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RESEARCH ARTICLE | *Translational Physiology*

Local pulmonary drug delivery in the preterm rabbit: feasibility and efficacy of daily intratracheal injections

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Salaets T, Gie A, Jimenez J, Aertgeerts M, Gheysens O, Vande Velde G, Koole M, Murgia X, Casiraghi C, Ricci F, Salomone F, Villetti G, Allegaert K, Deprest J, Toelen J. Local pulmonary drug delivery in the preterm rabbit: feasibility and efficacy of daily intratracheal injections. *Am J Physiol Lung Cell Mol Physiol* 316: L589–L597, 2019. First published January 24, 2019; doi:10.1152/ajplung.00255.2018.—Recent clinical trials in newborns have successfully used surfactant as a drug carrier for an active compound, to minimize systemic exposure. To investigate the translational potential of surfactant-compound mixtures and other local therapeutics, a relevant animal model is required in which intratracheal administration for maximal local deposition is technically possible and well tolerated. Preterm rabbit pups (born at 28 days of gestation) were exposed to either hyperoxia or normoxia and randomized to receive daily intratracheal surfactant, daily intratracheal saline, or no injections for 7 days. At *day 7*, the overall lung function and morphology were assessed. Efficacy in terms of distribution was assessed by micro-PET-CT on both *day 0* and *day 7*. Lung function as well as parenchymal and vascular structure were altered by hyperoxia, thereby reproducing a phenotype reminiscent of bronchopulmonary dysplasia (BPD). Neither intratracheal surfactant nor saline affected the survival or the hyperoxia-induced BPD phenotype of the pups. Using PET-CT, we demonstrate that 82.5% of the injected radioactive tracer goes and remains in the lungs, with a decrease of only 4% after 150 min. Surfactant and saline can safely and effectively be administered in spontaneously breathing preterm rabbits. The described model and method enable researchers to evaluate intratracheal pharmacological interventions for the treatment of BPD.

bronchopulmonary dysplasia; intratracheal administration; local drug delivery; preterm rabbit; surfactant

INTRODUCTION

Despite significant advances in perinatal care, chronic respiratory morbidity remains an important sequel of preterm birth. Antenatal steroids and surfactant replacement have increased the survival of preterm neonates, but, since their introduction, rates of bronchopulmonary dysplasia (BPD) have been rising. Defined as oxygen dependency at 36 wk postmenstrual age or at discharge, BPD still occurs in ~45% of survivors of extremely preterm birth (<28 wk of gestational age) (39). Even beyond BPD, preterm birth results in significant lung function abnormalities and increased hospitalization rates for respiratory tract infections during infancy and childhood (8, 27). It has even been suggested that preterm lungs are prone to develop chronic obstructive pulmonary disease (COPD) in adulthood (25). The need for novel therapies thus remains high.

Many pharmacological interventions have been investigated to treat or prevent BPD; however, just a few products have shown therapeutic potential (20). For instance, studies on systemic corticosteroids in different regimens have shown potent effects in reducing oxygen dependency at a postmenstrual age of 36 wk. However, significant adverse events have been reported, including gastrointestinal perforations, growth failure, hypertrophic cardiomyopathy and, most importantly, a worsened neurocognitive outcome (9, 10).

Overall, newborns are more prone to develop adverse events, which could be reduced by avoiding systemic drug exposure (2). Therefore, local pulmonary delivery of novel drug candidates could be safer and reduce off-target organ exposure (14). In this regard, a recent and successful approach has been reported by Yeh et al. consisting of the combined intratracheal administration of a surfactant-budesonide mixture (46). Surfactant is a life-saving treatment for preterm neonates with established respiratory distress, but it may also serve as a carrier for pulmonary drug delivery, since it possesses unique biophysical properties to efficiently spread along the air-liquid interface (17). The use of surfactant as a drug carrier could result in increased local drug deposition in the lungs and decreased systemic side effects (16, 33). Along the same lines, also other carriers of active compounds (e.g., exosomes, polymeric carriers) are being considered for use in neonatal lungs, with the same purpose of reducing systemic exposure (16, 45).

Animal models play an indispensable role during the preclinical development of pharmacological treatments, including for BPD. Unfortunately, commonly used animal models have important limitations: mice and rat models are not preterm and do not allow

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easy tracheal access because of their small size, whereas lambs are very expensive, and the use of baboons is ethically questioned. The preterm rabbit model could be an advantageous alternative, since it includes the major driver of BPD, prematurity. Preterm rabbit pups born at *day 28* of gestation depict altered lung function at birth, reminiscent of neonatal respiratory distress syndrome (7), are born in the early saccular stage of lung development (28), and have immature defense mechanisms against oxidative stress (13). Furthermore, the animals combine prematurity with relatively low housing and care investments and have a size that allows for technical manipulations (32, 36). When exposed to hyperoxia, they develop morphological and functional manifestations comparable to human BPD. Previously, we evaluated the efficacy of several systemically administered compounds in this preterm rabbit model (21, 26, 31).

In this study we established a method for direct access to the airways, through transcutaneous intratracheal injections on spontaneously breathing preterm rabbits. We evaluated the feasibility and safety of daily intratracheal instillations of saline or surfactant and their effect on the phenotype of the preterm rabbit BPD model. Furthermore, we determined the pulmonary distribution of surfactant and saline following intratracheal administration.

METHODS

Cesarean section. Time-mated pregnant rabbits (New Zealand White and Dendermonde cross-breed) were provided through the animal facility of KU Leuven. All experiments were approved by the Ethics Committee for Animal Experimentation (project nos. P090/2016 and P080/2017) and conducted according to current guidelines on animal welfare. Cesarean section was performed at 28 days of pregnancy (early saccular stage of lung development, term = 31 days). Does were sedated with 35 mg/kg of intramuscular ketamine (Nimatek; Eurovet Animal Health, Bladel, The Netherlands) and 6 mg/kg of xylazine (XYL-M; VMD, Arendonk, Belgium). After adequate sedation, does were placed in the supine position and euthanized with a mixture of 200 mg embutramide, 50 mg mebezonium, and 5 mg tetracain hydrochloride (iv bolus of 1 ml T61; Intervet, Boxmeer, The Netherlands). Immediately afterward, the abdomen was opened, and all pups were extracted through hysterotomy.

Neonatal rabbit care. At delivery, the pups were dried, stimulated, and placed in an incubator at 32°C and 50% of humidity. Oxygen concentration was continuously monitored with a Palm O2 D% Analyzer (Analytical Industries). Neonatal rabbit care has been described previously (22, 32). Briefly, pups were fed two times daily via an orogastric tube with increasing quantities of a milk replacer (FoxValley 30/50, containing 10 g/100 ml fat, 6 g/100 ml protein, and $<$1 g/100 ml carbohydrates), supplemented with vitamins and probiotics (6 g/100 ml Biolapis; Protexin, Somerset, UK) and immunoglobulins on the first 2 days of life (4 g/100 ml Col-o-Cat; Sanobest, 's-Hertogenbosch, The Netherlands). Furthermore, intramuscular vitamin K on D2 (0.002 mg/kg Konakion pediatric; Roche) and antibiotics from D2-D7 [benzylpenicillin (20,000 IU/kg penicillin (Kela, Sint-Niklaas, Belgium) and amikacin (20 mg/kg amukin (Bristol-Myers Squibb)] were administered. The pups remained in the incubator for 7 days except for the feeding and interventions.

Randomization. After an initial 1-h adaptation period, surviving pups were weighed, numbered, and randomly assigned to one of the following six different groups, ensuring an equal distribution within nests: *1*) normoxia control group (21% oxygen; N; $n = 11$), *2*) hyperoxia control group ($\geq$95% oxygen; H, $n = 11$), *3*) hyperoxia group treated with daily intratracheal injections of 100 mg/kg of surfactant [Curosurf (Chiesi Farmaceutici, Parma, Italy); H_{surf}; $n = 13$], *4*) hyperoxia group treated with daily intratracheal injections of an equal volume of saline (H_{sal}; $n = 13$), *5*) normoxia group treated with daily intratracheal injections of 100 mg/kg of surfactant (N_{surf}; $n = 10$), and *6*) normoxia group treated with daily intratracheal injections of an equal volume of saline (N_{sal}; $n = 11$) as visualized in Fig. 1*A*. Sample size calculation was performed using GPower 3.1. To pick up a 1/3 correction of the effect of hyperoxia on tissue damping [historical data hyperoxia versus normoxia (22)], with a power of 80% and an α of 0.01 (Bonferroni correction for 5 comparisons) in a two-tailed *t*-test, eight pups in each group were needed. To obtain this number at *day 7*, 10–13 pups were randomized to each experimental group (Table 1).

Intratracheal injections. Rabbit pups were anesthetized with 2.5% isoflurane (ISO-VET; EuroVet, Heusden-Zolder, Belgium) in 2 liters oxygen/min and placed in supine position. We initially evaluated different techniques to obtain tracheal access (including intubation with curved catheters, intubation with a small scope, or nasal intubation) (38); however, these attempts failed in neonates because of the small size and long curvature of the rabbit snout (41). Eventually we chose the transcutaneous access because of its feasibility. The laryngeal and tracheal cartilage was stabilized with an Allis forceps, and catheterization of the trachea was performed transcutaneously with a 19-mm-long 26-gauge catheter (Neoflon; BD). Correct insertion of the catheter was checked by a water valve in the transparent catheter. Subsequently either 1.25 ml/kg of surfactant or saline was slowly injected with a 30-gauge blunt Hamilton needle inserted through the catheter (technique visualized in Fig. 1*C*). In postmortem test pups, this technique resulted in delivery above the carina. A gentle position check and instillation were used, since initial attempts using a slightly more aggressive strategy resulted in overinflation of lungs and mortality. In these optimization experiments, position in the trachea was checked by air aspiration, and, after administration of the surfactant, a fast air bolus of 0.5 ml was applied. This resulted in procedural mortality in the surfactant group, but not in the saline group. Necropsy of the animals dying after surfactant administration revealed plump and (over)expanded lungs.

Lung function testing. At *day 7*, invasive lung function testing was performed using the Flexivent system (FlexiVent 5.2; SCIREQ, Montreal, Canada), as previously described (32). After anesthesia with 35 mg/kg of ketamine and 6 mg/kg of xylazine, the trachea of the pups was surgically exposed. An 18-gauge metal needle was inserted in the trachea and connected to the ventilator with the following settings: 120 breaths/min at a tidal volume of 8 ml/kg. Thereafter, four respiratory manoeuvers were performed: a recruitment manoeuver (deep inflation), a pressure-volume loop (PVRV), a single-frequency forced oscillation manoeuver (Snapshot90v5.1), and a broadband forced oscillation manoeuver (Prime8). The mean of three consistent repetitions is reported for each parameter measured.

Morphometric assessment. After lung function testing, deeply anesthetized animals were euthanized by exsanguination. A thoracotomy was performed, and the lungs were removed "en bloc." Left lungs were fixed with 4% paraformaldehyde under a constant hydrostatic pressure of 25 cmH_2O for 24 h in the airway. Afterward, left lung volume was measured through water immersion (Scherle's principle) (18). Lungs were embedded in paraffin. A central sagittal cut per lung was stained with hematoxylin and eosin and Miller's elastic stain.

Lung sections were scanned with a slide scanner (AxioScan Slide Scanner; Zeiss, Oberkochen, Germany). Afterward, a purpose-designed Fiji plugin randomly selected fields of 500 $\times$ 500 μm. Lung morphometric measurements were semiautomatically performed with a self-designed Fiji plugin, based on overlapping a 64-point grid on 20 fields/lung. For each lung mean linear intercept, mean trans-sectional wall length, and parenchymal surface area corrected for body weight were calculated (18). The semiautomatic method was validated to manual counting for eight lungs using the STEPanizer tool (42). For vascular morphometry, at least 15 arteries with an external diameter of 30–100 μm were measured on Miller-stained lung sections to obtain the internal and external diameter of the tunica media (muscular

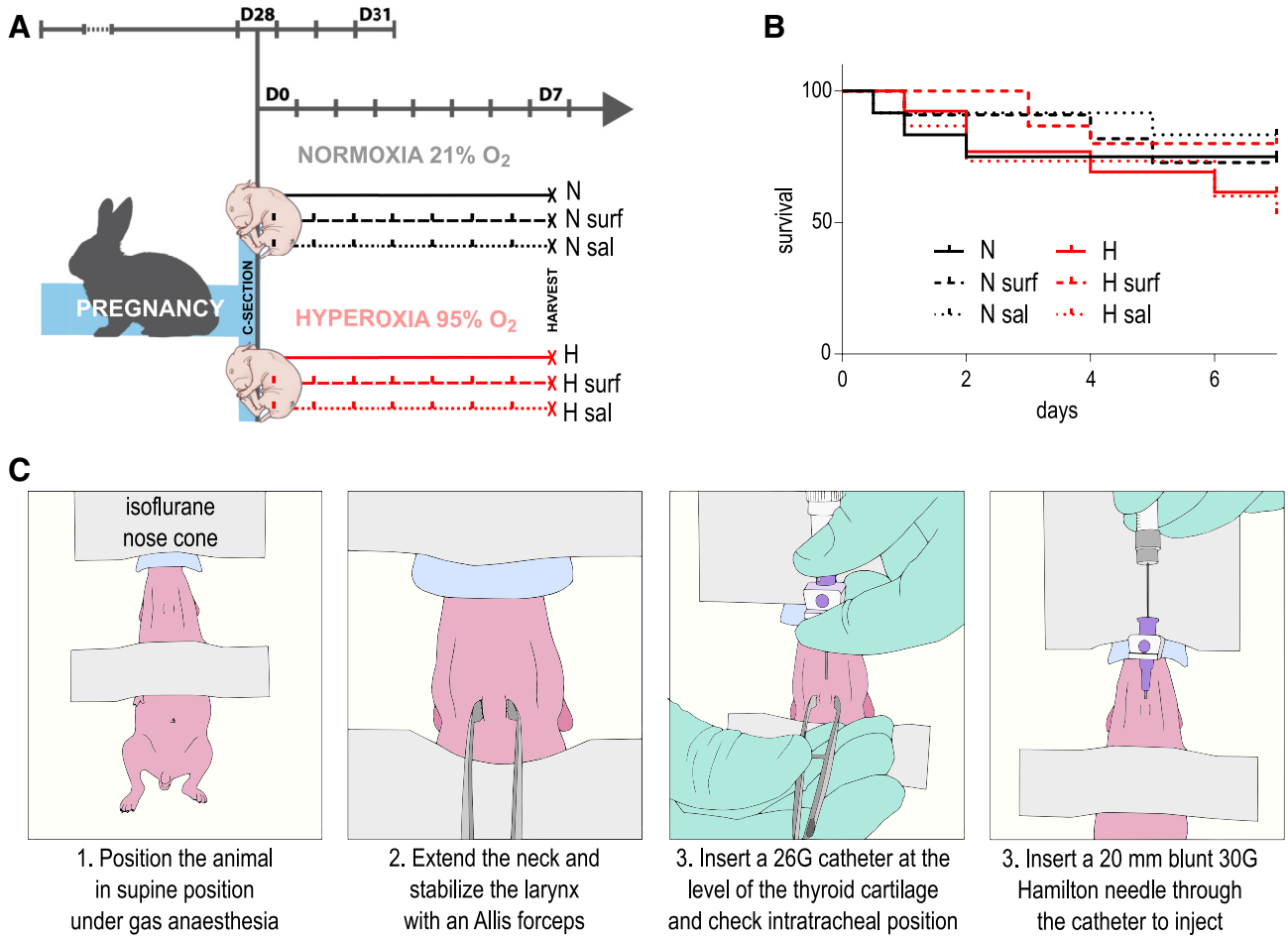

Fig. 1. Study design and survival. *A*: at 28 days of gestation, pups are delivered by cesarean section and randomized to 6 groups (n = 10–13). Three groups are raised in normoxia (21% oxygen): a control group (N, n = 11), a surfactant group (N surf, n = 10), and a saline group (N sal, n = 11) (both daily it administration). In parallel, 3 groups are raised in hyperoxia (>95% oxygen): control (H, n = 11), surfactant (H surf, n = 13), and saline (H sal, n = 13). All pups are harvested for functional and histological analysis at *day 7*. *B*: survival analysis does not reveal any differences between the study groups. *C*: illustration of the intratracheal injection strategy.

layer), to calculate medial thickness (MT%) as a ratio of the thickness of the tunica media to the vessel diameter (35).

Micro-PET-CT. Biodistribution of injected surfactant and saline was assessed with micro-PET-CT in a separate litter of six pups. Surfactant (100 mg/kg, 1.25 ml/kg) or equal volumes of saline were mixed with 2-deoxy-2-[^{18}F]fluoro-D-glucose ([^{18}F]FDG) (25 μCi, maximum 20% of the volume). Surfactant + [^{18}F]FDG (n = 3) or saline + [^{18}F]FDG (n = 3) was delivered intratracheally at *day 0* and *day 7*. PET-CT imaging was performed under isoflurane anesthesia (1.5–2% isoflurane in 100% oxygen) at both 15 and 150 min following intratracheal delivery (Fig. 4*A*). Each animal was fixed on an exchangeable Styrofoam bed in the prone position. First micro-PET was performed using a Concorde Focus 220 micro-PET (Siemens/Concorde Microsystems, Knoxville, TN) in a static protocol. Images were reconstructed with Fourier rebinning and 2D OSEM iterative reconstruction (ordered subsets expectation maximization, 16 subsets - 10 iterations) at zoom 2, resulting in a voxel size of 0.5 × 0.5 × 0.8 mm (40). Consequently, the Styrofoam bed was placed in a dedicated low-dose small-animal micro-CT scanner (SkyScan 1278; Bruker micro-CT, Kontich, Belgium) with the animal retained in the same

Table 1. *Survival and weight characteristics of included pups*

	N	H	H_{surf}	H_{sal}	N_{surf}	N_{sal}	*P* Value (ANOVA)
No. included	11	11	13	13	10	11	
Birth weight, g	38.7 (7.6)	36.8 (7.4)	36.9 (6.9)	35.3 (6.6)	34.5 (7.1)	34.0 (7.2)	0.64
No. survived, *n* (%)	9 (82)	8 (73)	11 (85)	8 (62)	7 (70)	9 (82)	
Birth weight, g	38.0 (6.1)	37.9 (3.9)	37.2 (5.4)	38.5 (5.3)	36.5 (6.6)	31.6 (4.5)	0.09
Body wt *day 7*, g	51.8 (9.7)	48.7 (5.1)	48.3 (9)	49.6 (6.6)	49.0 (8.6)	42.3 (7.6)	0.24
Relative weight gain, %	35 (7)	29 (8)	29 (12)	29 (9)	35 (9)	34 (12)	0.53

Values are means (SD); *n*, no. of animals. Shown are weights of the pups included in the experiment (n = 69) and of the pups harvested at *day 7* (n = 52). Data were analyzed with ANOVA. N, normoxia control group (21% oxygen); H, hyperoxia control group (≥95% oxygen); H_{surf}, hyperoxia group treated with daily it injections of 100 mg/kg of surfactant; H_{sal}, hyperoxia group treated with daily it injections of an equal volume of saline; N_{surf}, normoxia group treated with daily it injections of 100 mg/kg of surfactant; N_{sal}, normoxia group treated with daily it injections of an equal volume of saline.

position. Respiratory gated micro-CT images were acquired using the following parameters: 50 kV peak X-ray source voltage, 1 mm Al filter, 918 μA source current, 55 ms exposure time, 9 projection images/0.9° rotation step over a total angle of 180° in list mode and retrospectively gated. This resulted in four reconstructed 3D data sets with 50 μm isotropic reconstructed voxel size corresponding to four different phases of the breathing cycle (4D). Data reported here are at end of expiration. Software provided by the manufacturer (TSort, NRecon, DataViewer, TCONV, DicomCT, and CTan) was used to, respectively, gate, reconstruct, visualize, convert, process, and analyze μCT data (44). Fusion of the micro-PET and -CT images was done in PMOD 3.7, based on the position of four reference points on the animal bed. Consequently, lungs (right and left), upper airway, and stomach were manually delineated, and activity in these volumes of interest was expressed as percentage of total activity. For evaluation of internal distribution in the lungs, we defined the smallest possible volume containing 90% of the pulmonary activity, based on histograms, and expressed it as a fraction of the total delineated lung volume. Furthermore, the coefficient of variation (COV) for the activity of the voxels was calculated by dividing the SD by the average voxel activity.

Statistical analysis. All statistical analysis was performed in Prism (Graphpad Software, La Jolla, CA). A Grubbs' test with an α of 0.05 was used to identify outliers. For all lung function, morphometric, and vascular readouts, one-way ANOVA was used. With a Bonferroni-Sidak test for five comparisons, the uninjected hyperoxia group was compared with the uninjected normoxia group, and both injected groups were compared with the uninjected control group in the same condition (hyperoxia or normoxia). Survival was compared between groups with a log-rank (Mantel-Cox) test. Because the relative organ distribution of the PET-CT data is not normally distributed, nonparametric testing was performed (Wilcoxon signed-rank test, Kruskal-Wallis with Dunn multiple-comparison test). For the distribution of the activity within the lung, parametric tests were used (paired *t*-test and ANOVA with a Bonferroni-Sidak multiple-comparisons test).

RESULTS

Intratracheal injections do not increase mortality in preterm rabbit pups. A total of 98 pups were delivered from 9 different does. From these, 29 (30%) died during a 1-h adaptation period after respiratory distress symptoms or apnea. The remaining pups were randomized. Of them, 52 (75%) survived to harvest at 7 days. Baseline characteristics of the pups (birth weight, body weight) did not differ significantly between groups (Table 1). We did not observe any immediate mortality after intratracheal injections with surfactant or saline. Overall, survival was not significantly affected by surfactant or saline administration, or by hyperoxia exposure (Fig. 1*B*).

Intratracheal injections of surfactant or saline do not affect the development of the BPD phenotype. Seven days of hyperoxia affected inspiratory capacity, static elastance, dynamic compliance, and elastance, as well as tissue mechanics at forced oscillation (tissue damping and tissue elastance). No effect on airway resistance was observed. Intratracheal injections of surfactant or saline did not alter any of these parameters, neither in hyperoxia nor in normoxia groups (Table 2).

Hyperoxia exposure resulted in lower left lung volume at *day 7* (Fig. 2*B*). Morphometric assessment revealed a significantly increased mean transsectional wall length and a trend toward higher mean linear intercept, suggesting thicker septations and larger airspaces in the hyperoxia groups (Fig. 2, *A*, *C*, and *D*). In total, this resulted in a decreased parenchymal surface area corrected for body weight (Fig. 2*E*). The morphometric characteristics were not significantly influenced by intratracheal injections of surfactant or saline (Fig. 2).

A marked increase in pulmonary artery MT% was noted in the pups exposed to hyperoxia, reminiscent of pulmonary vascular disease in BPD. MT% was not significantly affected by daily intratracheal injections of surfactant or saline (Fig. 3).

Intratracheal injections result in high pulmonary deposition. PET-CT was used to evaluate the biodistribution of intratracheally delivered surfactant or saline (mixed with an [^{18}F]FDG radioisotope) in spontaneously breathing rabbit pups. The median fraction of radioactivity in the lungs was 78.3%, while 10.2% and 6.1% was detected in the stomach and upper airways, respectively (Fig. 4, *C* and *D*). No systemic absorption (activity in kidneys or bladder) was noted within the 150-min time frame. A significant, however small, decrease in the pulmonary fraction of activity occurred between the first scan, performed 10 min after injection, and a second scan at 150 min (median of 82.5 and 78.3%, respectively, $P = 0.02$; Fig. 4*D*). At both time points the pulmonary fraction did not differ significantly between injections with surfactant or saline at *day 0* or *7*.

About two-thirds of the pulmonary fraction (64.8 ± 15.7%) was located in the right lung, which is in line with the larger

Table 2. *Lung function readouts.*

	N	H	H_{surf}	H_{sal}	N_{surf}	N_{sal}
PV loop						
Inspiratory capacity, ml/kg	63.9 (14.3)	40.4 (6.0)*	43.6 (14.8)	48.6 (12.9)	66.5 (11.8)	72.2 (26.3)
Static compliance, $ml \cdot cmH_2O^{-1} \cdot kg^{-1}$	2.1 (0.4)	1.1 (0.3)	1.5 (1.1	1.3 (0.6)	2.8 (1.4	3.4 (2.7)
Static elastance, $cmH_2O \cdot kg^{-1} \cdot ml^{-1}$	0.47 (0.14)	1.06 (0.42)*	0.94 (0.75)	0.73 (0.24)	0.42 (0.17)	0.39 (0.22)
Single frequency oscillation						
Resistance, $cmH_2O \cdot s^{-1} \cdot ml^{-1}$	0.31 (0.07)	0.47 (0.11)	0.46 (0.12)	0.56 (0.33)	0.25 (0.08)	0.31 (0.12)
Dynamic compliance, $mL \cdot cmH_2O^{-1} \cdot kg^{-1}$	3.4 (0.8)	1.5 (0.6)*	1.9 (0.9)	1.8 (1.0)	3.6 (1.0)	4.0 (1.4)
Dynamic elastance, $cmH_2O \cdot kg^{-1} \cdot ml^{-1}$	0.31 (0.06)	0.63 (0.21)*	0.57 (0.36)	0.48 (0.15)	0.29 (0.08)	0.27 (0.08)
Forced oscillation						
Airway resistance, $cmH_2O \cdot s^{-1} \cdot ml^{-1}$	0.12 (0.05)	0.15 (0.06)	0.16 (0.08)	0.16 (0.06)	0.08 (0.04)	0.11 (0.08)
Tissue damping, cmH_2O/ml	1.6 (0.2)	2.5 (0.6)*	2.2 (0.5)	2.1 (0.3)	1.6 (0.5)	1.6 (0.2)
Tissue elastance, cmH_2O/ml	6.0 (1.1)	11.5 (3.8)*	9.8 (3.9)	8.4 (2.0)	6.0 (1.8)	6.0 (0.8)

Values are means (SD). N, normoxia control group (21% oxygen); H, hyperoxia control group (≥95% oxygen); H_{surf}, hyperoxia group treated with daily it injections of 100 mg/kg of surfactant; H_{sa}l, hyperoxia group treated with daily it injections of an equal volume of saline; N_{surf}, normoxia group treated with daily it injections of 100 mg/kg of surfactant; N_{sal}, normoxia group treated with daily it injections of an equal volume of saline. Hyperoxia controls are compared with normoxia controls; both saline- and surfactant-injected groups are compared with the controls in the same condition (hyperoxia or normoxia) (n = 7–11): *$P < 0.05$, adjusted for multiple comparisons according to Bonferroni-Sidak.

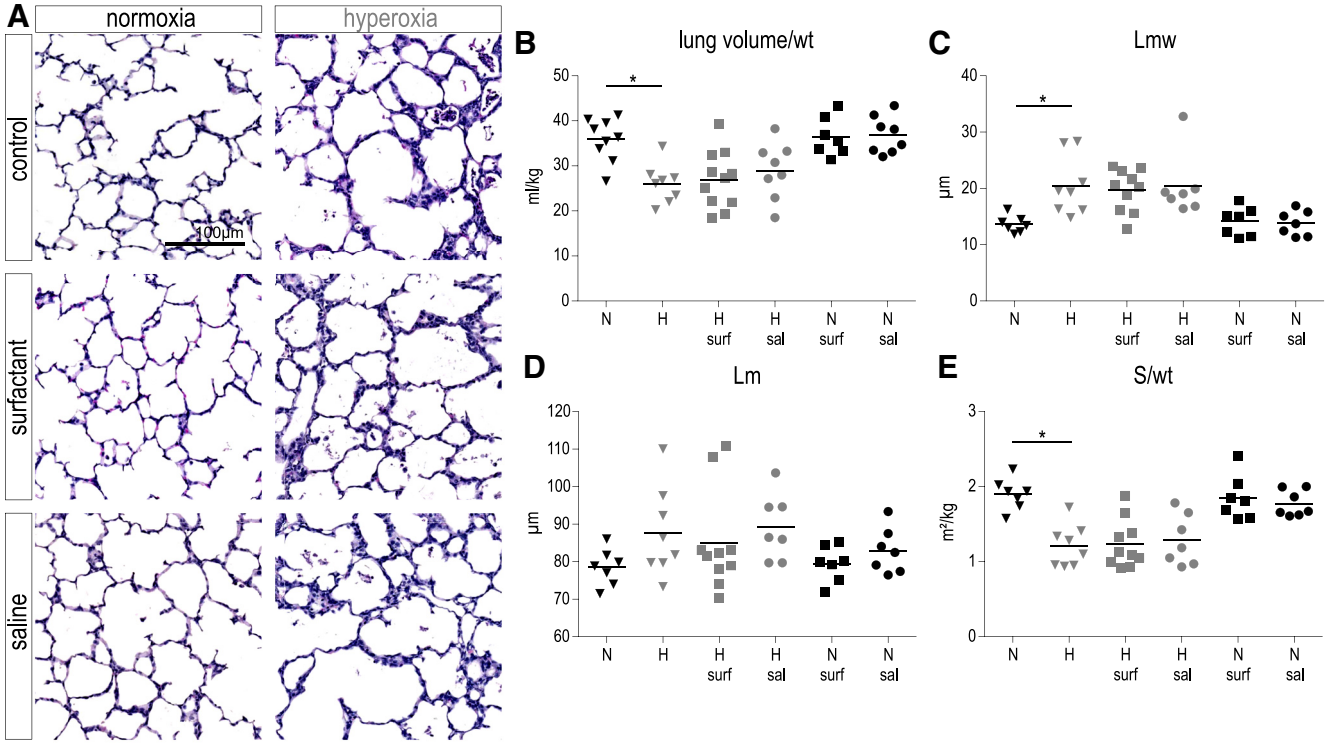

Fig. 2. Lung volume and alveolar morphometry. *A*: representative images of hematoxylin and eosin-stained lung slides. *B*: left lung volume as measured by water immersion is decreased in animals exposed to hyperoxia and unaffected by daily surfactant or saline it administration (n = 7–11). *C*: mean transactional wall length (L_{mw}) is increased in animals exposed to hyperoxia, suggesting thicker alveolar walls, but is unaffected by daily surfactant or saline it administration (n = 7–10). *D*: mean linear intercept (L_m) tends to increase in hyperoxia, suggesting larger airspaces, but is unaffected by daily surfactant or saline it administration (n = 7–10). *E*: parenchymal surface area is decreased in hyperoxia but unaffected by daily surfactant or saline it administration (n = 7–10). *$P < 0.05$ (significance level adjusted for multiple comparisons according to Bonferroni-Sidak).

volume of the right lung (58.3 ± 5.3%; Fig. 4*B*). There was no significant difference in activity between either lung, when corrected for total lung volume (P = 0.11). Right-to-left lung distribution did not change between the first scan at 10 min or the second scan at 150 min (P = 0.37).

On average, we observed that 90% of the activity was present in 44.6 ± 5.9% of the lung volume. This corresponds to a coefficient of variation of 1.38 ± 0.22. Visual inspection of the images suggests a rather central than peripheral deposition in all animals (Fig. 4*C*). The internal lung distribution was not significantly changed between the first and the second scans (43.9 ± 6.3 and 45.2 ± 5.7%, respectively, P = 0.38). Also, for internal distribution, no significant differences were noted between injections with surfactant or saline at *day 0* or *7* (Fig. 4*E*). However, when data from the surfactant and saline groups were pooled, a significantly more homogenous distribution of the activity was noted at *day 0* compared with *day 7* (47.8 ± 5.0 and 39.2 ± 4.1%, respectively, P = 0.01).

DISCUSSION

From a clinical and research perspective, the interest in local pulmonary delivery of molecules to promote the long-term respiratory outcome in preterm neonates is increasing. The

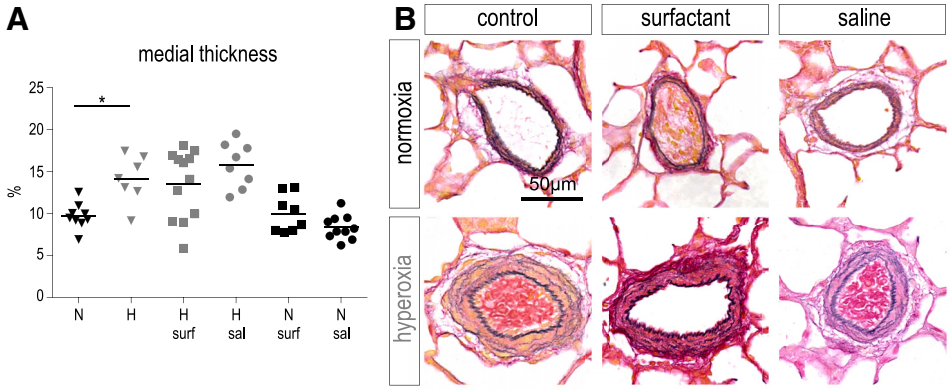

Fig. 3. Vascular morphometry. *A*: medial thickness, or thickness of the tunica media corrected for vessel diameter is increased by hyperoxia but remains unaffected by daily surfactant or saline it administration (n = 7–11). *B*: representative images of lung arteries on Miller-stained lung sections. *$P < 0.05$ (significance level adjusted for multiple comparisons according to Bonferroni-Sidak).

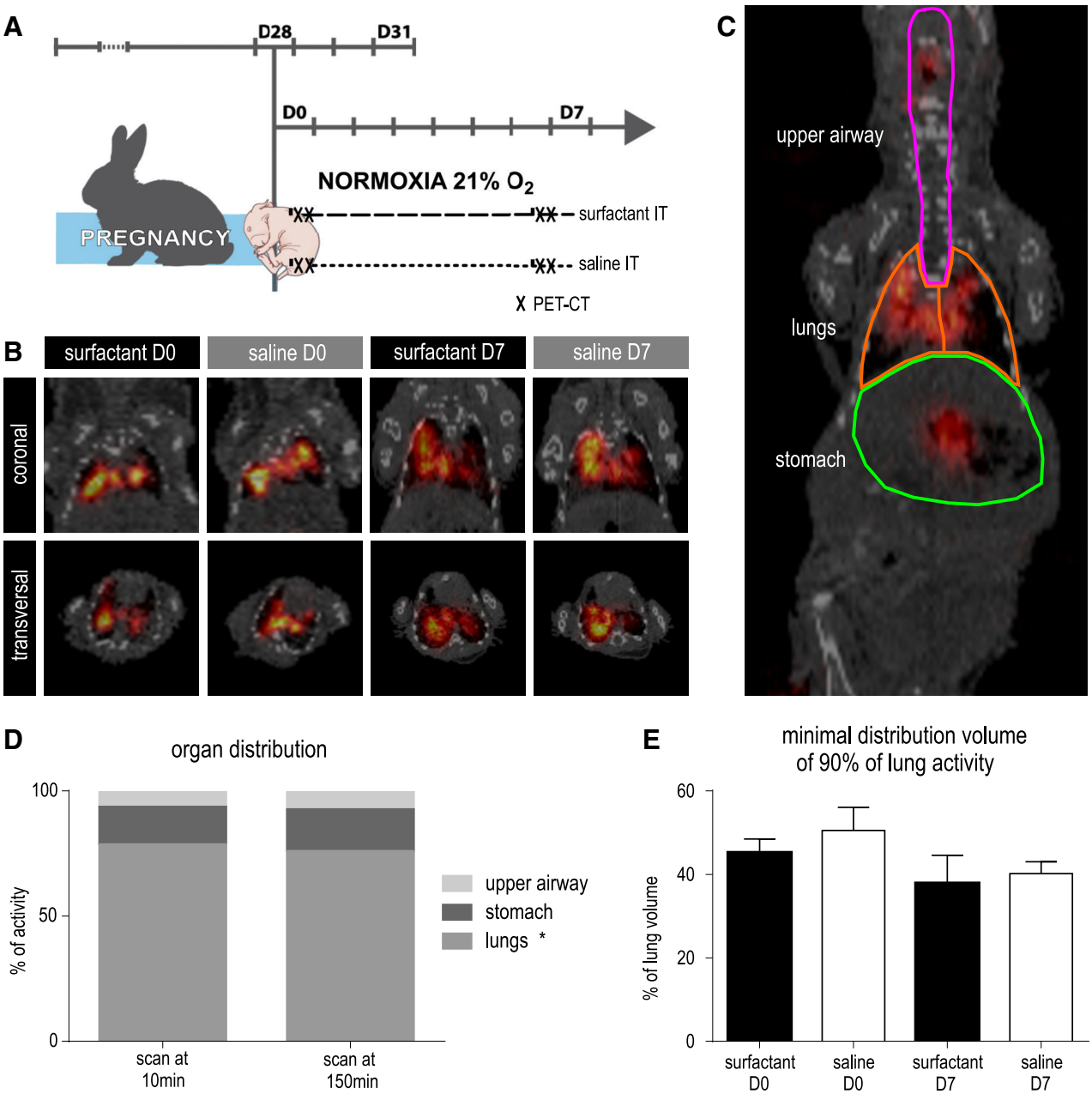

Fig. 4. PET-CT distribution data. *A*: a separate litter was delivered at 28 days of gestation (by cesarean section), housed in normoxia, and randomized to either it injections with surfactant + 2-deoxy-2-[^{18}F]fluoro-D-glucose ([^{18}F]FDG) or saline + [^{18}F]FDG (n = 3). Injections were performed at both *day 0* and *day 7*, followed by a PET-CT scan after 10 and 150 min. *B*: representative image of a full-body coronal section. Manually, the different volumes of interest containing [^{18}F]FDG activity (upper airway, right and left lung, stomach) were delineated on all scans. *C*: representative lung images of pups in both groups at *day 0* and *day 7*, taken at 10 min after injection, suggesting an unequal and predominantly central distribution in all groups. *D*: organ distribution of the injected activity in all groups (n = 12), 10 and 150 min after injection. We observe a significant but small relative decrease in lung activity (82.5 to 78.3%): *P = 0.03 (nonparametric testing, adjusted for multiple comparisons according to Dunn). *E*: internal distribution within the lung. Graph indicates the minimal relative lung volume containing 90% of the total activity at 10 min after injection. In perfectly homogenous distributions, this would be 90% of the volume. No differences between individual groups (n = 3) but, when pooled, higher at *day 0* compared with *day 7* (n = 6, P = 0.01).

current study validates the preterm rabbit model for research on this emerging topic. This small animal model combines prematurity and oxygen toxicity and results in a structural and functional phenotype mimicking human BPD (36). Until now, a preclinical evaluation of surfactant-compound mixtures would only have been possible in larger animal models like adult rabbits, preterm lambs, or baboons, at a higher financial and ethical cost (1, 29, 47). The combination of prematurity in a small animal model with tracheal access, as described in this paper, opens opportunities to investigate novel intratracheal therapeutics. The preterm rabbit obviously also has some limitations such as the limited availability of commercial reagents and antibodies for the explorative analysis of rabbit tissue. Additionally, there is no reliable visible assessment of male or female phenotype, making it difficult to evaluate the role of gender on preterm lung disease and therapy. Also, this

model cannot replace experiments in the more human-like ventilated or noninvasively supported large animal models. A final disadvantage of the model is the need for a transcutaneous puncture, since intubation through the normal route is technically very challenging.

This study provides arguments for the use of surfactant as a safe and efficient carrier for drugs targeting the neonatal lung. Because the large majority of preterm infants at risk of BPD are anyway receiving surfactant for RDS at birth, the addition of a therapeutic molecule in the surfactant emulsion to improve long-term respiratory outcome is a promising strategy. This study was set up to evaluate the feasibility, safety, and distribution of daily intratracheal administration of surfactant, as a potential drug carrier, in the preterm rabbit model.

A first essential prerequisite for a drug carrier is the absence of a noxious effect. The direct effect of surfactant on chronic lung disease has been a matter of debate for many years. The introduction of surfactant supplementation in the care of extreme preterm babies has been associated with higher rates of BPD (37, 39), a finding that is generally explained by the survival of infants with more immature lungs. Furthermore, in prophylactically treated infants, increased incidences of BPD or death have been observed compared with infants treated in a rescue strategy (34). Additionally researchers have raised concerns on the potential acute side effects of surfactant administration, such as ventilation disturbances in formerly normally functioning lung regions (4). On the other hand, long-term beneficial effects of repetitive instillations of surfactant have been described in ventilated infants [TOLSURF and CURDYS trials (15, 24)]. The rationale is that exogenous surfactant is necessary to overcome endogenous surfactant inactivation by oxidation and protein leak due to respiratory support.

Our study suggests in a relevant (nonventilated) preterm animal that repetitive surfactant administration has no direct effect on the evolution toward chronic lung disease. Despite older data suggesting surfactant inhibition in neonatal rabbits exposed to hyperoxia (5), daily exogenous surfactant did not result in in vivo improvement in lung function in this model. Surfactant administration neither altered the alveolar nor vascular architecture.

We did not investigate the acute effects of surfactant administration on a possible RDS phenotype, but earlier work has shown beneficial effects on the overall lung mechanics of preterm rabbits (30). The increased mortality in our optimization experiments, however, illustrates that surfactant should be used with caution. The absence of any procedural mortality in the final pressureless method demonstrates the importance of the administration strategy.

A second prerequisite for a good drug carrier is an adequate distribution to and in the target organ, in this case the lung. With the use of the technique described above, the vast majority of the injected activity reached the lungs (82.5%) and stayed there at least 150 min after injection (78.3%). The slight but significant decrease possibly reflects a small esophageal fraction that is impossible to distinguish from the pulmonary fraction and that descends to the stomach at the second time point. This study suggests that tracheal administration of drugs or surfactant, even in the absence of a closed ventilation system and positive pressure, leads to a very high pulmonary deposition, with minimal loss. This is a clear advantage over inhalation strategies in neonates, where low lung deposition and high loss (buccal mucosa, stomach, face, and device; up to 2%) have been observed (12). Intratracheal administration thus seems to result in less systemic exposure, limiting the potential for side effects.

We conclude that the internal distribution within the lung is acceptable, even if it is not perfect. A perfectly homogenous distribution would result in 90% of the activity present in 90% of the lung volume, while in our study this amount is present in ~45% of the lung volume. Previous studies did also show a patchy or rather central distribution of surfactant after intratracheal delivery, which is comparable to our data (6, 11, 43). Because these studies used less precise quantification methods, it is, however, impossible to exactly benchmark our findings.

From a drug delivery perspective, a more homogenous distribution might be desirable; however, established pulmonary drug delivery strategies, such as inhalation, have been proven to be efficient, despite comparable imperfect distribution (3, 12). Furthermore, the need for anesthesia during the PET-CTs might have negatively influenced the distribution of the tracer through hypoventilation. If allowed a longer recovery period, distribution toward the periphery of the lung might improve; however, this could not be tested because of the short half-life of [^{18}F]FDG. Additionally, in our study, repetitive injections (on *day 0* and *day 7*) in the same animal resulted in different distribution patterns, increasing the total amount of lung tissue exposed. Finally, it should be noted as a limitation that this proof of concept study uses [^{18}F]FDG, since the behavior of this tracer molecule does not necessarily reflect the biophysical and biological behavior of a given investigational drug (e.g., interaction with surfactant, viscosity in solution, and eventual uptake by the epithelium).

The rationale to use surfactant as a carrier is the idea that it would act synergistically by improving the internal distribution of a drug within the lung. In this study, we did not see any difference in intrapulmonary distribution of [^{18}F]FDG between the groups in which surfactant or saline was used as a carrier. We have to acknowledge that our study was not powered to perform this comparison ($n = 3$). We did see a trend toward improved distribution on *day 0* versus *day 7* in both the saline- and surfactant-treated animals. This is in line with previous observations in a sheep model that surfactant distribution is better in fluid-filled lungs (23).

Our data are in line with observations from Fajardo et al. in surfactant-depleted mechanically ventilated adult rabbits. They reported a comparable central distribution of radioactively labeled budesonide, not affected by surfactant (11). On the other hand, Huang et al. report on improved distribution of a fluorescent dye after intratracheal injection in a surfactant mixture in spontaneously breathing adult mice, most likely by altering the biophysical characteristics of the injected liquid (19). This discrepancy nicely illustrates that the effect of surfactant on the distribution of a drug will depend, like mentioned above, on the biophysical and biological properties of the specific drug.

In conclusion, we developed a model that allows the preclinical evaluation of intratracheal therapeutics in a BPD context. Here, we used this model to mimic intratracheal surfactant administration, a common clinical procedure, from bedside to bench. In our experiments, we provided support for the safety and efficacy of surfactant as a drug carrier, since its use *1*) did

not result in noxious effects on the lung and *2*) resulted in high pulmonary deposition with an acceptable internal distribution. Future perspectives include the use of this model and methods to translate innovative localized treatment strategies for BPD from the bench toward bedside.

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AUTHOR CONTRIBUTIONS

T.S., O.G., G.V.V., and J.T. conceived and designed research; T.S., A.G., J.J., and M.A. performed experiments; T.S., M.A., and M.K. analyzed data; T.S., A.G., C.C., F.S., and J.T. interpreted results of experiments; T.S. prepared figures; T.S. and J.T. drafted manuscript; T.S., A.G., J.J., G.V.V., C.C., F.R., F.S., K.A., J.D., and J.T. edited and revised manuscript; T.S., A.G., J.J., M.A., O.G., G.V.V., M.K., X.M., C.C., F.R., F.S., G.V., K.A., J.D., and J.T. approved final version of manuscript.

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