

RESEARCH ARTICLE

β_3 -adrenoceptor agonism exerts lung protection in a rat model of bronchopulmonary dysplasia

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Abstract

Background and Purpose: Bronchopulmonary dysplasia (BPD) affects premature newborns, particularly those receiving supplemental oxygen therapy. Exposure of underdeveloped lungs to oxygen levels higher than the intrauterine environment causes oxidative stress, impairing alveolarisation, vascularisation and causing fibrosis. The β_3 -adrenoceptor could represent a promising target for BPD treatment because of its oxygen-dependent regulation, ability to reduce oxidative stress and protection against intestinal hyperoxia-induced damage.

Experimental Approach: In this study, neonatal rat pups were reared under hyperoxia for 2 weeks to induce a BPD-like disease and treated with the β_3 -adrenoceptor agonist BRL37344 (1, 3 or 6 mg kg⁻¹).

Key Results: Through oxidative stress, hyperoxia decreases pulmonary volume, impairs alveolarisation and vascularisation, and causes fibrosis and type II pneumocyte hyperplasia, thereby reducing survival rates. BRL37344 at 3 mg kg⁻¹ counteracted these alterations, doubling survival rates.

Conclusions and Implications: Our findings demonstrate a new role for β_3 -adrenoceptors in protecting the lungs from hyperoxic injury, suggesting its pharmacological activation as a strategy for treating Bronchopulmonary dysplasia of prematurity and, possibly, other lung fibrosis-related disorders.

KEYWORDS

β_3 -adrenoceptor, bronchopulmonary dysplasia, lung, oxidative stress, PDGRF, prematurity

Abbreviations: BPD, bronchopulmonary dysplasia; CEPCs, circulant endothelial progenitor cells.

The authors confirm that the PI for this paper is Professor Brenda Gibson and that she had direct clinical responsibility for patients.

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1 | INTRODUCTION

Bronchopulmonary dysplasia (BPD) is a chronic lung disease primarily affecting premature newborns, especially those who have undergone mechanical ventilation and supplemental oxygen therapy as a result of respiratory distress syndrome (Sung et al., 2015). Although these interventions are crucial for supporting respiratory function in preterm infants, they can contribute to altering the normal development of lung architecture. The premature exposure of underdeveloped lungs to ambient oxygen levels, compounded by supplemental oxygen therapy and positive pressure ventilation, results in oxidative stress and inflammation, which in turn, cause incomplete alveolarisation, reduced vascularisation and, in the long term, excess collagen deposition. These alterations impair pulmonary mechanics and gas exchange, further compromising lung function (Thébaud et al., 2019). As a consequence, infants diagnosed with BPD are more vulnerable to both short-term complications, such as respiratory infections and pulmonary hypertension, and long-term complications, including neurocognitive defects and developmental delays.

Few treatments are currently available to improve the prognosis for BPD, namely exogenous surfactant, **caffeine** (Thébaud et al., 2019) and corticosteroids (Boscarino et al., 2024), and their efficacy is limited. Moreover, although they may symptomatically improve BPD symptoms in neonates, their use, especially for long-term corticosteroid therapy, is associated with adverse effects on the developing brain and other organs (Jensen & Watterberg, 2023). This highlights the urgent need for the identification of new pharmacological targets and effective, disease-modifying therapies. Among the various candidates, the **β_3 -adrenoceptors** holds promise. This is part of the G-protein-coupled family of **adrenoceptors**, which includes α_1A , α_1B , α_1D and α_2A , α_2B , α_2C and finally, β_1 , β_2 , β_3 -subtypes. Research on adrenoceptors has predominantly focussed on β_1 and β_2 , while β_3 has only recently attracted interest because of its pharmacological properties, peculiar expression profile and oxygen-dependent regulatory mechanisms (Dal Monte et al., 2013). At variance with β_1 and β_2 , this receptor is more resistant to agonist-induced desensitisation (Schena & Caplan, 2019), making it a suitable target for chronic therapies. β_3 -adrenoceptor is highly expressed during intrauterine hypoxic life and its expression drops after birth upon exposure to higher environmental oxygen levels (Filippi et al., 2022), remaining restricted to specific organs (Nardini et al., 2024). Moreover, increasing experimental evidence underscores the importance of β_3 -adrenoceptor in organ development. In particular, β_3 agonism has been shown to protect against neonatal hyperoxia-induced gut damage by preserving epithelial integrity, promoting vascularisation and supporting neuronal differentiation, and to favour prompt closure of the ductus arteriosus at birth (Cammalleri et al., 2023; Filippi et al., 2023; Nardini et al., 2024; Pini et al., 2020). Moreover, β_3 activation has been shown to markedly reduce the levels of reactive oxygen species (ROS), thereby exerting protection against oxidative stress (Bundgaard et al., 2017).

The present experimental study in a rat model of BPD-like disease was designed to explore the potential role of β_3 in lung development and to determine whether its pharmacological activation could reduce

What is already known?

- Bronchopulmonary dysplasia (BPD) a chronic lung disease affecting premature newborns has few treatments available.
- β_3 -adrenoceptors have oxygen-dependent regulation, reduce oxidative stress and provide protection against intestinal hyperoxia-induced damage.

What does this study add?

- BRL37344, a commonly used β_3 -agonist, improved survival in a rat hyperoxia-induced model of BPD.
- BRL37344 preserved alveolarisation and vascularisation against hyperoxia-induced lung damage.

What is the clinical significance?

- β_3 -adrenoceptor activation may provide a novel treatment for BPD and other lung fibrosis-related disorders.

lung oxidative stress and inflammatory response induced by premature oxygen exposure. The expected results could provide the basis for a new pharmacological approach using β_3 agonists to counteract the onset and progression of BPD by supporting normal pulmonary development and reducing oxygen-related damage in premature infants.

2 | METHODS

2.1 | Oxygen exposure and drug administration

All animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). This study was also authorised by the Ethical Committee of Florence University and the Italian Ministry of Health (No. 453/2021). Pregnant Sprague-Dawley rats (CrI:CD [SD]) were obtained from Charles River Laboratories and housed under controlled conditions. The litters were assigned to either an oxygen-enriched atmosphere (95%, hyperoxia) or an ambient oxygen level (21%, normoxia). Hyperoxia was maintained in an animal chamber (BioSpherix, Parish, NY, USA) equipped with a ProOx P110 probe to monitor oxygen levels (BioSpherix). The selective β_3 agonist **BRL37344** was administered subcutaneously (s.c.) to pups at three different doses (1, 3 and 6 mg kg⁻¹) based on previous literature (Milano et al., 2024; Nardini et al., 2025; Niu et al., 2012), every 12 h from birth (P0) to day 14 (P14). The pups were randomly assigned to six groups: (1) normoxia controls: pups reared on ambient

oxygen, untreated; (2) normoxia + BRL37344 3 mg kg⁻¹: pups reared in ambient oxygen, treated with 3 mg kg⁻¹ BRL37344; (3) normoxia + BRL37344 6 mg kg⁻¹: pups reared in ambient oxygen, treated with 6 mg kg⁻¹ BRL37344; (4) hyperoxia controls: pups reared on 95% oxygen, untreated; (5) hyperoxia + BRL37344 1 mg kg⁻¹: pups reared on 95% oxygen, treated with 1 mg kg⁻¹ BRL37344; (6) hyperoxia + BRL37344 3 mg kg⁻¹: pups reared on 95% oxygen, treated with 3 mg kg⁻¹ BRL37344; and (7) hyperoxia + BRL37344 6 mg kg⁻¹: pups reared in 95% oxygen, treated with 6 mg kg⁻¹ BRL37344. Body weight was measured daily. Dam rotation was performed every 24 h to avoid oxygen toxicity. At P8 and P14, the pups were killed by i.p. **pentobarbital** (800 mg kg⁻¹) and the lungs were collected for analysis. For morphologic evaluations, lung samples were fixed by tracheal instillation (Qu et al., 2015). Briefly, after exsanguination the trachea was cannulated, the lungs and heart were removed, washed in 0.1 M phosphate-buffered saline (PBS) at pH 7.4, and instilled via the cannula with a 4% formalin solution at a constant fluid pressure of 25 cm H₂O for 10 min. The heart and respiratory tree were removed, and different lung lobes were excised and assigned to specific analysis. For biochemical and cytofluorimetric analyses, the lungs were excised from the abdomen, washed in 0.1 M PBS pH 7.4 and immediately stored at -80°C. Eight to 10-week-old pregnant rats were housed individually, and after delivery with the litters, in standard cages under a regular 12/12-h light/dark cycle at room temperature (21–24°C) and 40–60% humidity, with ad libitum access to food (commercial chow) and water. The sex of the litters was not considered in this study.

2.2 | Lipid peroxidation assessment

Cell membrane lipid peroxidation was measured by determining malondialdehyde levels in lung samples according to the manufacturer's instructions (MAK568-1KT, Merck Millipore). Briefly, 10 mg of the caudal lung lobe was homogenised on ice in the lysis buffer provided in the kit and then centrifuged at 13000 g for 10 min. The supernatants were mixed with thiobarbituric acid and the absorbance was read at 532 nm. The results are expressed as nmol ml⁻¹.

2.3 | Blood sample collection and 5-Epi-5-F2t-IsoP measurement

At P8 and P14, 100 µl of blood were collected from the tail vein in ethylenediaminetetraacetic acid (EDTA)-containing tubes and centrifuged at 1500 × g for 10 min at 25°C to isolate the plasma, which was subsequently stored at -80°C. Before storage, the antioxidant butylhydroxytoluene (BHT, 15 mg ml⁻¹ in methanol, 1:100) was added to inhibit lipid peroxidation. After thawing, eventually uncleared samples were discarded. The plasma level of free isoprostane 5-epi-5-F2t-IsoP, a marker of oxidative stress (Dyall et al., 2022), was quantified using a Micro-Extraction by Packed Sorbent liquid chromatography–tandem mass spectrometry platform (MEPS-LC-MS/MS), as reported (Biagini et al., 2022). Blood samples were collected from at least one pup per

experimental group in each section, contingent upon litter size. Specific sections were performed to collect blood samples at P8.

2.4 | Lung myofibroblast isolation

The rats from all experimental groups were killed at day 7 and myofibroblasts were isolated through enzymatic digestion of finely minced lung tissue collected following bronchoalveolar lavage and vascular perfusion with PBS, as previously described (Reese et al., 2025). Tissue specimens were digested in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mg ml⁻¹ collagenase I (Roche Diagnostic, Rotkreuz, Switzerland), 2.5 mg ml⁻¹ trypsin and 2 mg ml⁻¹ DNase I (Roche Diagnostic) at 37°C. Then, the cells were filtered through sterile gauze, pelleted by centrifugation at 400 g for 10 min and resuspended in DMEM supplemented with 10% foetal bovine serum (FBS). The cells were cultured for 12 h and then used for the transwell migration assay and immunofluorescent analysis.

2.5 | Transwell migration assay

After 12 h of isolation, 30,000 cells were resuspended in DMEM supplemented with 0.5% FBS and seeded into the upper chamber of inserts with an 8-µm pore size. In the lower chamber, 600 µl of DMEM supplemented with 2% FBS was added. Following a 24-h incubation, the cells in the upper compartment were gently removed and the cells that had migrated to the lower chamber were fixed and stained using Diff-Quik dye kit (9990700; Eprelia, Portsmouth, New Hampshire). Migration was quantified by counting five independent, non-overlapping fields.

2.6 | Flow cytometric analysis

Flow cytometry analysis was performed on lung cells from at least 10 rats per group. The tissues were homogenised using the Multi Tissue Dissociation Kit 2 (#130-110-203, Miltenyi Biotec, Bergisch Gladbach, Cologne, Germany) by gentleMACS Octo Dissociator (Miltenyi Biotec), with the lung-specific '37C_Multi_C' programme. At the end of the protocol, the samples were filtered with pre-separation filters (MACS SmartStrainers 70 µm, Miltenyi Biotec®) to remove large particles and cell aggregates. Dead cells were removed from the total cell suspension before flow cytometric analysis using the Dead Cell Removal Kit (Miltenyi Biotec) to assess only the live cell population. Single cells were then isolated from the cell suspension and immunostained with appropriately diluted combinations of fluorochrome-conjugated antibodies (Table 1) for different cell lineage markers. To assess the presence of lipofibroblasts, the cells were also stained with HCS LipidTOX green (H34475, Invitrogen; Waltham, MA, USA). The compensation was performed before sample acquisition to correct for spectral overlap between the fluorochromes used. For each conjugated antibody, single-stained controls were prepared using the MACS® Comp Bead Kit,

TABLE 1 Antibodies used for flow cytometric analysis.

Antigen	Fluorophore	Catalogue number	Source	RRID
CD45	AF405	sc-1178	Santa Cruz Biotechnology	AB_627074
CD326	AF647	bs-1513R	Bioss antibodies	AB_10855636
CD31	PE-Cy5	bs-0195R	Bioss antibodies	AB_11089130
CD140a	Cy3	bs-0231R	Bioss antibodies	AB_11090910
HCS LipidTOX	AF488	H34475	Invitrogen	
ACTA2	PE-Cy7	bsm-33187M	Bioss antibodies	AB_2910163
β_3 -adrenoceptor	APC-Cy7	bs-10921R	Bioss antibodies	AB_2927635
SP-B	AF488	sc-133143	Santa Cruz Biotechnology	AB_2285686
CD34	AF594	sc-7324	Santa Cruz Biotechnology	AB_2009969
CD133	AF647	sc-365537	Santa Cruz Biotechnology	AB_3699281

anti-rat Igk (Miltenyi Biotec). Each control was acquired on the MACSQuant[®] 10 and used to generate the compensation matrix in MACSQuantify Software. The final matrix was applied to all samples acquired in the same experimental session. The stained cells were analysed using a MACSQuant Analyser 10 Flow Cytometer (Miltenyi Biotec) and the data were processed with Flowlogic 8.7 software (Miltenyi Biotec). The whole-lung single-cell suspensions were analysed by gating for specific markers:- CD45⁺/CD326⁺/CD31⁺/CD140⁺ to identify the PDGFR α ⁺ fibroblast population (19); CD45⁺/CD326⁺/CD31⁺/CD140a⁺ and Lipidtox⁺ for the characterisation of lipofibroblasts (19); CD45⁺/CD326⁺/CD31⁺/CD140a⁺ and ACTA2 (actin alpha 2, smooth muscle)⁺ to identify myofibroblasts (19); CD45⁺/SP-B⁺ for alveolar type II epithelial cells; and CD45⁺/CD31⁺/CD133⁺/CD34⁺ for early circulating endothelial precursor cells (CEPCs) (Asante et al., 2024). Each population was also gated for β_3 -adrenoceptors to assess its expression on the individual cell type examined.

The frequencies shown in the graphs are calculated on the CD45⁺ population.

2.7 | Three-dimensional morphometric analyses of the lungs

Whole intermediate lobes of the right lung were treated using the iDISCO⁺ protocol with eosin staining (Laurino et al., 2023). Briefly, samples were dehydrated in graded methanol while gently shaking at room temperature (RT), stained with Eosin Y for 24 h and, after eosin removal, incubated for 3 h at RT in a solution composed of dichloromethane (DCM; 250 ml) and methanol. Optical clearing was performed by dibenzyl-ether incubation. Cleared samples were imaged using a high-resolution light-sheet microscope with real-time autofocus (Silvestri et al., 2021). For quantification, images were downsampled to an isotropic voxel size of $5 \times 5 \times 5 \mu\text{m}^3$ and separated into blocks of 1 mm^3 . Segmentation of cavities and septa was performed using Ilastik (Berg et al., 2019). To isolate alveoli, we used a custom script written in Python. The segmented cavities were skeletonised and then represented as a tree. The 'leaves' (final branches) were isolated as the skeletons of the alveoli. Starting from them, the full alveolar shape

was obtained by a watershed algorithm applied to the cavities mask with the alveolar skeleton as a seed.

2.8 | Morphometric evaluation of elastic fibres

The left lungs were fixed overnight at 4°C in 4% paraformaldehyde, followed by dehydration in graded ethanol and embedding in paraffin. Five micrometre-thick sections were stained with paraldehyde fuchsin to assess elastic fibres morphometrically. Fifteen microphotographs from each sample were randomly captured using a digital camera connected to a Zeiss Axioskop light microscope (Zeiss, Mannheim, Germany) equipped with a 10X objective. The images were taken by independent observers (M.M. and P.N.) blinded to the experimental groups. The quantitative evaluation was performed using ImageJ software (NIH, Bethesda, MD, USA) to analyse both the paraldehyde fuchsin-positive area and the total lung area, after appropriate thresholding. The results were expressed as the percentage of the positive area relative to the total area of the region of interest (ROI).

2.9 | Morphometric assessment of intracellular lipid content

The caudal lobe of the right lung was included in the optimal cutting temperature (OCT) compound (Leica Microsystems, Wetzlar, Germany) and 5- μm -thick frozen sections were cut using a cryostat (Leica Microsystems). The lung slices were stained with a freshly prepared working solution of Sudan Black to assess the intracellular lipid content morphometrically. After incubation, the slides were washed with PBS, then mounted in an anti-fade aqueous medium (FluoroshieldTM with DAPI). Fifteen microphotographs from each sample were randomly captured using a digital camera connected to a Zeiss Axioskop light microscope (Zeiss, Mannheim, Germany) equipped with a 40X objective. The images were taken by independent observers (M.M. and P.N.) blinded to the experimental groups. The quantitative evaluation was performed using ImageJ software (NIH, Bethesda, MD, USA) to analyse the black-stained-positive area and the total lung area after appropriate

thresholding. The results were expressed as the percentage of the positive area relative to the total area of the ROI.

2.10 | Immunofluorescence analysis

All the immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018). The caudal lobe of the right lung was included in the OCT compound (Leica Microsystems, Wetzlar, Germany) and 5- μ m-thick frozen sections were cut using a cryostat (Leica Microsystems). To assess the protein nitrosylation and the number of alveolar type II epithelial cells and blood vessels, single immunofluorescent staining was conducted on tissue slices using rabbit anti-nitrotyrosine (NT; 06-284, Merck Millipore; RID:AB_310089), rabbit anti-proSP-C (Merck Millipore) and goat anti-CD31 (AF3628, R&D; McKinley Place NE, MN, USA; AB_2161028), as the primary antibodies, respectively. The sections were rehydrated in PBS to reduce non-specific binding and subsequently blocked with 1.5% bovine serum albumin (BSA; AppliChem, Darmstadt, Germany) in PBS. For proSP-C staining, the sections were additionally blocked and permeabilized using 1.5% BSA in 0.1% Triton X in PBS. The primary antibodies were diluted in PBS with 1% BSA at ratios of 1:200, 1:50 and 1:2000 respectively, and incubated overnight at 4°C. Then, the sections were washed, treated at RT for 2 h with appropriate Alexa Fluor 488-conjugated secondary antibodies and mounted in an anti-fade aqueous medium (Fluoroshield™ with DAPI).

For the double immunofluorescent staining, the isolated myofibroblasts were fixed with 4% paraformaldehyde, permeabilized and blocked using 3% BSA (81-053-3, Merck Millipore) in PBS containing 0.3% Triton X-100 for 1 h. The cells were incubated overnight at 4°C with a mixture of two primary antibodies, goat anti- α -smooth muscle actin (ab21027, Abcam, Cambridge, UK; AB_1951138) and rabbit anti-epidermal growth factor receptor (ab52894, Abcam; AB_869579), and then treated for 2 h at RT with appropriate Alexa-Fluor secondary

antibodies. Subsequently, the specimens were rinsed with PBS and mounted in an anti-fade aqueous medium (Fluoroshield™ with DAPI). Omission of the primary antibody served as the negative control. The specimens were examined under a Leica Stellaris 5 confocal microscope (Leica Microsystems), using the Leica Plan Apo 20X/NA and 63X/1.43 NA objectives (Leica Microsystems). The nitrotyrosine levels and the density of alveolar type II epithelial cells were evaluated on digitalized z-stack composite mosaic images of the entire lung slice by measuring the positive immunoreactive area for nitrotyrosine or by counting proSP-C-positive cells with ImageJ software (NIH), after first establishing appropriate parameters. The results were expressed as a percentage of proSP-C positive cells relative to the lung parenchymal area. Vascular density was evaluated on five non-overlapping parenchymal z-stack images from the lung sections of each animal. The number of blood vessels (20–50 μ m in diameter) was independently measured by two blinded observers (A.P. and M.M) in each region of interest (ROI), as previously described (Sammour et al., 2016). A total of two slices per animal from at least 10 animals per group were evaluated.

2.11 | Western blot analysis and ELISA assay

The intermediate lobe was homogenised in ice-cold lysis buffer (Cell Signaling) supplemented with 1 mM phenylmethanesulfonyl fluoride for western blot analysis or in a specific lysis buffer (Abcam) for VEGF ELISA assay. Upon centrifugation at 13,000 g for 15 min at 4°C, the supernatants were collected and the total protein content was quantified using Pierce BCA Protein Assay (Invitrogen). For western blot analysis, 60 μ g of total protein was electrophoresed on a mini Protean precast 4–12% gel (Bio-Rad Laboratories; Hercules, CA, USA) and blotted onto polyvinylidene difluoride (PVDF) membranes using the Trans Blot Turbo system (Bio-Rad Laboratories). The membranes were blocked with BSA or non-fat dry milk for 1 h at RT and incubated overnight at 4°C with primary antibodies listed in Table 2. Results were revealed by incubating the

TABLE 2 Antibodies used for Western blot analysis.

Antigen	Species	Source	Catalogue number	Concentration	RRID
β -actin	Mouse	Sigma-Aldrich (St. Louise, MO, USA)	A2228	1:4500	AB_476697
PTHrP	Rabbit	GeneTex (Irvine, CA, USA)	GTX00631	1:1000	AB_3714897
proSP-C	Rabbit	Merck Millipore (Darmstadt, Germany)	AB3786	1:750	AB_91588
TGF- β	Mouse	Invitrogen (Waltham, MA, USA)	MA1-21595	1:5000	AB_561657
Tubulin	Mouse	Merck Millipore (Darmstadt, Germany)	T5201	1:1000	AB_609915
VEGFR-2	Mouse	Santa Cruz Biotech (Santa Cruz, CA, USA)	sc-6251	1:1000	AB_628431
Peroxidase-conjugated AffinePure goat anti-rabbit IgG	Goat	Jackson ImmunoResearch (Ely, UK)	111-035-003	1:7500	AB_2313567
Peroxidase-conjugated AffinePure goat anti-mouse IgG	Goat	Jackson ImmunoResearch (Ely, UK)	115-035-044	1:7500	AB_2338503

membrane with Immobilon ECL Ultra Western HRP solution (Merck Millipore) and using a Bio-Rad Chemidoc (Bio-Rad Laboratories) as the detection system. Densitometric analysis of the bands was performed using ImageJ software (NIH) and the values were normalized to β -actin or tubulin. Original western blot images are reported in the Figures S1–S4).

The VEGF levels in the lungs were measured by ELISA (Ab100787, Abcam) according to the manufacturer instructions.

2.12 | Transmission electron microscopy

Lung fragments of the cranial right lobe were fixed overnight in Karnovsky's fixative (15720; Electron Microscopy Sciences, Hatfield, PA, USA) at +4°C, post-fixed in 1% OsO₄ (18451, Ted Pella, Redding, CA, USA) in phosphate buffer for 2 h at RT, dehydrated in graded acetone and embedded in epoxy resin (45359-1EA-F, Sigma Aldrich). Ultrathin sections were examined under a JEM-1010 electron microscope (Jeol, Tokyo, Japan) at 80 kV and photographed using a MegaView III high-resolution digital camera equipped with AnalySIS 2.2 imaging software (Soft Imaging System, Muenster, Germany).

2.13 | Lung hydroxyproline analysis

The hydroxyproline content was measured using a Hydroxyproline Assay Kit (MAK008, Merck Millipore) following the manufacturer's guidelines on accessory lobes that had been lyophilised for 12 h.

2.14 | Morphometric evaluation of lung fibrosis

The left lungs were fixed and paraffin-embedded as previously described. Five micrometre-thick sections were stained with collagen-specific Sirius red to assess lung fibrosis morphometrically. Twenty microphotographs from each sample were randomly captured using a digital camera connected to a Zeiss Axioskop light microscope (Zeiss) equipped with a 20X objective. The images were taken by independent observers (M.M. and P.N.) blinded to the experimental groups. The quantitative evaluation was performed using ImageJ software (NIH) to analyse both the Sirius-positive area and the total lung area, after appropriate thresholding. The results were expressed as the percentage of the Sirius red-positive area relative to the total lung tissue area.

2.15 | Statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2025). Data are expressed as mean $\pm$ SD. Statistical analysis was performed using a one-way analysis of variance (ANOVA) test followed by Tukey's multiple comparison test. For the survival analysis, the Kaplan–Meier method was used. Calculations were carried out using GraphPad Prism 9.0 software (GraphPad, San Diego, CA, USA) and

$P < 0.05$ was considered significant. Sample sizes subjected to statistical analyses were at least 10 animals per group. For each analysis, at least seven independent measurements were obtained.

2.16 | Materials

The following compounds were used in the study: BRL 37344 (Sigma-Aldrich; St. Louis, MO, USA), pentobarbital solution (Millipore, Merck KGaA, Darmstadt, Germany), phosphate-buffered saline (PBS; Sigma-Aldrich), 4% paraformaldehyde solution (PAF; Sigma-Aldrich), ethylenediaminetetraacetic acid (EDTA)-containing tubes (Greiner Bio-One, Frickenhausen, Germany), Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich), foetal bovine serum (FBS, Sigma-Aldrich), Eosin Y (Bio-Optica, Milan, Italy), dichloromethane (DCM; 250 ml) and methanol (Millipore, Merck KGaA, Darmstadt, Germany), butylhydroxytoluene (BHT; Millipore, Merck KGaA), dibenzyl-ether (Sigma-Aldrich), ethanol (Carlo Erba Reagents; Cornaredo, Milan, Italy), paraffin (Bio-Optica), paraldehyde fuchsin (Sigma-Aldrich), Sudan Black (Sigma-Aldrich), Fluoroshield™ with DAPI (4',6-diamidino-2-phenylindole, dihydrochloride; Sigma-Aldrich), Triton X-100 (Thermo Fisher Scientific, Waltham, MA, USA), phenylmethanesulfonyl fluoride (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), acetone (Sigma-Aldrich), Sirius red (Sigma-Aldrich), a VEGF ELISA assay (Abcam, Cambridge, UK), and non-fat dry milk (VWR, Avantor, Radnor, PA, USA).

Details of other materials and suppliers are provided in the specific sections and in Tables 1 and 2.

2.17 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY <http://www.guidetopharmacology.org> and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/24 (Alexander, Christopoulos, et al., 2023; Alexander, Fabbro, et al., 2023).

3 | RESULTS

3.1 | Survival rate

The findings of our study confirmed the previous literature, indicating that the used animal model mimics severe bronchopulmonary dysplasia (BPD), characterised by a low survival rate (O'Reilly & Thébaud, 2014), which was significantly reduced to one third of the pups reared under normoxia. The hyperoxia-exposed rats treated with BRL37344 at 1 and 3 mg kg^{−1} exhibited a significantly higher overall survival rate than the untreated controls, even though the compound at both doses was unable to restore the survival rate of normoxic animals (Figure 1). In contrast, BRL37344 at the highest dose of

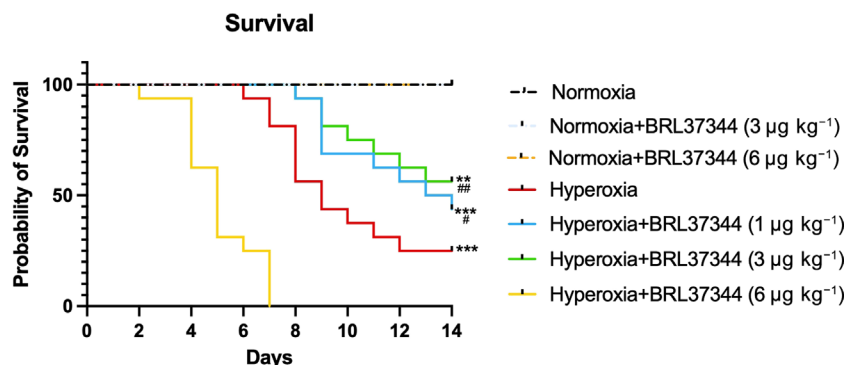


FIGURE 1 Survival rates of the different experimental conditions. Hyperoxia decreased the survival rate to one-third of the normoxic controls ($P < 0.001$). The administration of BRL37344 at 1 and 3 mg kg^{-1} resulted in a significantly increased survival rate ($P < 0.05$) compared to the untreated control, although the compound at both doses failed to restore it to normoxic levels. Conversely, BRL37344 at the highest dose of 6 mg kg^{-1} exhibited synergistic effects with the harmful impact of severe hyperoxia, leading to the death of all pups raised in hyperoxia within the first week. Based on these findings, the 6 mg kg^{-1} dose was excluded from the subsequent studies. Group size at P1: normoxia, normoxia + BRL37344 3 mg kg^{-1} , hyperoxia + BRL37344 6 mg kg^{-1} 25 pups; hyperoxia 100 pups; hyperoxia + BRL37344 1 mg kg^{-1} 50 pups; hyperoxia + BRL37344 3 mg kg^{-1} 42 pups; and normoxia + BRL37344 6 mg kg^{-1} 10 pups. Values are expressed as mean $\pm$ SD. *** $P < 0.001$ versus normoxia; §§ $P < 0.01$ and §§§ $P < 0.001$ versus hyperoxia.

6 mg kg^{-1} caused the death of all pups reared in hyperoxia within the first week, thereby precluding further analysis (Figure 1). This lethal outcome resulted from a synergistic interaction between the detrimental effects of severe hyperoxia and the high dosage of BRL37344, rather than from the compound inherent toxicity, as supported by the absence of mortality in animals administered with the same dose under normoxic conditions. Based on these results, our investigations focussed on the effects of BRL37344 at 1 and 3 mg kg^{-1} and did not include the 6 mg kg^{-1} dose.

3.2 | Oxidative stress evaluation

The hyperoxic BPD model is known to be associated with elevated oxidative stress levels (Dankhara et al., 2023). Given the ability of β_3 -adrenoceptors to reduce ROS levels (Bundgaard et al., 2017), we aimed to investigate whether the administration of BRL37344 could effectively decrease oxidative stress levels. We quantitatively measured immunoreactivity to nitrotyrosine (NT), a marker of protein tyrosine nitrosylation (Masini et al., 2006) and levels of malondialdehyde (MDA), an end-product of cell membrane lipid peroxidation, as hallmarks of local oxidative stress (Lucarini et al., 2016) in lung samples from pups at P14. As shown in Figure 2, hyperoxia exposure significantly increased both nitrotyrosine and malondialdehyde levels. Administration of BRL37344 demonstrated a dose-dependent protective effect against oxidative stress, lowering both biomarkers. However, their levels did not completely return to those of the normoxic controls (Figure 2a–g). No significant differences were observed under normoxia (Figure 2a–g).

We also evaluated plasma concentrations of 5-*epi*-5- F_{2t} -IsoP, a member of 5-series F_2 -isoprostanes (F_2 -IsoPs), as a marker of systemic oxidative stress at P8 and P14. Our findings demonstrated

that exposure to hyperoxia resulted in a significant increase in plasma levels of 5-*epi*-5- F_{2t} -IsoP at both time points (Figure 2h and i). The administration of BRL37344 at 3 mg kg^{-1} prevented this alteration only at P8 but not at P14 (Figure 2h and i). In contrast, the 1 mg kg^{-1} dose was ineffective at both time points (Figure 2h and i). As expected, no differences in 5-*epi*-5- F_{2t} -IsoP levels were detected in the plasma of pups reared in normoxia, treated or not with BRL37344 (Figure 2h and i) at any time points. Blood samples were collected from at least one pup per experimental group in each section, depending on the litter size. Therefore, the number of examined animals for 5-Epi-5- F_2t -IsoP quantification does not match the survival rate data.

3.3 | Lung morphology

Exposure to hyperoxia impairs the later stages of pulmonary development, leading to a marked reduction in lung volume and alveolarisation (O'Reilly & Thébaud, 2014; Thébaud et al., 2005). Our findings demonstrated that in the untreated rats exposed to hyperoxia, the ratio of lung-to-body weight (LW/BW) was significantly decreased compared to the control pups raised in normoxia. The administration of BRL37344 at a dose of 3 mg kg^{-1} prevented the decrease in lung volume, while the lower 1 mg kg^{-1} dose was ineffective (Figure 3a).

Analysis of the lungs from the untreated pups exposed to hyperoxia by 3D morphometry revealed a significant increase in the alveolar volume-to-surface area ratio and decrease in the alveolar volume density (Figure 3b–d). These findings indicate that elevated oxygen levels impair alveologenesi. The administration of BRL37344 at 3 mg kg^{-1} effectively counteracted both these alterations, although it did not revert them to the normoxic conditions. Conversely, the lower

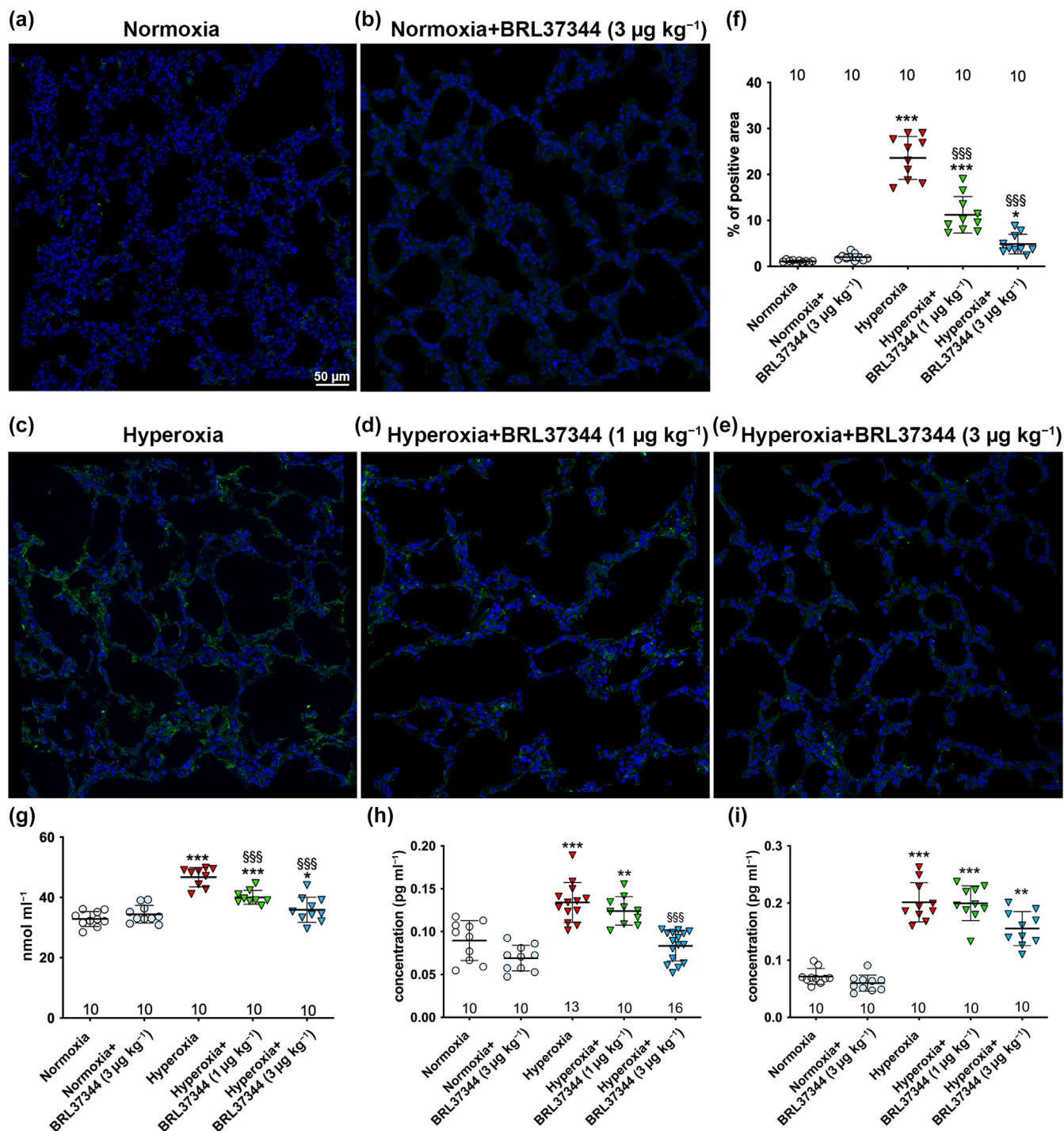


FIGURE 2 Oxidative stress evaluation. (a–e) Representative images and (f) morphometric quantification of the immunofluorescent expression of nitrotyrosine (NT) (green), a marker of local oxidative stress. (g) Pulmonary malondialdehyde (MDA) levels. Hyperoxia significantly increased both lung MDA and NT levels, indicating increased oxidative stress, while BRL37344 showed a dose-dependent protective effect (a–g). Sample size included at least 10 pups per experimental group. For each sample, seven independent measurements were obtained. The hyperoxia-exposed controls showed higher plasma levels of isoprostane 5-epi-5-F2t-IsoP than the normoxic pups at both (h) P8 and (i) P14. Treatment with BRL37344 at 3 mg kg⁻¹ entirely prevented this alteration at P8 but not at P14, whereas the 1 mg kg⁻¹ dose was ineffective at any time points. Under normoxia, no differences were noted in the pups treated or not with BRL37344. Values are expressed as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus normoxia; §§§ $P < 0.001$ versus hyperoxia.

1 mg kg⁻¹ dose was only effective in improving the alveolar volume density (Figure 3b–d). Administration of BRL37344 to the rats under normoxia had no significant effects on these lung parameters,

indicating that its protective action is limited to the harmful effects of hyperoxia (Figure 3b–d). No adverse effects of BRL37344 were observed in the normoxic pups.

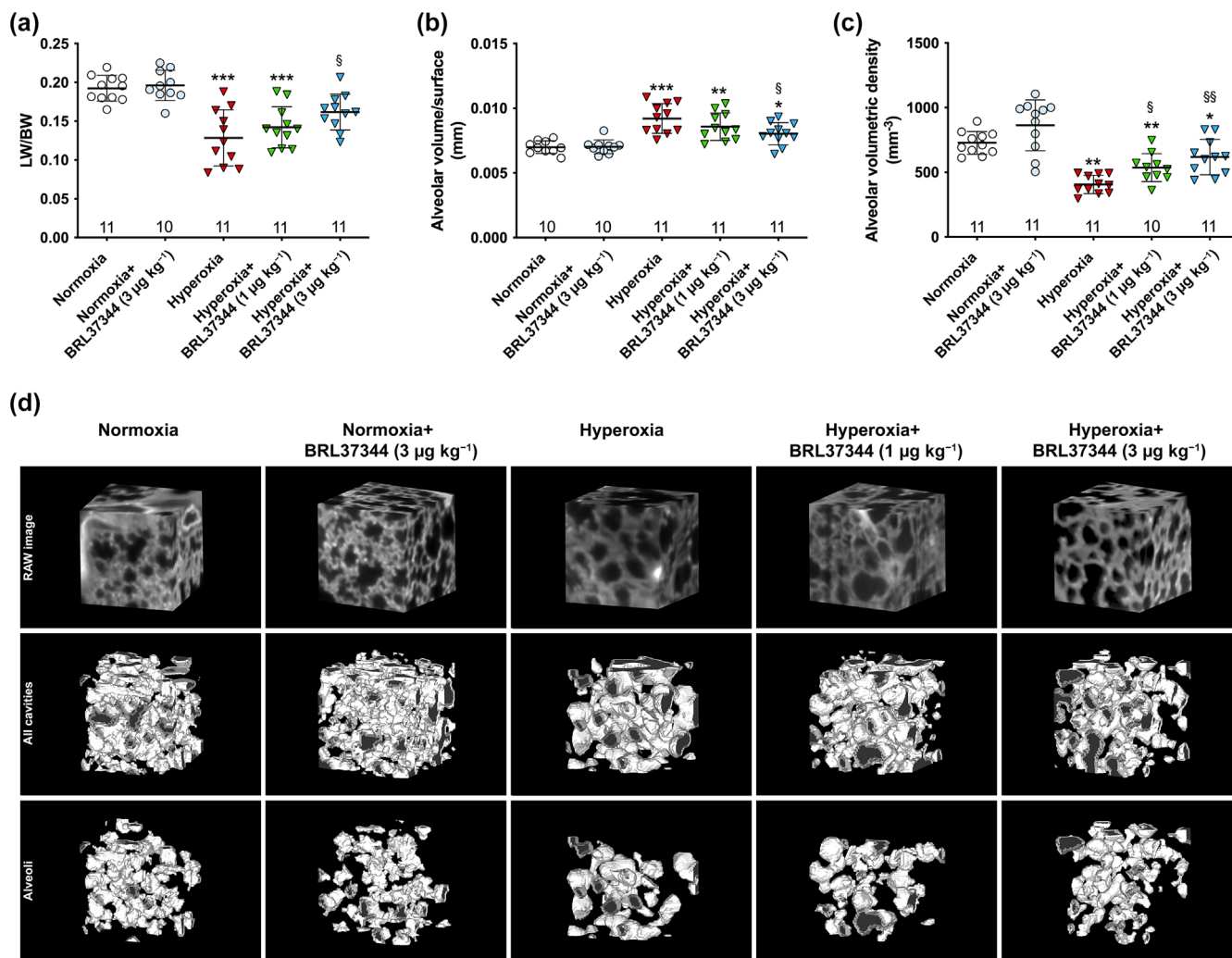


FIGURE 3 Effect of BRL37344 on lung morphology. (a) At P14, hyperoxia resulted in a decreased ratio of lung-to-body weight (LW/BW) compared to the normoxic controls, which was preserved by administration of 3 mg kg⁻¹ BRL37344. (b, c) Morphometric analysis of the three-dimensional volumetric renderings of (d) terminal airspaces showed impairment of alveolarisation induced by hyperoxia, which was partially counteracted by 3 mg kg⁻¹ BRL37344. Sample size included at least 10 pups per experimental group. For each sample, seven independent measurements were obtained. Values are expressed as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus normoxia; § $P < 0.05$ and §§ $P < 0.01$ versus hyperoxia.

3.4 | β_3 -adrenoceptor expression by lung cells

We next sought to identify the pulmonary cells expressing β_3 -adrenoceptor by flow cytometry on whole-lung single-cell suspensions. As inflammatory cells are known to express this receptor constitutively, we used the pan-haematopoietic marker CD45 to label and exclude them. Then, the CD45-cell population was gated for CD326-/CD31-/PDGFR α + to identify fibroblasts, further distinguished as CD326-/CD31-/PDGFR α + /Lipidtox+ lipofibroblasts, or CD326-/CD31-/PDGFR α + /ACTA2+ myofibroblasts (Endale et al., 2017). SP-B+ gating was applied to identify alveolar type II epithelial cells and CD31+/CD34+/CD133+ gating was applied to identify circulating early endothelial progenitors (CEPCs) (Asante et al., 2024). These populations were further analysed for the presence of β_3 -adrenoceptors. This

analysis first demonstrated that β_3 -adrenoceptor is expressed on PDGFR α + fibroblasts, both lipofibroblasts and myofibroblasts, as well as CEPCs, whereas it is absent on alveolar type II epithelial cells (Figure 4).

3.5 | Effects of BRL37344 on lung fibroblast subpopulations

Lipofibroblasts and myofibroblasts are distinct subpopulations of PDGFR α + fibroblasts, involved in both normal lung development (Endale et al., 2017) and the progression of BPD (Lecarpentier et al., 2019). This is because of the key roles of lipofibroblasts in synthesising the lipid component of surfactant and of myofibroblasts

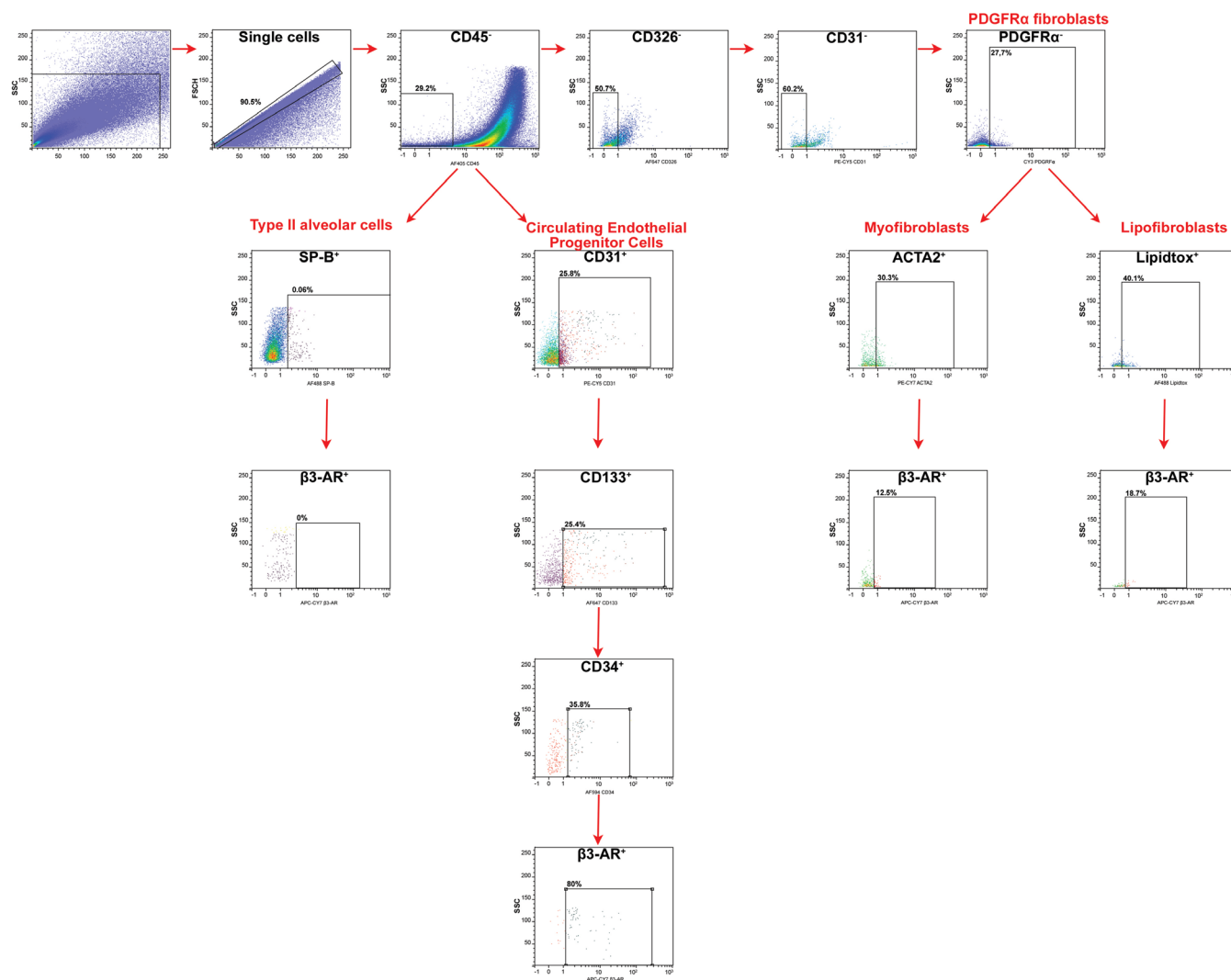


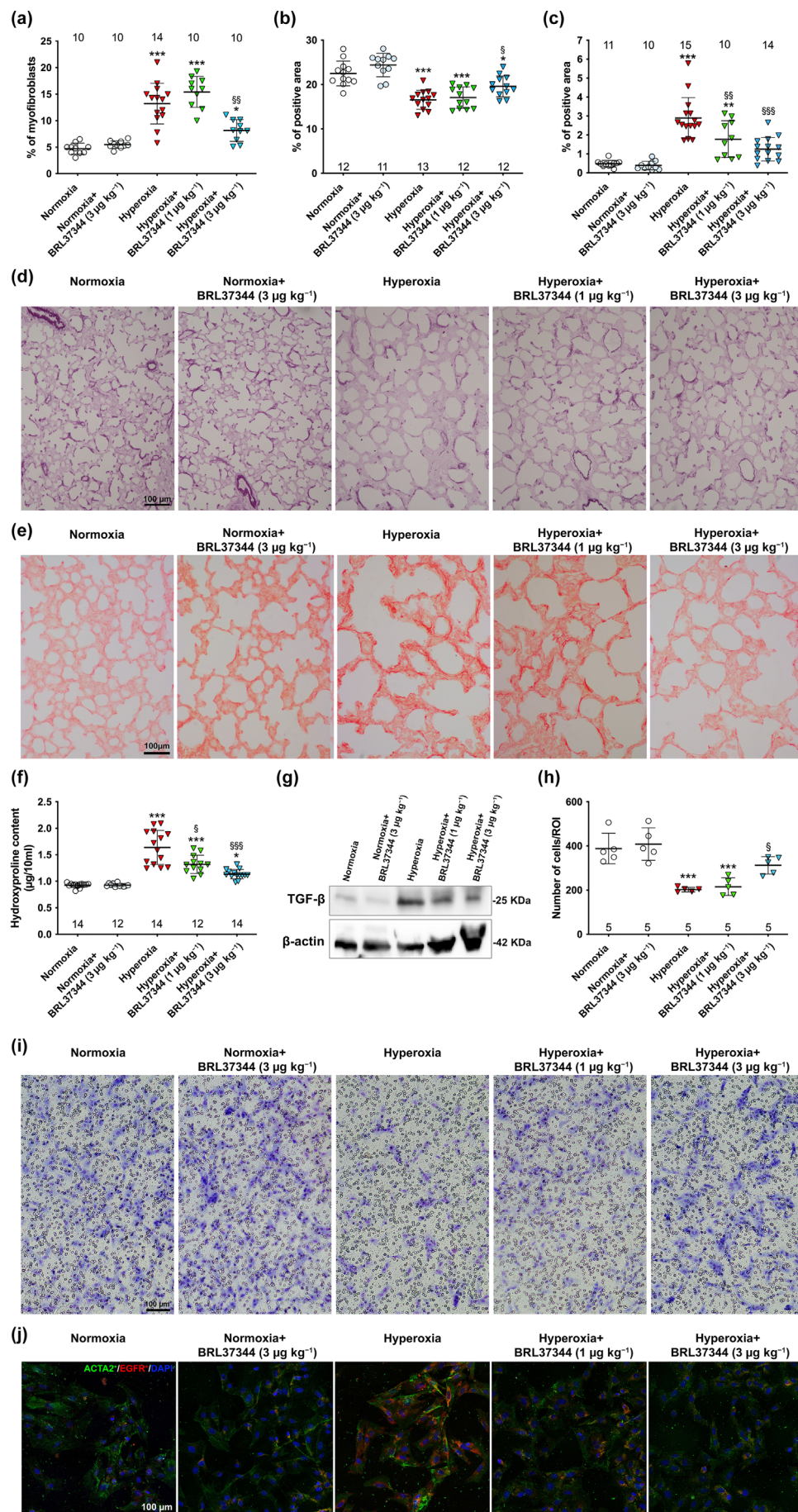
FIGURE 4 Overview of the gating strategy used for flow cytometric analysis of β_3 -adrenoceptor (β_3 -AR) expression on lung cells. Exemplificative strategy from the normoxic control at P14. Single-cell suspensions were immunolabelled for various lineage markers and subsequently analysed for β_3 -adrenoceptor expression. The frequencies shown on the plots were calculated from the parent population. The applied gating protocol revealed that the β_3 -adrenoceptor is expressed by lipofibroblasts (CD326[−]/CD31[−]/PDGFR α ⁺/LipidTox⁺), myofibroblasts (CD326[−]/CD31[−]/PDGFR α ⁺/ACTA2⁺) and early circulating endothelial progenitors (CD31⁺/CD34⁺/CD133⁺). Notably, this receptor was absent in alveolar type II epithelial cells.

in promoting alveologenesis and interstitial lung fibrosis. Since both fibroblast subtypes express β_3 -adrenoceptor, we next investigated the effects of BRL37344 on these cells by flow cytometry using the above-described gating protocol.

Myofibroblasts – Hyperoxia led to an increase in the number of myofibroblasts, which was blunted by the administration of 3 mg kg^{−1} BRL37344, whereas the 1 mg kg^{−1} dose was ineffective (Figure 5a). Myofibroblasts are crucial for alveologenesis, as they migrate to the septal tips (Li et al., 2024) and lay down elastic fibres, which are essential for the development of new septal walls (Branchfield et al., 2016). Given that hyperoxia is known to shift myofibroblasts towards a pathological, pro-fibrotic phenotype (Asante et al., 2024), we assessed whether BRL37344 could maintain the physiological function of myofibroblasts. To this aim, we evaluated the content of elastic fibres, the

degree of fibrosis and the migratory ability of myofibroblasts in a trans-well assay. Morphometric analysis of lung sections stained with elastin-specific paraldehyde fuchsin revealed that the alveolar elastic fibre network was significantly impaired in the untreated hyperoxic pups compared with the normoxic controls (Figure 5b, d). Treatment with BRL37344 at 3 mg kg^{−1} partially preserved the elastic fibres, whereas the lower 1 mg kg^{−1} dose was ineffective (Figure 5b, d). Morphometric analysis of lung sections stained with collagen-specific Sirius red, together with the dosage of hydroxyproline content in lung lysates, provided evidence of the anti-fibrotic properties of BRL37344. The hyperoxia-exposed rats showed significant increases in Sirius red-positive lung tissue areas and in hydroxyproline levels, both key markers of fibrosis, as compared to the normoxic controls (Figure 5c, e, f). In contrast, the pups treated with BRL37344

FIGURE 5 Effects of BRL37344 on lung myofibroblasts. (a) Flow cytometric analysis of myofibroblasts in whole-lung single-cell suspensions. (b) Morphometric analysis of paraldehyde fuchsin-stained sections (d) showed that hyperoxia reduced the elastic fibre network, while treatment with 3 mg kg^{-1} BRL37344 prevented this alteration. (c) Densitometric assessment of (e) Sirius red-stained sections and (f) hydroxyproline assay showed that hyperoxia increased lung collagen deposition, while BRL37344 exhibited a dose-dependent protective effect. (g) Western blot analysis revealed that exposure to hyperoxia led to elevated levels of TGF- β , while administration of BRL37344 at both doses significantly reduced this alteration. Original western blot images are reported in Figure S1. (h) Quantification and (i) representative images of the transwell migration assay showing that hyperoxia impaired myofibroblast migration, while 3 mg kg^{-1} BRL37344 partially prevented this effect. (j) Representative images of ACTA2 (green) and EGFR (red) double-immunolabelled lung myofibroblasts in culture. Nuclei are counterstained blue with DAPI. Compared with the normoxic controls, myofibroblasts from the hyperoxia-exposed pups showed increased EGFR expression, which was partially inhibited by BRL37344 at 3 mg kg^{-1} but not at 1 mg kg^{-1} . No changes in any of these markers were observed in the normoxic rats treated or not with BRL37344. Sample size included at least 10 pups per experimental group. For each sample, seven independent measurements were obtained. Scale bars = $100 \mu\text{m}$. Values are expressed as mean $\pm$ SD. * $P < 0.05$ and *** $P < 0.001$ versus normoxia; § $P < 0.05$, §§ $P < 0.01$ and §§§ $P < 0.001$ versus hyperoxia.



demonstrated a dose-dependent protective effect against hyperoxia-induced lung fibrosis (Figure 5c, e, f). Consistent with these findings, western blot analysis revealed that the level of the pro-fibrotic cytokine **transforming growth factor beta (TGF- β)** in lung homogenates was increased in the lungs of the hyperoxic rats as compared to the normoxic controls and was partly, albeit significantly, reduced by BRL37344 at both doses (Figure 5g).

The trans-well migration assay on isolated lung myofibroblasts showed that hyperoxia induced a significant decrease in the number of migrated cells, whereas 3 mg kg⁻¹ BRL37344 partially restored their migratory ability (Figure 5h, i). Given the role of **epidermal growth factor receptor (EGFR)** in myofibroblast migration (Li et al., 2016; Li et al., 2024), we next evaluated its immunofluorescent expression by isolated lung myofibroblasts from the different experimental groups. Hyperoxia markedly increased EGFR immunofluorescence, while 3 mg kg⁻¹ BRL37344 partially prevented this alteration (Figure 5j).

Lipofibroblasts – By flow cytometry, we observed that hyperoxia significantly increased the lipofibroblast subpopulation. Treatment with 3 mg kg⁻¹ BRL37344 prevented this change, whereas the 1 mg kg⁻¹ dose was ineffective (Figure 6a). To assess lipofibroblast function, we next evaluated their ability to accumulate cytoplasmic lipid droplets through histochemical and ultrastructural analyses of lung tissue samples. Morphometry of Sudan black-stained lung sections revealed that hyperoxia significantly reduced the lipid droplets in lipofibroblasts, while 3 mg kg⁻¹ BRL37344 partially counteracted this decrease (Figure 6b, c). Ultrastructural analyses also support this observation. As shown in Figure 6, lipofibroblasts and type II alveolar cells were closely associated, suggesting that they are functionally linked to produce surfactant. Lipofibroblasts in hyperoxic controls contained few, small lipid droplets in their cytoplasm, while those in hyperoxic rats treated with 3 mg kg⁻¹ BRL37344 had more abundant, larger lipid droplets, similar to those in normoxic pups (Figure 6d). The lower 1 mg kg⁻¹ BRL37344 was ineffective. Our findings indicate that hyperoxia impaired the capacity of lipofibroblasts to accumulate lipids necessary for surfactant synthesis, inducing an increase in lipofibroblast subpopulation as a compensatory response of the lung. BRL37344 at an appropriate dose appears to counteract this alteration.

3.6 | Effects of BRL37344 on alveolar type II epithelial cells

Our findings show that the β_3 -adrenoceptor was not expressed by alveolar type II epithelial cells, suggesting that its pharmacological stimulation cannot directly affect these cells. However, it has been established that a cross-talk between lipofibroblasts, which express β_3 -adrenoceptors, and alveolar type II epithelial cells is essential for surfactant production and alterations of such interactions are associated with impaired lung development and surfactant deficiency (Rubin et al., 2004). Therefore, we extended our investigations to alveolar type II epithelial cells.

The morphometric analysis performed on lung sections immunostained for the alveolar type II cell marker prosurfactant protein C (proSP-C) revealed that the hyperoxia-exposed controls displayed hyperplasia of these cells (Figure 7a, b), a typical histopathologic feature of human BPD (Dankhara et al., 2023; Fehrenbach, 2001). Administration of 3 mg kg⁻¹ BRL37344 partially prevented this alteration, although being not able to completely restore the normoxic lung architecture (Figure 7a, b). Again, the lower 1 mg kg⁻¹ dose had no effects (Figure 7a, b). The indirect impact of BRL37344 on preserving alveolar type II epithelial cell function was then assessed by evaluating the levels of proSP-C, the precursor of the surfactant protein C, and the **parathyroid hormone-related protein (PTHrP)**, which is essential for surfactant lamellar body formation (Rubin et al., 2004) and is known to reduce the trans-differentiation of lipofibroblasts into myofibroblasts (Oruqaj et al., 2016). The western blot analysis on lung homogenates demonstrated no significant differences in proSP-C levels among the hyperoxic groups, regardless of the treatment with BRL37344 (Figure 7c). In contrast, the administration of 3 mg kg⁻¹ BRL37344 partially mitigated the decrease in PTHrP levels caused by hyperoxia, whereas the lower dose of 1 mg kg⁻¹ was ineffective (Figure 7d). These findings suggest an indirect role of BRL37344 in supporting the function of alveolar type II epithelial cells exposed to hyperoxia, likely mediated through lipofibroblasts.

3.7 | Effects of BRL37344 on lung endothelial cells and circulating endothelial progenitor cells (CEPCs)

As the flow cytometric analysis demonstrated β_3 -adrenoceptor expression on both lung endothelial cells and circulating endothelial progenitor cells (CEPCs), we assessed the effect of BRL37344 treatment on lung vasculature. We evaluated blood vessel density by morphometry of tissue sections immunostained for endothelial CD31 and CEPC number by flow cytometry on lung cell suspensions gated for CD31+/CD34+/CD133+. The exposure of developing lungs to hyperoxia significantly reduced the number of blood vessels (Figure 8a, b). Treatment with BRL37344 at both doses significantly prevented this alteration, although not to the levels of the normoxic controls (Figure 8a, b), confirming the role of β_3 agonism in promoting lung vascularisation. Of note, BRL37344 had no effect under normoxia, indicating that it does not induce lung angiogenesis per se (Figure 8a, b). Although hyperoxia significantly reduced the number of pulmonary vessels, it unexpectedly did not affect the number of CEPCs (Figure 8c). Notably, BRL37344 treatment significantly increased the number of CEPCs compared with control groups in both normoxia and hyperoxia, confirming the role of β_3 -adrenoceptor in precursor cell mobilisation (Calvani et al., 2020; Melecchi et al., 2024; Méndez-Ferrer et al., 2010; Nastasi et al., 2022). We next investigated the canonical signalling pathway involved in vascularisation. It is well known that high oxygen significantly impairs the growth of lung vasculature by interfering with **vascular endothelial growth factor (VEGF)** signalling pathways (Baker & Abman, 2015). Our findings confirmed that hyperoxia significantly reduced lung VEGF levels, as

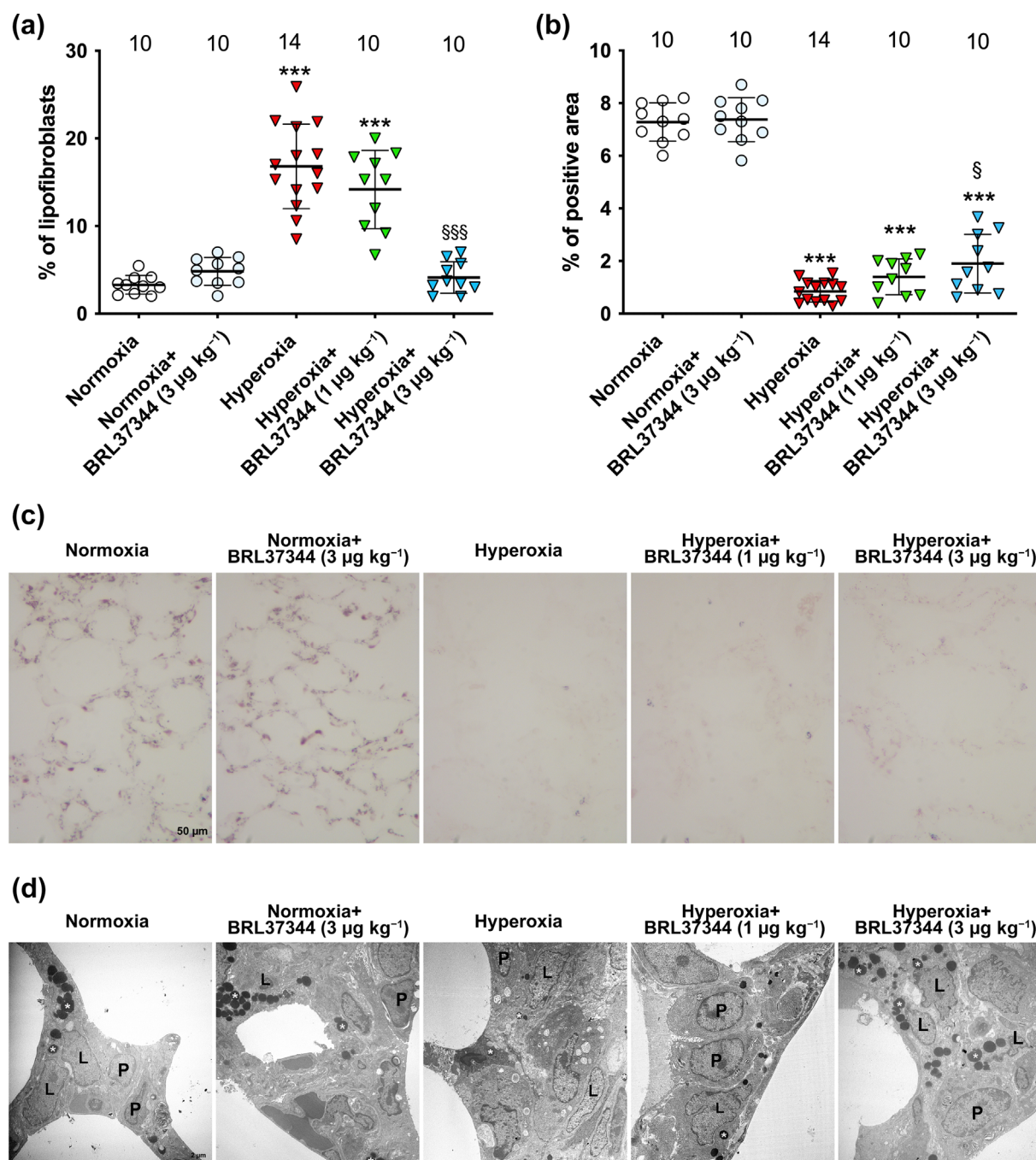


FIGURE 6 Effects of BRL37344 on lung lipofibroblasts. (a) Flow cytometric analysis of lipofibroblasts in whole-lung single-cell suspensions. (b) Morphometric analysis of (c) Sudan black-stained sections to detect cytoplasmic lipid droplets. Scale bar = 50 μm (d) Representative ultrastructural images of the alveolar region of the right upper lung lobe. Lipofibroblasts (L) with lipid droplets (some labelled by asterisks) are present close to type II alveolar cells (P). Scale bar = 2 μm. (a) Hyperoxia caused a significant increase in the number of lipofibroblasts, with (b–d) scant cytoplasmic lipid droplets. Treatment with BRL37344 at 3 mg kg⁻¹, but not at 1 mg kg⁻¹, reduced lipofibroblast hyperplasia (a) and partially prevented the loss of lipid bodies (b–d), resembling the lung features in the normoxic pups. No differences in the lipofibroblast subpopulation were found under normoxia (a–d). Sample size included at least 10 pups per experimental group. For each sample, seven independent measurements were obtained. Values are expressed as mean ± SD. *** $P < 0.001$ versus normoxia; § $P < 0.05$ and §§§ $P < 0.001$ versus hyperoxia.

assayed by enzyme-linked immunosorbent assay (ELISA, Figure 8d). The administration of 3 mg kg⁻¹ BRL37344 partially counteracted this effect, whereas the lower 1 mg kg⁻¹ dose was ineffective (Figure 8d). By western blotting, we also evaluated the levels of **vascular**

endothelial growth factor receptor 2 (kinase insert domain receptor; VEGFR-2), known to be the primary VEGF receptor involved in the pathogenesis of BPD (O'Reilly & Thébaud, 2014). Our results confirmed that hyperoxia significantly decreased VEGFR-2 levels, while

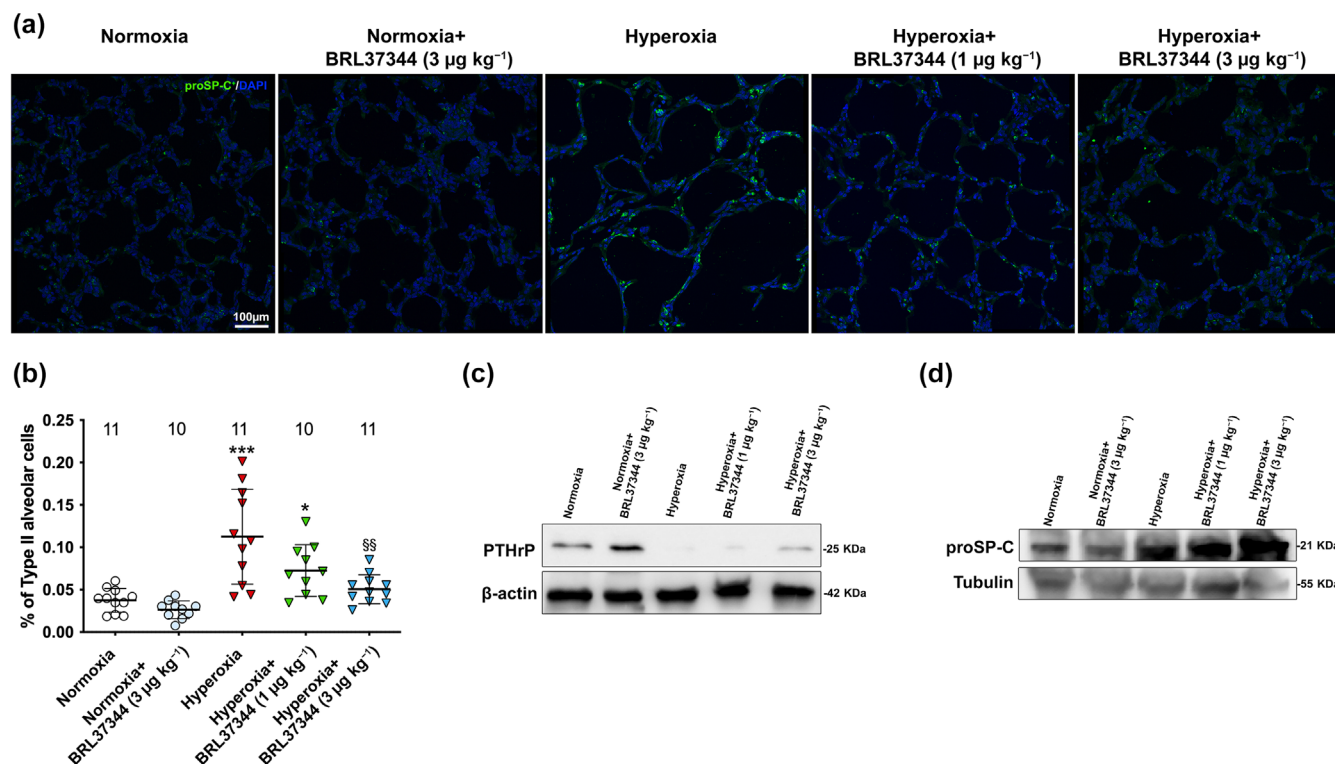


FIGURE 7 Effect of BRL37344 on alveolar type II epithelial cells. (a) Representative images and (b) morphometric quantification of the immunofluorescent expression of proSP-C (green), a marker of alveolar type II epithelial cells. Nuclei are counterstained with DAPI (blue). Scale bar = 100 μm . Quantification of proSP-C⁺ cells revealed that hyperoxia induced hyperplasia of alveolar type II epithelial cells, while treatment with BRL37344 at 3 mg kg^{-1} , but not at 1 mg kg^{-1} , effectively prevented this alteration. (c, d) Representative western blots of lung lysates from all experimental groups (β -actin or tubulin was used as a loading control). Hyperoxia caused a reduction of PTHrP expression and an increase in proSP-C levels. Treatment with BRL37344 at 3 mg kg^{-1} partially prevented the reduction of PTHrP but was ineffective against the alteration of proSP-C. Original western blot images are reported in Figures S2 and S3. Sample size included at least 10 pups per experimental group. For each sample, seven independent measurements were obtained. Values are expressed as mean $\pm$ SD. * $P < 0.05$ and *** $P < 0.001$ versus normoxia; §§ $P < 0.01$ versus hyperoxia.

treatment with BRL37344 at 3 mg kg^{-1} , but not at 1 mg kg^{-1} , counteracted this reduction (Figure 8e).

4 | DISCUSSION

This study provides the first evidence for the role of β_3 -adrenoceptors in a rat model of hyperoxia-induced bronchopulmonary dysplasia (BPD)-like disease, designed to mimic the authentic BPD of preterm infants. In this model, rat pups were exposed to very high oxygen concentrations (95%) during the first 14 days after birth to reproduce a condition of severe BPD, characterised by a low survival rate. Indeed, in the clinical care of preterm newborns, the oxygen levels administered, tailored to the infant's specific requirements, rarely exceed 60% for prolonged periods. However, previous studies indicate that such concentrations do not cause substantial lung damage in rat pups, likely because of their potent antioxidant systems during early neonatal life (O'Reilly & Thébaud, 2014). Conversely, exposure to 95% oxygen closely simulates the lung damage observed in preterm infants with BPD. Furthermore, given the oxygen-dependent regulation of β_3 -adrenoceptors, their expression and activation are likely to be

significantly reduced under extreme hyperoxic conditions. We selected the model with the highest sustainable oxygen concentration to evaluate agonist effectiveness in the most challenging conditions. This approach allows us to hypothesise that a positive response to the agonist under extreme conditions, where β_3 -adrenoceptor expression might be minimised, suggests its potential effectiveness in moderate hyperoxia, similar to that experienced by preterm infants, where receptor expression may be substantially maintained.

The first relevant finding of our study is that the treatment with the commonly used β_3 agonist BRL37344 at an appropriate dosage (3 mg kg^{-1}) significantly preserves the lung structural integrity, thereby nearly doubling the pups' survival rate.

The second relevant finding of the study indicates that BRL37344 significantly reduced hyperoxia-induced pulmonary oxidative stress at P14, confirming its protective effect against ROS. Moreover, our findings showed that BRL37344 at both doses significantly decreased systemic hyperoxia-induced oxidative stress levels at P8, as evaluated by the plasma concentrations of 5-*epi*-5-F_{2t}-IsoP, a byproduct of arachidonic acid peroxidation and a reliable oxidative stress marker (Nechuta et al., 2014). However, this beneficial effect disappeared by P14. In our previous study on an animal model of

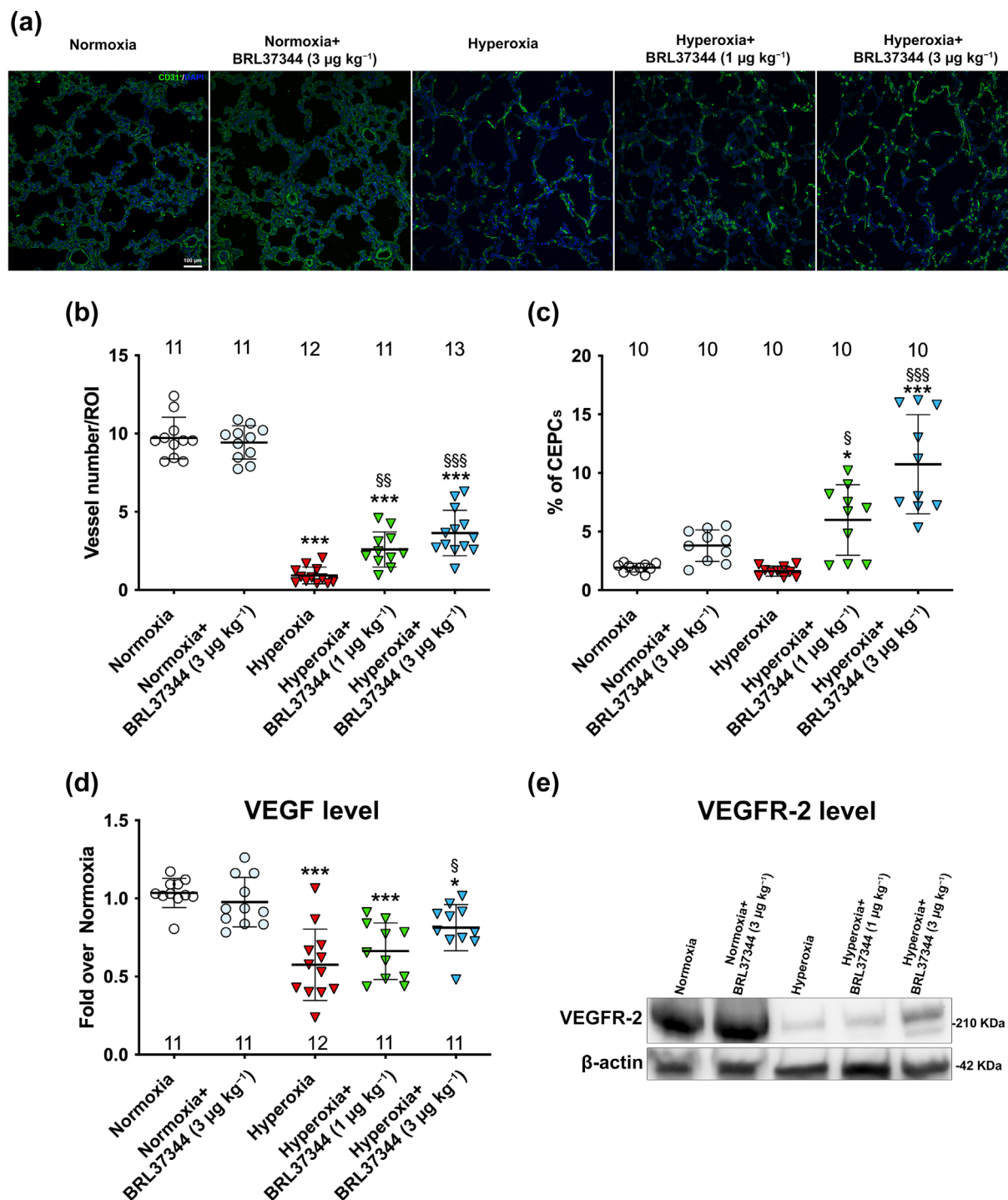


FIGURE 8 Effect of BRL37344 on lung vasculature. (a) Representative images showing CD31⁺ expression (green), a marker of endothelial cells. Nuclei are counterstained with DAPI (blue). Scale bar = 100 μ m. (b) Morphometrical analysis of blood vessel density. The administration of BRL37344 at both doses partially mitigated the reduction in vascular density induced by hyperoxia. No changes were observed in the normoxic rats treated or not with BRL37344. (c) Administration of BRL37344 at both doses significantly increased the number of CEPCCs compared to the other experimental groups. (d) ELISA assay of VEGF lung levels. Pups exposed to hyperoxia exhibited lower VEGF levels compared to the normoxic controls. Administration of 3 mg kg⁻¹ BRL37344 effectively prevented this decrease, while the 1 mg kg⁻¹ dose was ineffective. Under normoxia, no significant changes were observed upon treatment with BRL37344. (e) Representative western blots of lung lysates from the different experimental groups, showing decreased VEGFR-2 levels induced by hyperoxia, which were partially prevented by 3 mg kg⁻¹ BRL37344. β -actin was used as a loading control. Original western blot images are reported in Figure S4. Sample size included at least 10 pups per experimental group. For each sample, seven independent measurements were obtained. Values are expressed as mean $\pm$ SD. Values are expressed as mean $\pm$ SD. * $P < 0.05$ and *** $P < 0.001$ versus normoxia; § $P < 0.05$, §§ $P < 0.01$ and §§§ $P < 0.001$ versus hyperoxia.

hyperoxia-induced bowel damage, requiring a lower 85% oxygen concentration, treatment with 3 mg kg⁻¹ BRL37344 effectively reduced the levels of 5-*epi*-5-F_{2t}-IsoP increased by hyperoxia by P14 (Nardini et al., 2024). This discrepancy suggests that the protective effects of BRL37344 under the extreme hyperoxic conditions (95%) characteristic of the BPD-like model cannot persist beyond the first week of the experiment. However, P8 corresponds to the peak of alveologenesis in normal lung development (Branchfield et al., 2016), a time point when BRL37344 has demonstrated its protective efficacy.

The third major finding of our study is that the administration of BRL37344 effectively prevented the structural lung alterations induced by hyperoxia. The remarkable increase in the lung-to-body weight ratio observed following treatment with this β_3 agonist suggests that pulmonary development is preserved. The partially preserved ratio of alveolar volume-to-surface area and the increase in alveolar volume density induced by BRL37344 treatment indicate improved alveolarisation and preserved surface area of the respiratory epithelium. Until now, β_3 -adrenoceptor has only been identified in bronchial epithelial cells (Bossard et al., 2011). Here, we first report that this receptor is also expressed by myofibroblasts and lipofibroblasts, which are known to play crucial roles in alveologenesis (Endale et al., 2017), as well as by CEPs, which are involved in angiogenesis (Qi et al., 2013). Interestingly, we found that β_3 -adrenoceptor is absent in alveolar type II epithelial cells, which are a critical component of the alveolar epithelium, as they are both the source of surfactant and the stem cell reservoir for alveolar repair (Fehrenbach, 2001). Alveologenesis is a complex biphasic process primarily driven by myofibroblasts (Branchfield et al., 2016), consisting of a first wave where new septal walls are formed and a second wave where septae are lifted off existing walls (Mund et al., 2008). During the initial phase, myofibroblasts migrate to the forming septae (Li et al., 2024) and lay down a matrix of elastic fibres, which are continuously remodelled to facilitate the septal formation and stabilise the newly formed structures. At P14, when the first wave ends, myofibroblasts decrease their expression of α -SMA, while the elastin content peaks because of the deposition of an intricate network of thin elastic fibres within the inter-alveolar stroma (Branchfield et al., 2016). Hyperoxia is known to alter several signalling pathways that regulate myofibroblast function, leading them to shift from physiological alveolar wall build-up to pathological fibrosis (Berger & Bhandari, 2014; Branchfield et al., 2016; Nelson & Kugler, 2024). Among them, the EGFR signalling pathway, which is activated by ROS (Lecarpentier et al., 2019), plays a critical role in the migratory capability of myofibroblasts and is overexpressed in animal models of BPD-like disease and in patients with BPD (Li et al., 2024). Moreover, abnormal activation of EGFR has disruptive effects on normal lung morphogenesis and promotes lung fibrosis (Vallath et al., 2014). In this context, our findings suggest that β_3 agonism may help preserve alveologenesis by maintaining normal myofibroblast function. We have demonstrated that cultured lung myofibroblasts exposed to hyperoxia exhibited EGFR overexpression and a diminished migratory ability. The lung histological analysis has shown that, despite myofibroblast hyperplasia, the hyperoxia-exposed pups showed a reduction in elastic fibres accompanied by an increase

in collagen production at P14. Conceivably, hyperoxia-damaged myofibroblasts with impaired migratory capability cannot reach the septal tips to sustain alveologenesis and thus persist in the lung interstitium, where they give rise to fibrosis. In contrast, the cultured myofibroblasts isolated from hyperoxic rats treated with 3 mg kg⁻¹ BRL37344 exhibited EGFR expression levels and migratory capabilities similar to those isolated from the normoxic controls. In the lung tissue, 3 mg kg⁻¹ BRL37344 caused a reduced number of myofibroblasts compared to the untreated hyperoxic pups, along with a preserved elastic fibre network and reduced collagen deposition. A reduction in the profibrotic cytokine TGF- β was also observed. Taken together, our findings indicate that β_3 agonism plays a pivotal role in preserving the physiological function of lung myofibroblasts against the adverse effects of hyperoxia during alveologenesis, thus exerting a marked antifibrotic effect. It is tempting to speculate that β_3 agonism may inhibit the well-documented trans-differentiation of lipofibroblasts into myofibroblasts induced by hyperoxia (Torday & Rehan, 2016), although further experiments are required to clarify this point.

The presence of β_3 -adrenoceptors on lipofibroblasts may also help explain the protective effect of BRL37344 against hyperoxia-induced lung damage. Lipofibroblasts are a fibroblast subpopulation specialised to accumulate neutral lipids within cytoplasmic droplets (Tahedi et al., 2014; Torday & Rehan, 2016). They are present in the alveolar septa and support the synthesis of surfactant by alveolar type II cells nearby by supplying triglycerides through a mechanism known as neutral lipid trafficking (NLT) (Torday et al., 1995). Although newborn rats are surfactant-sufficient (Berger & Bhandari, 2014), it is known that hyperoxia disrupts the cross-talk between lipofibroblasts and alveolar type II cells, leading to neutral lipid trafficking alteration (Lecarpentier et al., 2019) and defective surfactant production and effectiveness. In the hyperoxia-exposed pups, we observed lipofibroblasts characterised by significantly fewer lipid droplets compared to the normoxic controls, suggesting an impairment of neutral lipid trafficking and surfactant production. Moreover, an increased number of lipofibroblasts was observed, which may reflect a compensatory response to breathing distress and elevated ROS levels, given their ability to enhance resilience against oxidative stress (Tahedi et al., 2014; Torday & Rehan, 2016). Notably, the hyperoxia-exposed pups also exhibited alveolar type II cell hyperplasia, likely in an attempt to compensate for surfactant deficiency and alveolar damage (Carvalho & Stevenson, 2022; Honda et al., 2003). Moreover, in these animals, Western blotting revealed almost undetectable levels of PTHrP, the key paracrine molecule involved in the cross-talk between these cells and lipofibroblasts required for surfactant production (Torday & Rehan, 2016). Conversely, the hyperoxic rats treated with 3 mg kg⁻¹ BRL37344 exhibited a nearly preserved lipid droplet content in lipofibroblasts and PTHrP levels similar to those of the normoxic pups, with no hyperplasia of lipofibroblasts and alveolar type II cells. These findings suggest that β_3 agonism exerts a protective effect on neutral lipid trafficking and surfactant production. The observed increase in proSP-C in all hyperoxic pups, irrespective of whether they were treated or not with BRL37344, is consistent with the absence of β_3

adrenoceptors on alveolar type II cells and may suggest that β_3 agonism only preserves the lipid component of surfactant while being ineffective on the protein fraction from type II cells.

Another remarkable result from this study is that BRL37344 (3 mg kg^{-1}) partially prevented the hyperoxia-induced impairment in lung vascularisation, a typical characteristic of BPD. Vascular development is primarily regulated by VEGF signalling, whose dysregulation is a crucial factor in the pathogenesis of BPD (O'Reilly & Thébaud, 2014; Thébaud et al., 2005) and other conditions of prematurity, such as retinopathy of prematurity (ROP) and necrotising enterocolitis (NEC) (Dal Monte et al., 2013; Klerk et al., 2021). VEGF signalling in the lungs encompasses not only the maintenance of vascularisation but also alveologenesis, which is shown to depend on angiogenesis (O'Reilly & Thébaud, 2014). While there are some discrepancies regarding the effect of hyperoxia on VEGF, most studies report diminished lung VEGF levels in pups exposed to hyperoxia 9 days after birth (Meller & Bhandari, 2012). Additionally, pharmacological inhibition of VEGF signalling during the first 2 weeks post-birth has been shown to reduce the number of lung capillaries (Galambos et al., 2002), while its exogenous administration alleviates hyperoxia-induced lung injury in BPD-like animal models (Kunig et al., 2005). Furthermore, in these models, a down-regulation of VEGFR-2 expression was found (Thébaud et al., 2005). Our findings demonstrate that β_3 agonism counteracts the down-regulation of both VEGF and VEGFR-2 induced by hyperoxia, thus partially preventing vascularisation impairment. We previously observed a similar, even more remarkable effect in a model of hyperoxia-induced gut injury, where the administration of 3 mg kg^{-1} BRL37344 completely reverted the negative impact of excess oxygen on vascular growth (Nardini et al., 2024). Moreover, in a model of retinopathy of prematurity, BRL37344 was shown to facilitate the revascularisation of the central retina, restoring physiological condition and counteracting the hyperoxia-induced damage (Melecchi et al., 2024).

The present study also demonstrates that CEPCs express the β_3 -adrenoceptor. This observation aligns with previous findings in infantile haemangiomas (Bassi et al., 2022). CEPCs are key players in vascularisation and their reduction has been implicated in the pathogenesis of BPD (Qi et al., 2013). In a mild BPD model, moderate hyperoxia exposure has been shown to impair pulmonary vascularisation and to reduce the CEPC population in the bone marrow and lungs of 11-day-old mice (Balasubramaniam et al., 2007). However, the same oxygen levels may exert age-dependent effects on CEPC numbers. In adult mice, moderate hyperoxia has been demonstrated to double the number of pulmonary CEPCs without reducing lung vessel density, suggesting a potential role for these cells in maintaining vascular integrity (Balasubramaniam et al., 2007). In our experimental setting, severe hyperoxia impaired vascular development without a corresponding decrease in pulmonary CEPCs in 14-day-old rats. Aside from potential differences among rodent species, our findings suggest that higher oxygen levels and longer exposure may worsen vascular injury compared to moderate hyperoxia, potentially accelerating the mobilisation of CEPCs to the lung to sustain the vasculature. Nevertheless, further investigation is required to validate this hypothesis,

including quantifying CEPCs under different oxygen conditions in lungs, peripheral blood and bone marrow, as well as detailed phenotypic characterisation of CEPC subtypes. Although the definitive role of CEPCs across various BPD models remains to be elucidated, our data indicate that β_3 agonism enhances CEPC recruitment to the lung. This hypothesis aligns with observations that β_3 -adrenoceptor activation is required for mobilising haematopoietic stem cells—the CEPC ancestors—from the bone marrow into the bloodstream in rodents (Méndez-Ferrer et al., 2010). It could be speculated that β_3 agonism may be a potential therapeutic approach to enhance the regenerative capacity of the lung vasculature, especially in hyperoxic conditions (Filippi et al., 2024).

A particular aspect that deserves attention is the dramatic mortality rate of the pups administered BRL37344 at the highest dose (6 mg kg^{-1}) under hyperoxia. A similar outcome was observed in our study on a rat model of hyperoxia-induced gut alterations (Filippi et al., 2023). Considering the absence of mortality in animals administered with the same dose under normoxic conditions, it is conceivable that BRL37344, when administered at such a high dose, exhibits a synergistic lethal effect with the noxious effects of severe hyperoxia. Indeed, treatment with BRL37344 at the effective dose of 3 mg kg^{-1} did not show any toxic effects or interference with any of the assayed parameters, suggesting a favourable therapeutic window at this lower dose.

This study has some limitations. First, β_3 agonism was achieved using BRL37344, a compound with greater selectivity for β_3 than both β_1 and β_2 -adrenoceptors in rodents (Schena & Caplan, 2019). Its suitability for subcutaneous administration makes it preferable for the neonatal rat model than other highly selective, second-generation β_3 agonists, such as mirabegron, which is administered via oral gavage and is less practical for newborn rodents. To note that BRL37344 has been shown to act through β_2 -adrenoceptors in some tissues, such as skeletal muscle (Ngala et al., 2009). Therefore, it cannot be totally ruled out that some of the beneficial effects of BRL37344 observed in our study may depend, at least in part, on β_2 agonism. Nevertheless, a recent *in vivo* study investigating the role of β_3 -adrenoceptors in the pathogenesis of retinopathy of prematurity revealed that the effects of BRL37344 were specifically mediated through β_3 -adrenoceptors, because they were completely blocked by the β_3 antagonist **SR59230A** (Melecchi et al., 2024). Furthermore, recent studies have questioned the high selectivity of mirabegron, because its relaxant effects on urinary bladder detrusor appear to be mediated by either β_2 agonism or α_1 antagonism (Erdogan et al., 2022; Lim & Chess-Williams, 2022). Hence, BRL37344 still stands as a validated β_3 agonist.

A second limitation of this study is the absence of lung functional assessments, which would have required a more complex experimental protocol. In view of potential translational investigations to explore the actual therapeutic potential of β_3 agonism in BPD, such functional studies are necessary and could represent a future research direction in this field.

The last limitation of this study pertains to the rat BPD-like disease. Although exposing newborn rodents to hyperoxia is considered a reliable model to mimic BPD in preterm infants, this model differs

from the actual human disease because it shows early development of lung fibrosis, which is rather characteristic of 'old' BPD. This discrepancy may be because of the harmful effects of the very high oxygen levels used in the model. Additionally, human BPD has a multifactorial aetiology and pathogenesis (O'Reilly & Thébaud, 2014). Therefore, the lack of an optimal animal model encompassing all the factors contributing to BPD remains an unavoidable limitation, which can only be partially addressed through the refinement of study methodologies.

5 | CONCLUSION

This *in vivo* study in a rat model provides new insights into the role of β_3 -adrenoceptors in lung development and the pathogenesis of bronchopulmonary dysplasia (BPD). By promoting alveologenesis, counteracting vascular impairment and reducing fibrosis, β_3 agonism plays a protective role against the onset and progression of hyperoxia-induced BPD-like disease in newborn rats, ultimately leading to increased survival. Although caution is necessary when extrapolating findings from animal studies to humans, our research suggests that pharmacological β_3 agonism warrants further investigation as a potential therapeutic strategy for BPD, potentially improving lung growth and differentiation in premature newborns. If confirmed clinically, β_3 agonists may help reduce the duration of oxygen therapy, improve outcomes for premature infants and minimise the long-term sequelae of BPD. Moreover, the antifibrotic effect of β_3 agonism could also justify exploring its therapeutic efficacy in other adult lung disorders, such as idiopathic pulmonary fibrosis and chronic obstructive pulmonary disease. It is worth noting that effective and safe β_3 agonists are already registered as therapeutics in the neonatal medicine repertoire, which may favour an easier bench-to-bedside translation.

AUTHOR CONTRIBUTIONS

Conceptualisation: Luca Filippi, Alessandro Pini and Patrizia Nardini. **Methodology:** Luca Filippi, Alessandro Pini, Patrizia Nardini, Maura Calvani, Daniele Bani and Fabio di Francesco. **Investigation:** Virginia Zizi, Marta Molino, Francesco Carrozzo, Denise Biagini, Daniele Guasti, Elisa Imbimbo, Alessandra Franceschini, Ludovico Silvestri and Rachele Amato. **Visualisation:** Patrizia Nardini, Marta Molino and Virginia Zizi. **Funding acquisition:** Luca Filippi and Alessandro Pini. **Project administration:** Luca Filippi and Alessandro Pini. **Supervision:** Luca Filippi, Alessandro Pini and Patrizia Nardini. **Writing—original draft:** Alessandro Pini. **Writing—review and editing:** Alessandro Pini, Luca Filippi and Daniele Bani.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for [Design & Analysis](#), [Immunoblotting and Immunochemistry](#), and [Animal Experimentation](#), and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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SUPPORTING INFORMATION

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