LOCAL PULMONARY DRUG DELIVERY IN THE PRETERM RABBIT: FEASIBILITY AND EFFICACY OF DAILY INTRATRACHEAL INJECTIONS

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Intratracheal injections in the preterm rabbit

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ABSTRACT

Recent clinical trials in newborns have successfully used surfactant as a drug carrier for an active compound, in order to minimize systemic exposure. To investigate the translational potential of surfactant-compound mixtures and other local therapeutics, a relevant animal model is required where intratracheal (IT) 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 IT surfactant, daily IT 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 microPET-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 BPD. Neither IT 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 radio-active tracer goes and remains into the lungs, with a decrease of only 4% after 150 minutes. Surfactant and saline can safely and effectively be administered in spontaneously breathing preterm rabbits. The described model and method enable researchers to evaluate IT pharmacological interventions for the treatment of BPD.

KEY WORDS

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

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 weeks' postmenstrual age or at discharge, BPD still occurs in about 45% of survivors of extremely preterm birth (<28 weeks 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 weeks. However, significant adverse events have been reported, including gastro-intestinal 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 intra-tracheal (IT) 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, as 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 the 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 rats models are not preterm and do not allow easy tracheal access due to their small size, while lambs are very expensive and the use of baboons is ethically questioned. The preterm rabbit model could be an advantageous alternative as 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, IT injections on spontaneously-breathing preterm rabbits. We evaluated the feasibility and safety of daily IT 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 IT administration.

METHODS

Cesarean Section

Time-mated pregnant rabbits (New Zealand White and Dendermonde cross-breed) were provided through the animal facilityof KU Leuven. All experiments were approved by the Ethics Committee for Animal Experimentation (project numbers 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 BV, Bladel, The Netherlands) and 6 mg/kg of xylazin (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 (intravenous bolus of 1 mL T61®; Intervet International BV, Boxmeer, The Netherlands). Immediately afterwards 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, Pomona, US). Neonatal rabbit care has been described before (22, 32). Briefly, pups were fed twice daily via an orogastric tube with increasing quantities of a milk replacer (FoxValley 30/50, Arizona, USA, containing 10g/100ml fat, 6g/100ml protein and <1g/100ml carbohydrates), supplemented with vitamins and probiotics (6g/100ml Biolapis, Protexin, Somerset, UK) and immunoglobulins on the first 2 days of life (4g/100ml Col-o-Cat, Sanobest, ‘s Hertogenbosch, The Netherlands). Furthermore, intramuscular vitamin K on D2 (0.002 mg/kg Konakion pediatric, Roche, Belgium) and antibiotics from D2-D7 (benzylpenicillin (20,000 I.U./kg Penicilline, Kela, Sint-Niklaas, Belgium) and amikacin (20 mg/kg, Amukin, Bristol-Myers Squibb, New York, US)) were administered. The pups remained in the incubator for 7 days except for the feeding and interventions.

Randomization

After an initial 1-hour adaptation period, surviving pups were weighed, numbered, and randomly assigned to one of six different groups, ensuring an equal distribution within nests: 1) normoxia control group (21% oxygen; N; n = 11), 2) hyperoxia control group (≥95% oxygen; H, n = 11), 3) hyperoxia group treated with daily intratracheal (IT) injections of 100mg/kg of surfactant (Curosurf®, Chiesi Farmaceutici, Parma, Italy; Hsurf; n = 13), 4) hyperoxia group treated with daily IT injections of an equal volume of saline (Hsal; n = 13), 5) normoxia group treated with daily IT injections of 100mg/kg of surfactant (Nsurf; n = 10) and 6) normoxia group treated with daily IT injections of an equal volume of saline (Nsal; n = 11) as visualized in figure 1a. 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, 8 pups in each group were needed. In order 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 isoflurane 2.5% (ISO-VET®, EuroVet, Heusden-Zolder, Belgium) in 2 L 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), however these attempts failed in neonates because of the small size and long curvature of the rabbit snout. 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 G catheter (Neoflon®, BD, New Jersey, US). Correct insertion of the catheter was checked by water valve in the transparent catheter. Subsequently either 1.25 mL/kg of surfactant or saline was slowly injected with a 30 G blunt Hamilton needle inserted through the catheter (technique visualized in figure 1c). In post mortem test pups this technique resulted in delivery above the carina. A gentle position check and instillation

was used, as 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 0,5ml 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 6mg/kg of xylazin, the trachea of the pups was surgically exposed. An 18G metal needle was inserted in the trachea and connected to the ventilator with the following settings: 120 breaths/min at a tidal volume of 8mL/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 3 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 24h in the airway. Afterwards, 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 (HE) and Miller´s elastic stain.

Lung sections were scanned with a slide scanner (AxioScan® Slide Scanner, Zeiss, Oberkochen, Germany). Afterwards, a purpose-designed Fiji-plug-in randomly selected fields of 500x500 µm. Lung morphometric measurements were semi-automatically performed with a self-designed Fiji-plugin, based on overlapping a 64-point grid on 20 fields per lung. For each lung mean linear intercept (Lm),

mean trans-sectional wall length (Lmw) and parenchymal surface area (S) corrected for body weight were calculated (18). The semi-automatic method was validated to manual counting for 8 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 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 6 pups. Surfactant (100mg/kg, 1.25 mL/kg) or equal volumes of saline were mixed with 2-deoxy-2-(18F)fluoro-D-glucose (^{18}F-FDG) (25µCi, max 20% of the volume). Surfactant+^{18}F-FDG (n=3) or saline+^{18}F-FDG (n=3) was delivered IT 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 150min following IT delivery (figure 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, USA), 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 0,5x0,5x0,8mm (40). Consequently, the Styrofoam bed was placed in a dedicated low-dose small-animal micro-CT scanner (SkyScan 1278, Bruker micro-CT, Kontich, Belgium) while the animal remained in the same position. Respiratory gated micro-CT images were acquired using the following parameters: 50 kVp X-ray source voltage, 1 mm Al filter, 918 µA source current, 55 ms exposure time, 9 projection images per 0.9° rotation step over a total angle of 180° in list mode and retrospectively gated. This resulted in four reconstructed 3D datasets with 50 µm isotropic reconstructed voxel size corresponding to four different phases of the breathing cycle (4D). Data reported here is 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 4 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 standard deviation by the average voxel activity.

Statistical analysis

All statistical analysis was performed in Prism (Graphpad Software, La Jolla, CA, USA). A Grubbs' test with an α of 0.05 was used to identify outliers. For all lung function, morphometric and vascular read-outs, one-way ANOVA was used. With a Bonferroni-Sidak test for 5 comparisons, the uninjected hyperoxia-group was compared to the uninjected normoxia-group, and both injected groups were compared to the uninjected control group in the same condition (hyperoxia or normoxia). Survival was compared between groups with a log-rank (Mantel-Cox) test. As the relative organ distribution of the PET-CT data is not normally distributed, non-parametric testing was performed (Wilcoxon signed rank test, Kruskal-Wallis with Dunn multiple comparisons 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

IT 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-hour 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 (birthweight, body weight) did not differ significantly between groups (table 1). We did not observe any immediate mortality after IT injections with surfactant or saline. Overall survival was not significantly affected by surfactant or saline administration, or by hyperoxia exposure (figure 1b).

IT 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. IT 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 (figure 2b). Morphometric assessment revealed a significantly increased mean transsectional wall length (Lmw) and a trend towards higher mean linear intercept (Lm), suggesting thicker septations and larger airspaces in the hyperoxia groups (figure 2a, 2c and 2d). In total, this resulted in a decreased parenchymal surface area (S) corrected for body weight (figure 2e). The morphometric characteristics were not significantly influenced by IT injections of surfactant or saline (figure 2).

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

IT injections result in high pulmonary deposition

PET-CT was used to evaluate the bio-distribution of IT-delivered surfactant or saline (mixed with an ^{18}F-FDG radio-isotope) in spontaneously breathing rabbit pups. The median fraction of radio-activity in the lungs was 78.3%, while 10.2% and 6.1% was detected in the stomach and upper airways respectively (figure 4c and 4d). No systemic absorption (activity in kidneys or bladder) was noted within the 150min 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% to 78.3%, p=0.02, figure 4d). 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 volume of the right lung (58.3%±5.3%, figure 4b). There was no significant difference in activity between both lungs, when corrected for total lung volume (p=0.11). Right-to-left lung distribution did not change between the first scan at 10 min and 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 (figure 4c). 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 (figure 4e). 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 to 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 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 non-invasively supported large animal models. A final disadvantage of the model is the need for a transcutaneous puncture, as 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. As 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 IT 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

244 rates of BPD (37, 39), a finding that is generally explained by the survival of infants with more
245 immature lungs. Furthermore in prophylactically treated infants increased incidences of BPD or
246 death have been observed, compared with infants treated in a rescue strategy (34). Additionally
247 researchers have raised concerns on the potential acute side effects of surfactant administration,
248 such as ventilation disturbances in formerly normally functioning lung regions (4). On the other hand
249 long term beneficial effects of repetitive instillations of surfactant have been described in ventilated
250 infants (TOLSURF and CURDYS trials: (15, 24)). The rationale is that exogenous surfactant is necessary
251 to overcome endogenous surfactant inactivation by oxidation and protein leak due to respiratory
252 support.

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

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

263 A **second** prerequisite for a good drug carrier is an adequate distribution to and in the target organ,
264 in this case the lung. Using the technique described above, the vast majority of the injected activity
265 reached the lungs (82.5%) and stayed there at least 150 minutes after injection (78.3%). The slight,
266 but significant decrease possibly reflects a small esophageal fraction that is impossible to distinguish
267 from the pulmonary fraction, and that descends to the stomach at the second time point. This study
268 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%) has been observed (12). IT 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 about 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). As 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 towards 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, as 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 towards 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 radio-actively labeled budesonide, not affected by surfactant (11). On the other hand, Huang et al. report on improved distribution of a fluorescent dye after IT 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 IT 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 as 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 towards bedside.

ACKNOWLEDGEMENTS

We would like to thank Katrien Luyten and Jens Wouters for their technical assistance with the histology and micro PET-CT respectively.

GRANTS

Thomas Salaets is partially funded by the SAFEPEDRUG project (supported by the agency for innovation by Science and Technology in Flanders (IWT) SBO 130033). André Gie and Julio Jimenez are supported by the Erasmus+ Programme of the European Commission (2013-0040). Jan Deprest is partly funded by the Great Ormond Street Hospital Charity Fund. Greetje Vande Velde has received funding from KU Leuven (IF STG/15/024 en C24/17/061), and Fonds voor Wetenschappelijk Onderzoek (G.0691.15N).

DISCLOSURES

This study was funded by Chiesi Farmaceutici S.p.A. (Parma, Italy), which is the employer of authors CC, FR, FS, GV. The animal-derived surfactant Poractant alfa (Curosurf, 80 mg/ml) was also supplied by Chiesi Farmaceutici S.p.A. XM served as consultant for Chiesi Farmaceutici S.p.A. in this study.

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FIGURES

Figure 1. Study design and survival.

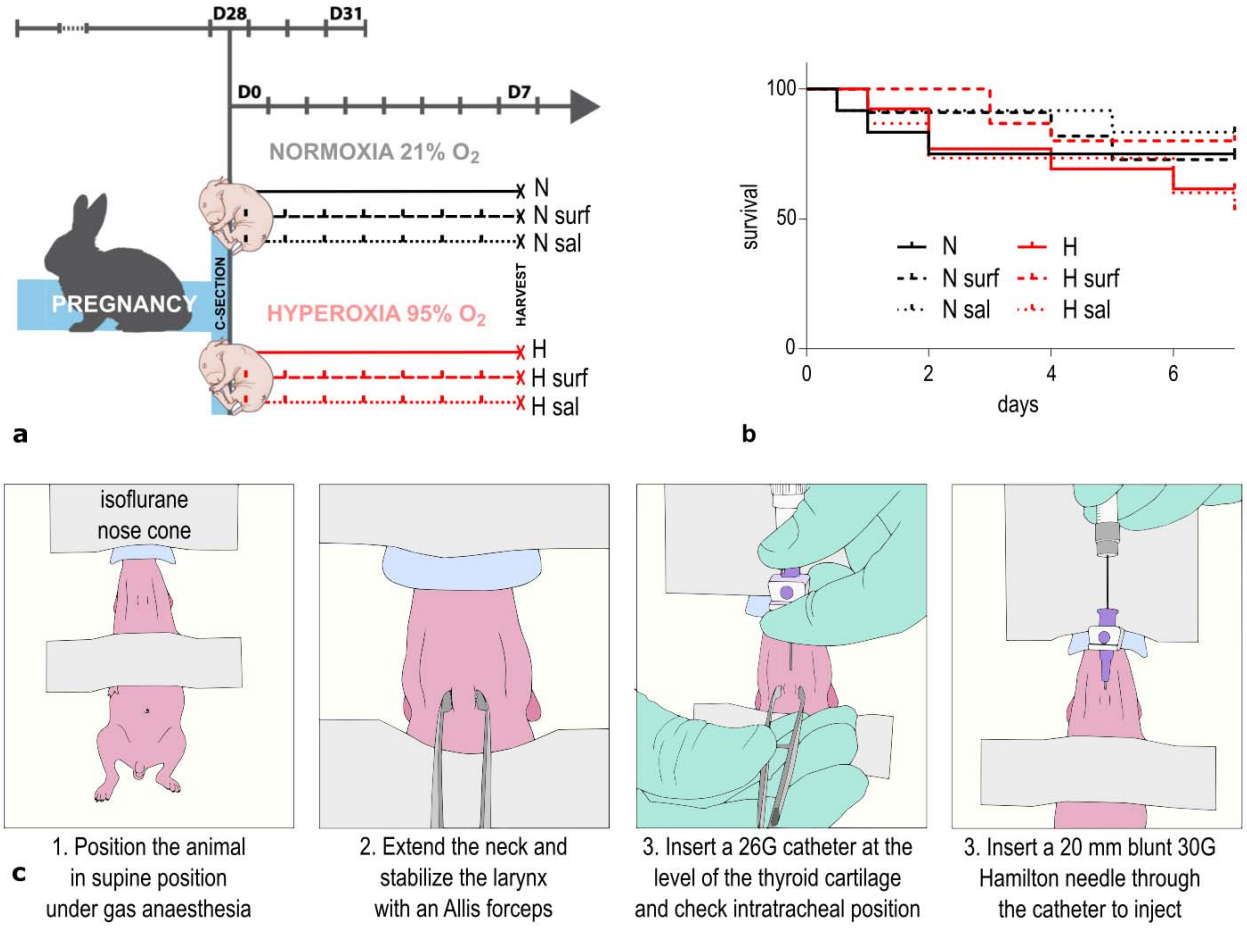


Figure 2. Lung volume and alveolar morphometry.

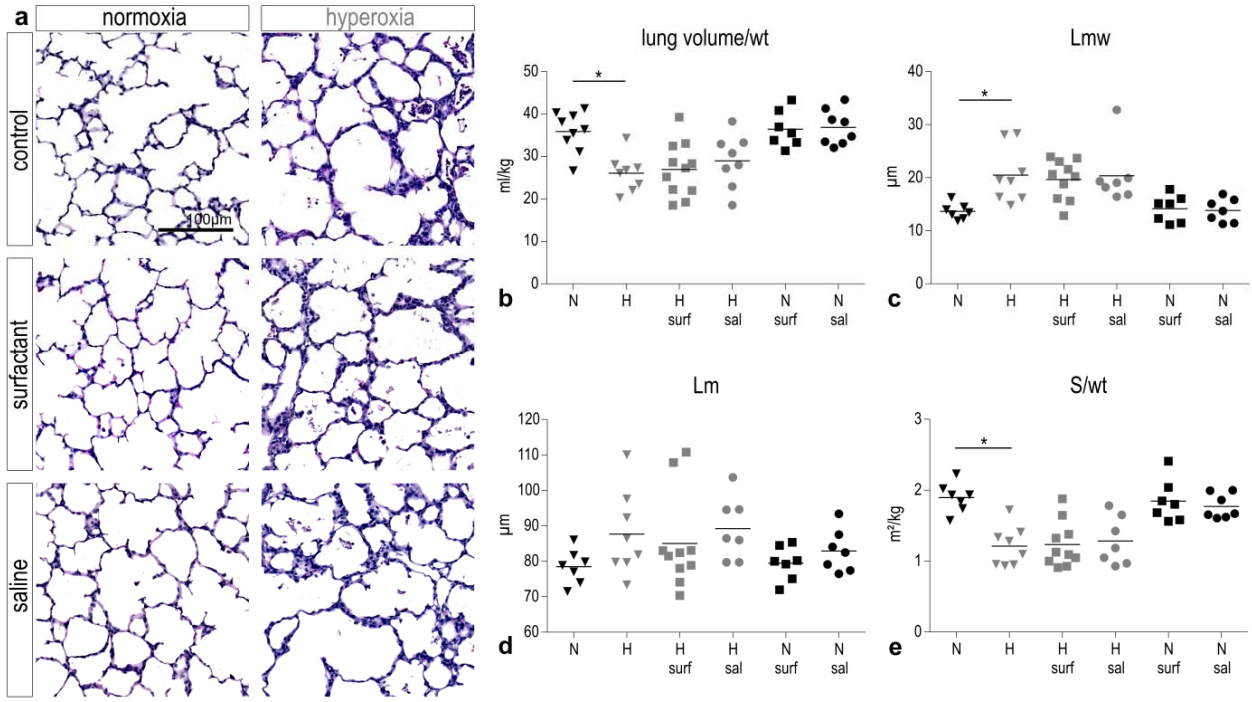


Figure 3. Vascular morphometry.

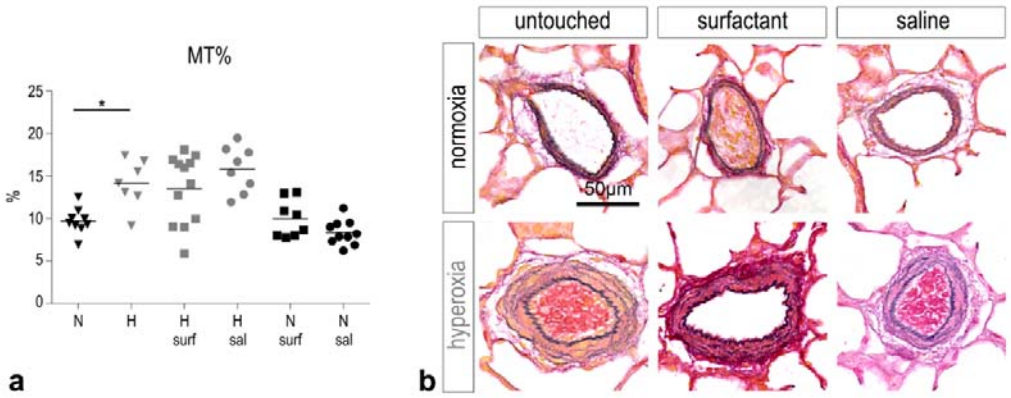


Figure 4. PET-CT distribution data.

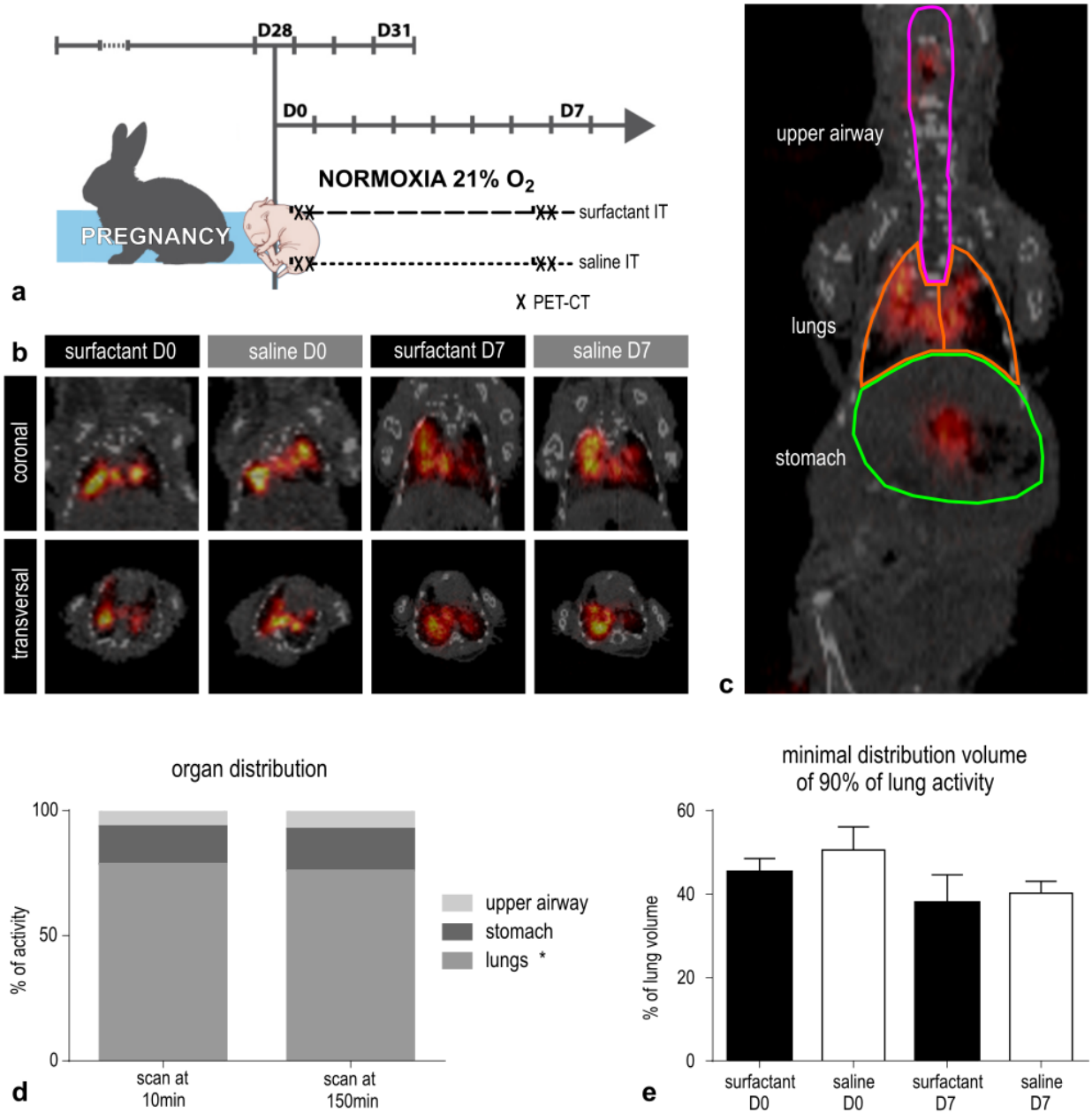


FIGURE LEGENDS

Figure 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

Figure 2. Lung volume and alveolar morphometry.

(A) Representative pictures of HE-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) Lmw (mean transactional wall length) 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) Lm (mean linear intercept) 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)*

Figure 3. Vascular morphometry.

(A) MT% (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 pictures of lung arteries on Miller-stained lung sections.

**p<0,05 (significance level adjusted for multiple comparisons according to Bonferroni-Sidak)*

Figure 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 + ^{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 to day 7 (n=6, p=0.01).

TABLES

Table 1. Survival and weight characteristics of included pups

	N	**H**	**H surf**	**H sal**	**N surf**	**N sal**	*p-value (ANOVA)*
Number 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*
Number survived	**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 weight 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*

Table 2. Lung function read-outs.

	N	H	H surf	H sal	N surf	N sal
PV-loop						
Vend/wt - 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
Cst - Static compliance (mL/cmH2O.kg)	2.1±0.4	1.1±0.3	1.5±1.1	1.3±0.6	2.8±1.4	3.4±2.7
Est - Static elastance (cmH2O.kg/mL)	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						
R - Resistance (cmH2O.s/mL)	0.31±0.07	0.47±0.11	0.46±0.12	0.56±0.33	0.25±0.08	0.31±0.12
C - Dynamic compliance (mL/cmH2O.kg)	3.4±0.8	1.5±0.6*	1.9±0.9	1.8±1.0	3.6±1.0	4.0±1.4
E - Dynamic elastance (cmH2O.kg/mL)	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						
Rn - Airway resistance (cmH2O.s/mL)	0.12±0.05	0.15±0.06	0.16±0.08	0.16±0.06	0.08±0.04	0.11±0.08
G - Tissue damping (cmH2O/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
H - Tissue elastance (cmH2O/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

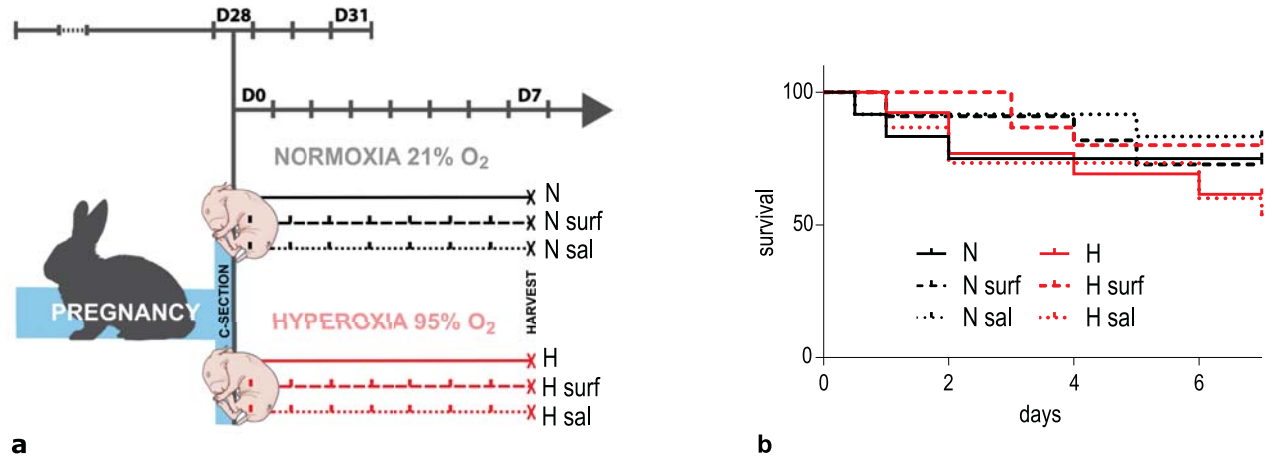
TABLE LEGENDS

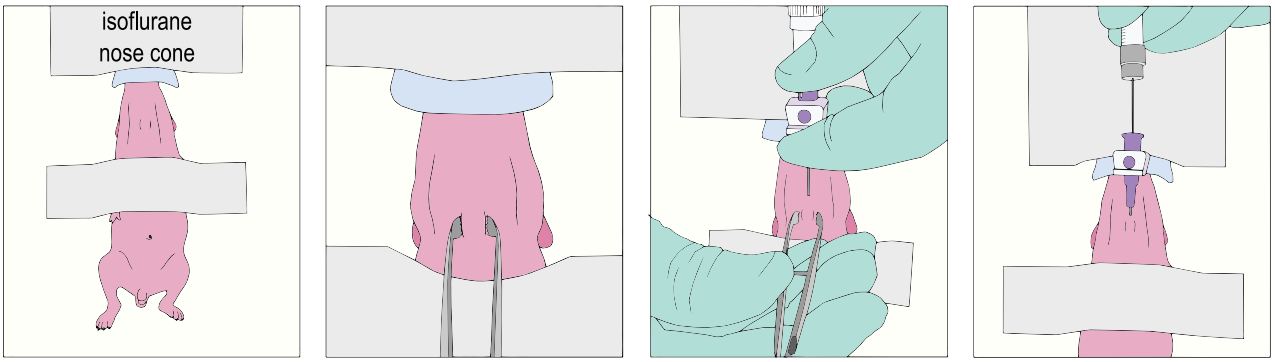
Table 1. Survival and weight characteristics of included pups

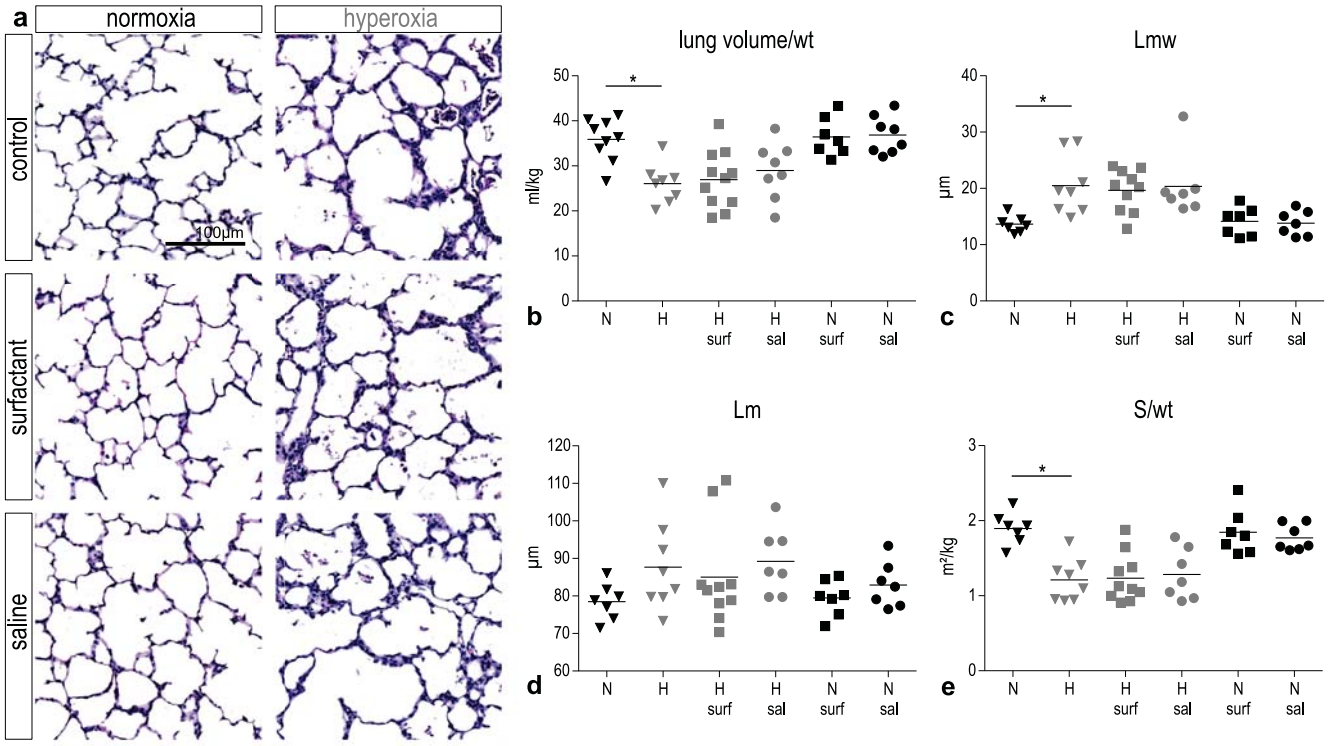
Weights of the pups included in the experiment (n=69), and of the pups harvested at day 7 (n=52). Mean ± SD are shown. Data analyzed with ANOVA.

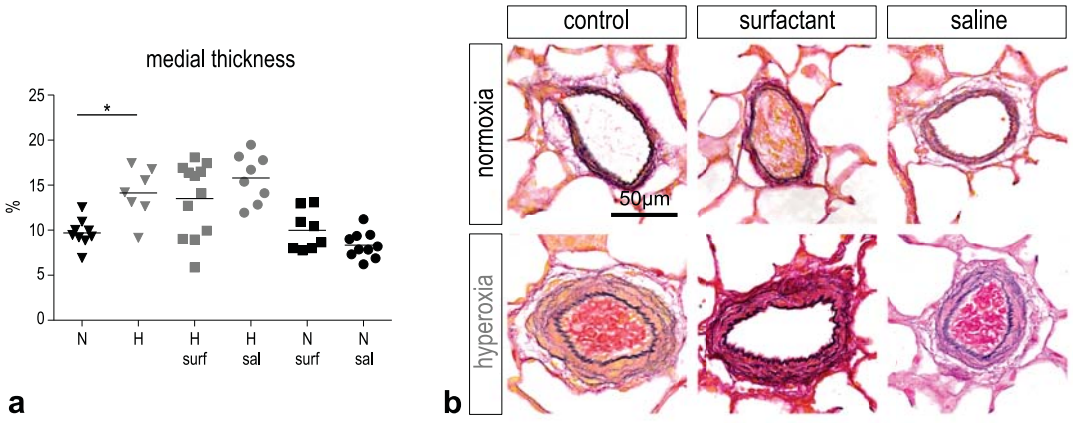
Table 2. Lung function read-outs.

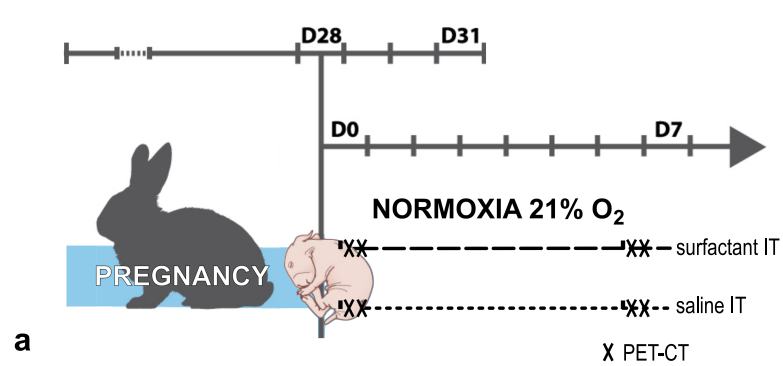
*Hyperoxia controls are compared to normoxia controls, both saline and surfactant injected groups are compared to the controls in the same condition (hyperoxia or normoxia) (n=7-11): *p<0,05 adjusted for multiple comparisons according to Bonferroni-Sidak.*











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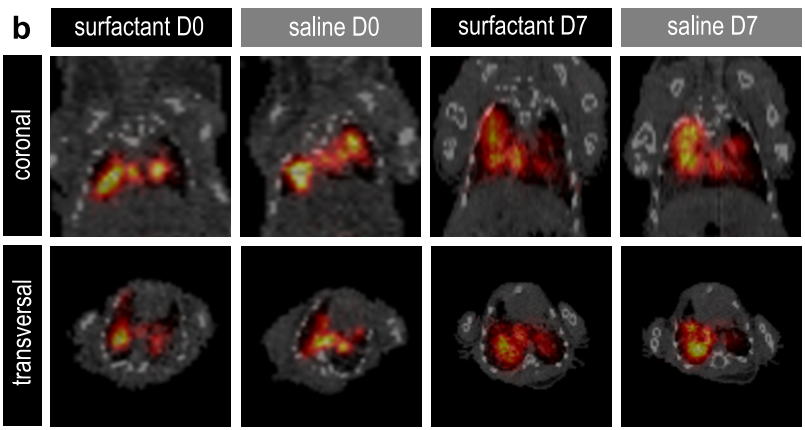

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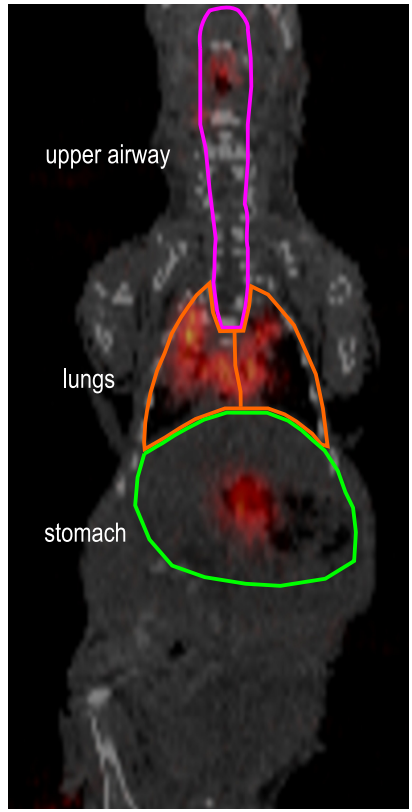

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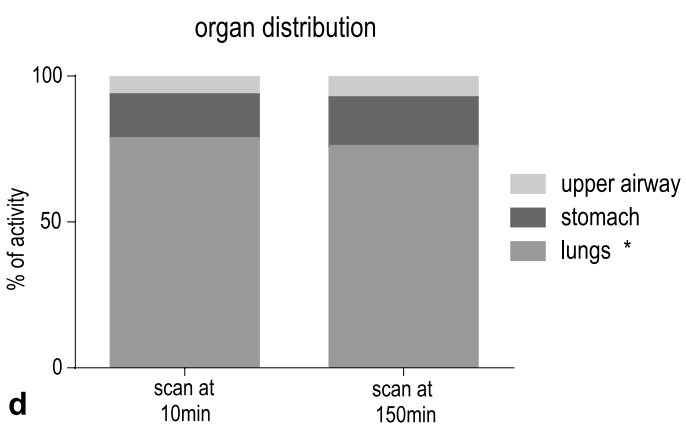

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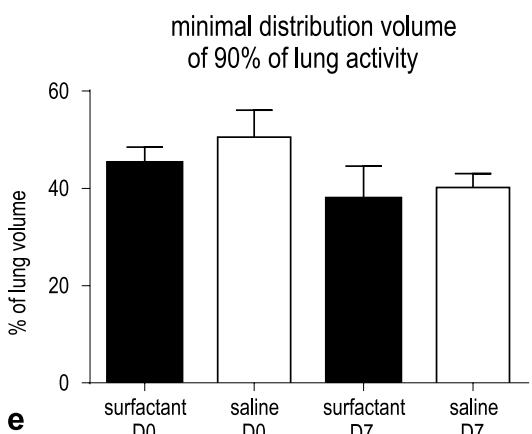

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