. 5 (**A**) Radial Alveolar Count (RAC) and (**B**) Acute Lung Injury score (ALI) from lung sections obtained at postnatal day 14 from age-matched term rabbit pups (Term age-matched, $n=11$) and from preterm rabbits managed with Normoxia (Preterm Nox, $n=29$), Hyperoxia 50% O_2 (Preterm Hox-50%, $n=29$), Preterm Hox-50% combined with a single lipopolysaccharide (LPS) intratracheal injection immediately after birth (Preterm Hox-50% + 1x LPS, $n=25$), Preterm Hox-50% combined with two LPS intratracheal injections, one immediately after birth and another one at day 5 (Preterm Hox-50% + 2x LPS, $n=14$), and hyperoxia 70% O_2 (Preterm Hox-70%, $n=32$). (**C**) Representative micrographs of lung slides stained with HE (scale bar = 250 µm). $^{*}P<0.05$ vs. Term age-matched; $^{\#}P<0.05$ vs. Preterm Nox; $^{\&}P<0.05$ vs. Hox-70%. One-way ANOVA

in preterm rabbits has been proposed to be the equivalent age at which BPD diagnosis is made in premature babies (i.e., 36 weeks post-menstrual age) [50]. Taking the seven-day hyperoxia model as a starting point, we implemented an oxygen-weaning protocol that, unfortunately, yielded a low survival at PND14 (< 20%) in pilot experiments, confirming the toxic effect and irreversible damage of 95% O_2 exposure in the first week of life.

Exposure to lower oxygen concentrations, at 50% and 70% O_2, achieved significantly higher survival at PND14 compared with the oxygen-weaning strategy. Notably, however, 50% O_2 did not show significant differences in any of the cardiopulmonary parameters compared with the preterm pups of the Nox group. In contrast, the Hox-70% group showed an enhanced BPD-like phenotype denoted by less alveolarization, lower lung function, and a higher lung injury score than the Nox group. Interestingly, 70% hyperoxia improved the survival of preterm pups. Thus, 70% hyperoxia seems to fulfill two apparently conflicting roles: on the one hand, it supports the transition to extrauterine life of rabbit pups, which display mild-to-moderate respiratory distress at delivery [26], and on the other hand, it enhances the BPD-like phenotype at PND14.

Subsets of animals in the Nox, Hox-70%, and Term groups were assessed using more sophisticated techniques such as design-based stereology and longitudinal micro-CT imaging after the observation from previous experimental sessions that Nox and Hox-70% showed a good compromise between PND14 survival and the generated BPD phenotype. Term pups were included as controls. Design-based stereology is the gold standard of lung morphometry, providing minimally biased quantitative data about the volume, surface area, length, and number of certain lung structures [40]. Stereology outcomes were suggestive of arrested development

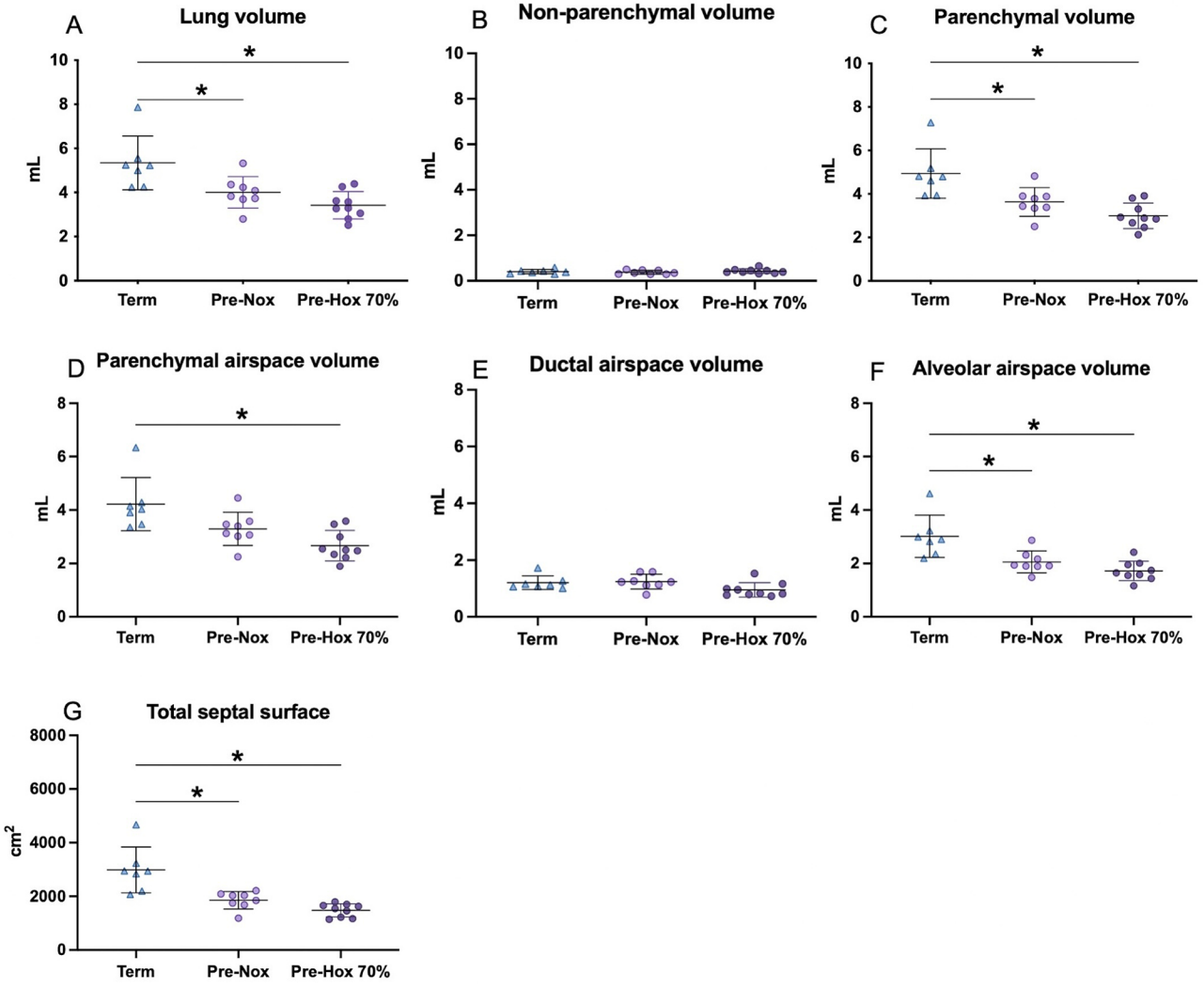

Fig. 6 Design-based stereology parameters from lungs obtained at postnatal day 14 from term rabbit pups (Term, $n=7$) and from preterm rabbits managed with Normoxia (Pre-Nox, $n=8$) or Hyperoxia 70% O_2 (Pre-Hox 70%, $n=9$). *$P<0.05$ vs. Term. One-way ANOVA

in normoxic preterm rabbits compared to age-matched term animals, which was further enhanced by hyperoxia. Preterm pups showed significantly lower lung volumes at PND14, driven by a lower volume of the parenchymal compartment without differences in the non-parenchymal volume. Similarly, the significantly lower parenchymal air volume found in preterm pups was driven by lower alveolar airspace volume without differences in the ductal volume. These findings are indicative of reduced alveolarization and are in line with the RAC.

Micro-CT imaging is a non-invasive technology that enables precise tridimensional lung measurements in the same pup at different time points. We acquired micro-CT scans at PND7 and PND13 using an anesthetic protocol established for spontaneously breathing term rabbits [42], confirming its safety profile and effectiveness also for preterm rabbit pups at the two indicated time points. Micro-CT-derived parameters confirmed a significantly lower total lung volume in preterm groups at PND13 compared to term rabbit pups as detected by stereological analyses. Nevertheless, no differences were observed among groups at PND7 in air/tissue components, suggesting that lung structural and morphological changes occurred during the second week of life, which is in agreement with the RAC quantification in the histological samples. However, functional CT-derived biomarkers, such as V_T and MV, were able to detect substantial differences across the three groups as early as PND7. Preterm animals, particularly those exposed to 70% oxygen, had lower V_T and MV than the Term group, demonstrating a reduction in pulmonary capacity and validating the role of supplemental oxygen in promoting the BPD-like phenotype. Micro-CT data were also corroborated by invasive respiratory function measurements. In fact, the reduction of FRC, V_T, and MV in preterm groups positively correlated with the diminishing of IC and static

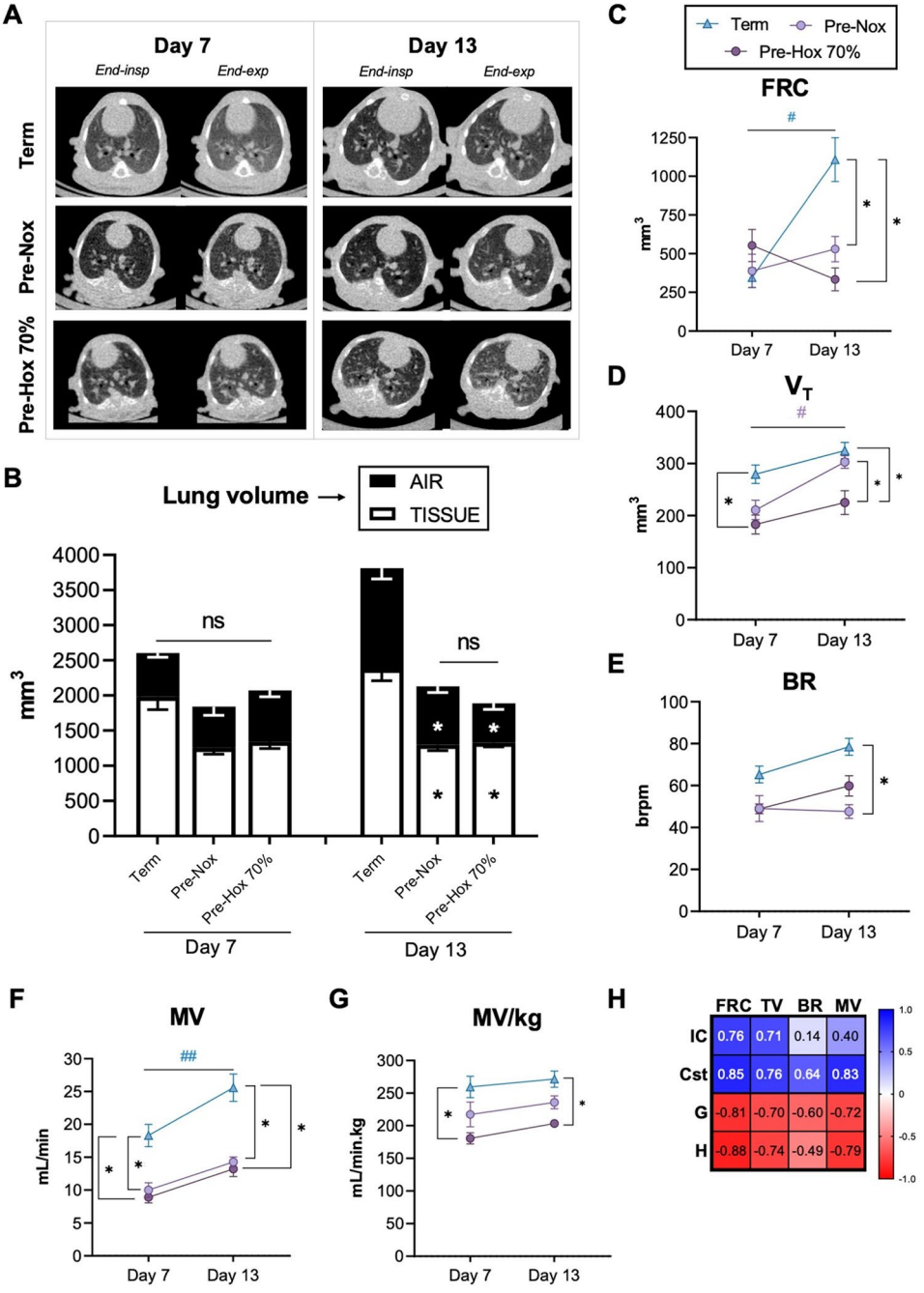

Fig. 7 (**A**) Representative micro-CT images and (**B**) lung volumes obtained at PND7 and PND13 from term rabbit pups (Term, $n=6$) and from preterm rabbits managed with Normoxia (Pre-Nox, $n=6$) or Hyperoxia 70% O_2 (Pre-Hox 70%, $n=6$). (**C**) Functional residual capacity (FRC), (**D**) tidal volume (V_T), (**E**) breath rate (BR), (**F**) minute ventilation (MV), and (**G**) MV normalized by body weight. (**H**) Shows the correlation matrix between lung function measurements obtained from flexiVent™ (inspiratory capacity, IC; static compliance, C_{st}; lung tissue damping, G; and lung tissue elastance, H) and micro-CT parameters; the values within the matrix correspond with the Spearman´s R for each comparison. *$P<0.05$ vs. Term; #$P<0.05$ between PND 7 and PND13 within the same group; one-way ANOVA. Mean ± SEM are shown

compliance and negatively with tissue damping and tissue elastance, indicating increased stiffness of the lung and impaired ventilation in normal breathing.

Besides the oxygen supplementation, the intratracheal delivery of LPS was also used as a hit to exacerbate the lung inflammatory response. The intratracheal instillation of LPS produces a rapid inflammatory response characterized by the alveolar infiltration of polymorphonuclear neutrophils and the release of mediators that play a fundamental role in inflammation and lung damage [51].

Intratracheal LPS administration has been used to induce pulmonary inflammation in intubated preterm lambs to mimic the inflammatory phase of BPD [52, 53]. Intratracheal LPS cannot be used in newborn rodents because of their small size at delivery. Instead, intraamniotic LPS has been widely used in rodents to induce experimental chorioamnionitis, which leads to alveolar simplification and arrested vascular development at PND14 [12]. Unlike in newborn rodents, the intratracheal delivery route is feasible in preterm rabbits [33] and was used to deliver LPS directly to the lungs. LPS was delivered in combination with exogenous surfactant to enhance its lung distribution. Intratracheal LPS injections were feasible without evidence of acute mortality associated with the intratracheal injection. The groups receiving intratracheal LPS achieved the highest lung injury scores. Interestingly, a single LPS administration immediately after birth was associated with a significantly higher injury score at PND14 compared to Nox and Hox-70% groups and increased tissue damping (G), a measure for the air resistance in the small airways and alveoli.

This study shows the feasibility of several strategies to extend the 28-day preterm rabbit model of BPD to PND14. However, the study has limitations. This study was not randomized; it combines the results from independent experimental sessions conducted sequentially over two years. Thus, the groups included in several experimental sessions (e.g., Nox and Hox-50% groups) have a higher sample size. We acknowledge that we could only investigate a limited number of hit combinations. Although preterm birth is the common denominator of all the proposed models, alternative hyperoxia levels (e.g., 60% or 80% O_2) and alternative hyperoxia-LPS combinations (e.g., hyperoxia 70% plus LPS) may eventually be explored in future studies. Additionally, antenatal strategies to induce experimental chorioamnionitis, placental insufficiency, or intrauterine growth restriction could also be explored in preterm rabbits. Lastly, age-matched term pups were naturally delivered and mother-reared, whereas premature pups were delivered via C-section and fed milk formula, as would be expected in a clinical setting. We acknowledge that these differences may partially influence the overall outcome; however, it has been shown that preterm disadvantage persists even when term rabbit pups are delivered via C-section and fed formula [24].

Conclusions

This work shows the feasibility of extending the preterm rabbit model of BPD to PND14. Preterm delivery at the saccular phase itself, even in the absence of other postnatal BPD hits, was associated with lung function deficits, delayed lung development, and cardiovascular abnormalities compared with age-matched term animals. Continuous exposure to moderate hyperoxia (70% O_2) and the intratracheal administrations of LPS can be used as additional hits to enhance the BPD-like phenotype of premature rabbits at PND14.

Abbreviations

ALI	Acute Lung Injury
BPD	Bronchopulmonary Dysplasia
BR	Breath Rate
C_{st}	Static Compliance
CT	Computed Tomography
FRC	Functional Residual Capacity
G	Lung Tissue Resistance
H	Lung Tissue Elastance
Hox	Hyperoxia
i.m.	Intramuscular
LPS	Lipopolysaccharide
MLA	Mean Lung Attenuation
MV	Minute Ventilation
Nox	Normoxia
PAT	Pulmonary artery acceleration time
PEEP	Positive-end Expiratory Pressure
PET	Pulmonary artery ejection time
PND	Postnatal Day
RAC	Radial Alveolar Count
ROI	Region of Interest
V_T	Tidal Volume

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Not applicable.

Author contributions

CC, GV, FFS, FR, CB, MZi, and FR conceived and planned the experiments. FS, ES, MS, AM, GA, EF, AG, LR, RC, MZo, and HS carried out the experiments. CC, GV, EF, FFS, FR, LR, CB, XM, MZi, and FR contributed to the interpretation of the results. CC, XM, and FR took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript. All authors have revised and approved the final version of the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethical approval

All experimental procedures involving animals were approved by the local animal ethics committee and met the standard European regulations on animal research (n°783/2019-PR).

Consent for publication

Not applicable.

Competing interests

CC, ES, MS, GA, GV, AG, FFS and FR are employees of Chiesi Farmaceutici S.p.A. XM served as a consultant for this study. The other authors have no conflicts of interest to declare.

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