Intratracheal Budesonide/Surfactant attenuates hyperoxia-induced lung injury in preterm rabbits

André G Gie[1*], Yannick Regin[1*], Thomas Salaets[1], Costanza Casiraghi[2], Fabrizio Salomone[2], Jan Deprest[1, 4], Jeroen Vanoirbeek[3], Jaan Toelen[1]

Author affiliations

[1] Department of Development and Regeneration, KU Leuven, Leuven, Belgium

[2] Chiesi Farmaceutici, R&D Department, Parma, Italy

[3] Centre for Environment and Health, Department of Public Health and Primary Care, KU Leuven, Leuven, Belgium

[4] Institute for Women's Health, University College London Hospital, London, United Kingdom

* These authors contributed equally to this work.

Running head: Intratracheal Budesonide/Surfactant prevents BPD

Key words: respiratory therapy, hyperoxia, bronchopulmonary dysplasia, surfactant, experimental animal model

Corresponding Author:

Jaan Toelen Phone: +321634380 Fax: Fax +32-16-343842

E-mail: jaan.toelen@kuleuven.be

Department of Development and Regeneration

O&N 4, Herestraat 49

3000 Leuven

Author contributions: AG, YR, TS, JD, JT designed the experiments. AG, YR performed the intratracheal budesonide/surfactant experiments, functional testing, histological evaluation, collected the data and made the figures. AG, YR analyzed and interpreted the intratracheal budesonide/surfactant data. AG, YR, TS, CC, FS, JT, JV, JT all contributed to the writing of the manuscript.

Abstract

Recent clinical trials have shown improvements in neonatal outcomes after intratracheal administration of combination budesonide/surfactant (ITBS) in infants at risk of bronchopulmonary dysplasia. However, the effect of ITBS on lung function and alveolar structure is not known. We aimed to determine the effect of ITBS on lung function, parenchymal structure and inflammatory cytokine expression in a relevant preterm animal model for bronchopulmonary dysplasia. Premature neonatal rabbits were administered a single dose of ITBS on the day of delivery and exposed to 95% oxygen. Following seven days of hyperoxia, *in vivo* forced oscillation and pressure-volume maneuvers were performed to examine pulmonary function. Histological and molecular analysis was performed to assess alveolar and extracellular matrix (ECM) morphology, along with gene expression of connective tissue growth factor (CTGF), IL-8 and CCL-2. ITBS attenuated the functional effect of hyperoxia-induced lung injury and limited the change to respiratory system impedance, measured using the forced oscillation technique. Treatment effects were most obvious in the small airways, with significant effects on small airway resistance and reactance. Additionally, ITBS mitigated the decrease of inspiratory capacity and static compliance. ITBS restricted alveolar septal thickening without altering the mean linear intercept and mitigated hyperoxia-induced remodeling of the ECM. These structural changes were associated with improved inspiratory capacity and lung compliance. Gene expression of CTGF IL-8 and CCL-2 were significantly down regulated in the lung. Treatment with ITBS shortly after delivery attenuated the functional and

structural consequences of hyperoxia-induced lung injury to day 7 of life in the preterm rabbit.

Introduction

Prematurity along with lung inflammation are central to the development of bronchopulmonary dysplasia (BPD) (2, 23, 24) . Premature infants are born prior to the functional maturation of the respiratory system and regularly develop respiratory distress. These infants frequently require supplemental oxygen and mechanical ventilation, both of which can increase lung inflammation and the risk of BPD (17).

Treatment options to prevent the development of BPD are limited (26). While high dose systemic steroids can decrease lung inflammation and the risk of BPD, increased risk of neurocognitive injury and systemic side effects have limited their use (11). Intratracheal steroid therapy is an emerging therapy that can be combined with exogenous surfactant administration to directly target the lung and avoid systemic effects (22). Intra-tracheal surfactant supplementation is commonly used in preterm infants with respiratory distress and provides an opportunity to use surfactant as a vehicle to deliver steroids to the peripheral lung. Budesonide combined with surfactant can modify or prevent pulmonary inflammation (3, 14, 15, 19, 20). Budesonide does not alter the biophysical properties of surfactant and has the additional benefits of prolonged pulmonary effect and rapid clearance of systemically absorbed drug (6, 19, 25).

Data on effectiveness of intratracheal steroid therapy to decrease the rate of BPD are sparse. Clinical studies are limited to a single randomized control trial and a single observational study examining intratracheal budesonide combined with surfactant. These studies have demonstrated the combination of intratracheal

budesonide (0.25mg/kg) and surfactant (100mg/kg) to decrease the incidence and severity of BPD in mechanically ventilated children (21, 37). Supporting evidence of the pulmonary benefits of intratracheal budesonide/surfactant (ITBS) has come from animal models. ITBS has been found to acutely improve gas exchange and limit lung and systemic inflammation (20, 27). However, due to limited lung function and structure data, the effect of ITBS on the preterm lung is not yet fully understood. Whether the acute changes seen with ITBS have a lasting effect on lung function and structure is yet to be determined.

This paper demonstrates that a single prophylactic treatment with ITBS has beneficial effects on the preterm lung exposed to hyperoxia. ITBS attenuated the functional and structural consequences of hyperoxia-induced lung injury and limited lung inflammation to day 7 of life.

Material & Methods

Experiments were approved by the Ethics committee for Animal Experimentation of KU Leuven (P081/2017) and performed in agreement with *Directive 2010/63*/EU concerning the protection of animals used for scientific purposes and the Declaration of Helsinki on animal use in biomedical research. An overview of the study design is given in figure 1.

Animal protocols: Time mated New Zealand White-Dendermonde hybrid rabbits were provided by the KU Leuven animal facility and housed in a temperature-controlled environment. Pups were delivered via Caesarean section on day 28 of gestation (term of 31 days) and placed in hyperoxia (95% O_2) for the first hour of

life. Surviving pups were randomized to normoxia (21% O_2, N), hyperoxia (95% O_2, H) or hyperoxia plus intratracheal budesonide/surfactant (ITBS) and housed in a custom humidity- and temperature-controlled incubator (Okolab, Pozzuoli, Italy). Pups were manually fed twice daily via an orogastric tube with stepwise increased volumes of milk (Day One®, Protein 30%, Fat 50%; FoxValley, Illinois, US) supplemented with probiotics (Bio-Lapis®; Probiotics International Ltd, Somerser, UK) and immunoglobulins (Col-o-Cat®, SanoBest, Hertogenbosch, Netherlands). Pups received a single dose of vitamin K1 (0.25 mg/kg BW, Konakion pediatrique®; Roche, Basel, Switzerland) intramuscularly on day 2.

Intratracheal drug delivery: Intratracheal injections were performed as previously described (30). Briefly, pups were anesthetized with isoflurane (2.5%; ISO-VET; EuroVet, Heusden-Zolder, Belgium) and the trachea transcutaneously cannulated with a 26 gauge catheter. Movement of fluid in the catheter with spontaneous respiration confirmed intratracheal placement, and an intratracheal injection with 1.25 ml/kg porcine derived surfactant (poractant alfa, Curosurf®, Chiesi Farmaceutici, Parma, Italy) mixed with 0.25 mg/kg budesonide (Pulmicort® 0.25 mg/ml, AstraZeneca, Cambridge, UK) performed. Following intratracheal injection, pups were returned to hyperoxia.

Pulmonary function testing (PFT): *In vivo* PFT was performed on day 7 using the FlexiVent system with FlexiVent module 2 (FlexiVent 8.0; SCIREQ, Montreal, Canada) as previously described (28). Pups were ventilated at a rate of 120 breaths/min, tidal volume 8ml/kg, with a PEEP 3cmH20. Pressure volume and forced oscillation PFT were performed following a recruitment maneuver to ensure lungs were fully inflated at the time of testing. A series of PFT were performed as follows, inspiratory capacity, single frequency oscillation (Snapshot

150: a single frequency measurement at 2.5 Hz), broadband oscillation maneuver (Primewave 8: measuring respiratory impedance from 0.5 Hz to 19.5 Hz) and a pressure-volume-maneuver (PVr-P: continuous increase of airway pressure to 10 cmH_2O). Small airway resistance and reactance was calculated as the difference in resistance between the lowest and highest frequencies tested in the primewave 8 broadband forced oscillation maneuver ($Z_R0.5$- $Z_R19.5$, $Z_x0.5$- $Z_x19.5$ respectively) (13). PFT maneuvers were repeated in triplicate and the mean calculated. PFT with a coefficient of determination <90% were excluded and the maneuver repeated following a recruitment maneuver.

Alveolar morphology: Following lung function testing, lungs were excised *en-bloc* and pressure-fixed with 4% paraformaldehyde at 25 cmH_2O hydrostatic pressure as previously described (28). Paraffin sections of 5µm thickness were stained with hematoxylin and eosin and scanned using a high-throughput slidescanner (Axio Scan® Slide Scanner, Zen Zeiss, Oberkochen, Germany). An in-house programmed ImageJ algorithm was used to select 20 random fields (500µmx500µm) per lung and calculate mean linear intercept (Lm), mean linear intercept of the alveolar airspace (Lma, reflecting size of the airspace) and mean transactional wall length (Lmw, reflecting alveolar septal thickness) semi-automatically (29). Radial alveolar count (RAC) was performed 20 times per lung (8). Parenchymal tissue and collagen content was assessed using digital image analysis on Sirius Red stained slides examining 20 random fields (500µmx500µm) per lung (32). A single blinded observer performed all histological evaluation.

Quantitative Real-Time PCR: Expression of CCL-2, IL-8, connective tissue growth factor (CTGF) mRNA was performed on whole lung homogenate and corrected to

the house-keeping gene HPRT as described previously (32). Male sex was identified by detection of the SYR-gene (34). Primer sequences can be found in supplementary table (https://figshare.com/s/398690bae2239e4ea497).

Statistical Analysis: Analysis was performed using GraphPad Prism 8.0 software (La Jolla, California, USA). Groups were compared by 1-way ANOVA with Dunnett's post hoc test (normoxia v hyperoxia; hyperoxia v hyperoxia plus intratracheal budesonide/Surfactant), unless stated otherwise. Gene expression analysis was performed using the ΔΔCT method, with statistical analysis performed on ΔΔCT and fold change used for visualization. A p-value <0.05 was considered significant.

Results

Hyperoxia exposure leads to growth restriction: the mean birth weight of pups was 34.8g with no statistically significant difference in birth weight between groups. Hyperoxia exposure restricted growth and by day 7 of life pups reared in hyperoxia had significantly lower body weight ($p<0.001$) and proportional growth ($p<0.001$)(see supplementary data table 2 https://figshare.com/s/c17ddc435a4483aebb2c). ITBS pup had significantly higher body weight by D7 of life compared to hyperoxia ($p<0.05$). Survival was similar in all groups.

ITBS limits hyperoxia-induced decline of lung function: hyperoxia exposure led to a significant decline in lung function (table 1, figure 2,3). Forced oscillation (FOT) PFT demonstrated hyperoxia to significantly alter both the resistance ($p=0.0001$) and reactance ($p<0.0001$) of the lung (figure 2). Of note, small airway resistance ($Z_R0.5$- $Z_R19.5$) and reactance ($Z_x0.5$- $Z_x19.5$) were both significantly altered by

hyperoxia. Pressure-volume (PV) PFT showed hyperoxia exposure decreased inspiratory capacity ($p<0.0001$) and static compliance ($p<0.0001$), while static elastance was increased ($p<0.0001$) (figure 3). PV curves were flattened, and hysteresis significantly decreased by hyperoxia ($p<0.0001$).

A single dose of ITBS mitigated the hyperoxia-associated lung function decline in both FOT-PFT and PV-PFT (table 1, figures 2,3). FOT based tests demonstrated both respiratory system resistance ($p<0.001$) and reactance ($p<0.001$) to benefit from ITBS. Additionally, hyperoxia associated disruption of small airway resistance and small airway reactance was mitigated by ITBS ($p<0.001$). Inspiratory capacity ($p<0.05$) and static elastance ($p<0.05$) were significantly increased compared to hyperoxia.

ITBS attenuates hyperoxia-induced alveolar injury: To evaluate the influence of hyperoxia on lung development we examined alveolar morphology. Hyperoxia exposure increased both the mean linear intercept, representing alveolar size (Lm)($p<0.05$), and mean transectional wall length, representing alveolar wall thickness (Lmw) ($p<0.0001$) (figure 4, supplementary table 3 https://figshare.com/s/c17ddc435a4483aebb2c). Additionally the RAC was decreased by hyperoxia ($p=0.004$)(supplementary data, table 3). The mean alveolar airspace (Lma) was not affected by hyperoxia. These findings indicate hyperoxia-exposure to increase the alveolar size by thickening the alveolar wall and not by increasing the alveolar airspace. The increase in Lmw and decrease in RAC was tempered by ITBS ($p<0.01$, $p=0.01$ respectively)(figure 4, supplementary

data, table 3). Overall alveolar size (p=0.63) and alveolar airspace (p>0.99) were unaffected by ITBS.

ITBS mitigates hyperoxia-induced remodeling of the extracellular matrix: hyperoxia exposure led to significant remodeling of the extracellular matrix of the lung. Total tissue content (p=0.03), total collagen content (p<0.0001) and the collagen:tissue ratio (p<0.0001) of the parenchyma were increased by hyperoxia exposure (figure 5). Treatment with ITBS was associated with significantly less parenchymal remodeling. Tissue content (p<0.05), collagen content (p=0.006) and collagen:tissue ratio (p=0.03) were all lower in the ITBS group than in hyperoxia.

ITBS minimizes hyperoxia-associated induction of acute phase response genes: to evaluate the acute phase response of the lung to hyperoxia we analyzed the gene expression of CTGF, CCL-2 and IL-8 in the lung. Hyperoxia led to a significant increase of gene expression of CTGF (p<0.05), CCL-2 (p<0.0001) and IL-8 (p<0.001) on day 7 of life. ITBS blunted the expression of CTGF (p<0.05), CCL-2 (p<0.001) and IL-8 (p<0.01) on day 7 (figure 4, 5).

Discussion:

We demonstrate intratracheal budesonide/surfactant (ITBS) to limit hyperoxia-associated lung injury in a preterm model of bronchopulmonary dysplasia. ITBS mitigated hyperoxia-induced loss of lung function, attenuated the disruption of parenchymal structure and limited the mRNA expression of CTGF, CCL-2 and IL-8 to day 7 of life.

To evaluate the effect of hyperoxia and ITBS on lung function we performed both forced oscillation (FOT) and pressure-volume (PV)-based pulmonary function tests (PFT). Hyperoxia significantly altered small airway function along with distal tissue mechanics, while central airway function was unaffected. PV-PFT demonstrate that hyperoxia exposure results in restrictive lungs. A single dose of ITBS limited the hyperoxia-associated loss of lung function. FOT-PFT revealed ITBS to significantly improve small airway function, decreasing small airway resistance and increasing small airway reactance. The improvement in small airway function by ITBS has not previously been described. Additionally, tissue mechanics of the peripheral lung, inspiratory capacity, static elastance and PV-curves were significantly improved.

Short-term animal experiments have previously demonstrated ITBS to improve *ex vivo* lung compliance, respiratory physiology and decrease lung injury (20, 27). However, there are no other data on the effect of ITBS on *in vivo* lung functions in either animal or human clinical trials. Whether the improvements in small airway function and inspiratory capacity by ITBS leads to improvement in the obstructive and restrictive lung disease of BPD survivors remains to be seen (33).

The FOT-PFT findings are especially relevant to modern neonatology. Forced oscillation lung function testing is becoming available for clinical use in neonates and can discriminate between healthy infants and those with pulmonary conditions such as transient tachypnea of the neonate (18). While not readily available, infant FOT would allow clinicians to evaluate the response of infants to respiratory therapy and identify those in need of additional treatment.

Similar to the disruption of lung function, hyperoxia exposure significantly disrupted the structure of the lung parenchyma. Alveolar structure, total lung tissue, lung collagen and collagen:tissue ratio were significantly altered. These structural changes led to functional consequences, such as decreased static lung compliance. Hyperoxia-associated parenchymal disruption and altered alveolar morphometry has been described in infants who demised from BPD as well as in animal models (9, 16, 28). However, to our knowledge this is the first correlation of altered alveolar structural to disrupted lung function in BPD.

ITBS administration minimized the disruption of hyperoxia-associated structural remodeling of the parenchyma. Following ITBS, pups exposed to hyperoxia had significantly less disruption of alveolar development and remodeling of the extracellular matrix. Additionally, we could correlate the limited disruption of lung development with improved lung function. Interruptions of alveolar development and lung fibrosis are key findings in BPD and therapy that minimizes disruption of normal parenchymal development is critically important. Early prophylactic therapy with prolonged structural effect on the lung could allow BPD survivors to reach their full lung function potential and prevent a premature decline in lung function.

Though the pathophysiology of BPD and preterm lung injury is not fully understood, inflammation plays a central role in disrupting lung development and the development of BPD (24). Prior transcriptome analysis of the hyperoxia preterm rabbit model of BPD has identified CCL-2 and IL-8 to be the key

293 inflammatory mediators (31). Similar to our findings, human and animal studies
294 have described elevated CCL-2 and IL-8 to be associated with the development
295 and severity of BPD and found intratracheal budesonide plus surfactant to
296 decrease IL-8 in the lung (19, 24, 35, 37).
297
298 We speculate that the benefit of ITBS is potentially related to the attenuation of
299 hyperoxia-associated CTGF gene expression in the lung. Hyperoxia induces CTFG
300 gene expression leading to parenchymal remodeling and loss of lung function. By
301 attenuating the induction of CTGF expression, ITBS tempers the functional and
302 structural consequences of hyperoxia exposure. Similar to our findings, increased
303 CTFG expression has been linked to disrupted lung development, remodeling of
304 the ECM, and induction of lung inflammation and IL-8 production(5, 7, 36, 38).
305 Furthermore, increased CTGF expression has been found in the lungs of neonates
306 with BPD (1). However the beneficial effect of ITBS may not be universal, as ITBS
307 did not alter CTGF or IL-8 expression in acute ventilator associated lung injury in
308 fetal lambs (20).
309
310 The decrease of CTGF, IL-8, and CCL-2 gene expression to day 7 of life is likely due
311 to the pulmonary pharmacokinetics of budesonide. Budesonide is conjugated to
312 intracellular fatty acid esters, in the airway which are gradually hydrolysed and
313 then slowly released as free budesonide, extending the effect of a single
314 administration (25). The extended suppression of lung inflammation by ITBS has
315 been demonstrated in preterm infants (37). However there are contrasting
316 results on the extended availability of budesonide esters in the lung. Despite
317 significant improvement in lung function and decreased lung inflammation

relatively little budesonide esters were found in the lungs of preterm lambs 6 hours after intratracheal the administration of intratracheal budesonide-surfactant (14). Although the mechanism of the prolonged effect of intratracheal budesonide remains uncertain, it's effect is attractive for the treatment of ill neonates, as a single dose can have a lasting effect avoiding the need for repeated airway manipulation.

Our study was able to combine prematurity with extensive *in vivo* lung function testing and alveolar structure evaluation. Additionally, we were able to evaluate the effect of ITBS beyond the acute response following administration. Limitations of the model include the single duration and concentration of oxygen used and the limited molecular and mechanistic insights possible in the rabbit. To limit the animals used we chose not to include groups treated with surfactant-alone or budesonide-alone nor did we examine the effect of ITBS in the absence of hyperoxia exposure.

Effective therapy to prevent the development of BPD following preterm birth is required. Once established, there is limited therapy for treatment or evidence to guide the management of BPD (12). Infants with BPD fail to reach their full lung function potential, and have diminished lung function throughout life potentially resulting in the early onset of COPD (4, 10). Treatment that improves small airway function may have lifelong advantages and limit the development of COPD. Targeted steroid delivery to the lung via intratracheal administration potentially limits or prevents the off-target side effects of systemic steroids while maintaining its advantageous effect on the lung. The co-administration of budesonide with

surfactant to preterm infants with respiratory failure is an attractive treatment strategy to prevent the development of BPD in these high-risk infants. Our study demonstrates that ITBS attenuated hyperoxia induced lung injury in the absence of mechanical ventilation and indicates that premature infants exposed to supplemental oxygen may benefit from ITBS. It remains unclear whether ITBS offers benefit in moderate levels of hyperoxia or in normoxic conditions. Future animal studies examining the effect of ITBS in moderate levels of hyperoxia, and clinical studies of premature infants exposed to supplemental oxygen and non-invasive ventilation will be of great interest in the search for interventions to limit the development of BPD.

Conclusion:

Intratracheal budesonide-surfactant on the day of birth limits hyperoxia-associated disruption of lung function and structure in a preterm model of BPD. A single dose of budesonide-surfactant attenuated the functional and structural consequences of hyperoxia-induced lung injury.

Statement of financial support: This research was supported by a C2 grant from KU Leuven (C24/18/101) and a research grant from the Research Foundation – Flanders (FWO G0C4419N). AG is supported by the Erasmus+ Programme of the European Commission (2013–0040). YR is holder of an FWO-SB fellowship (Research Foundation - Flanders, 1S71619N). JD is partly funded by the Great Ormond Street Hospital Charity Fund.

Conflict of interest statement: FS and CC are employees of Chiesi Farmaceutici S.p.A. The animal-derived surfactant Poractant alfa (Curosurf, 80 mg/ml) was supplied by Chiesi Farmaceutici S.p.A., which is the employer of authors FS and CC. None of the funding bodies was involved in the design of the study and in the collection, analysis, and interpretation of data. AG, YR, TS, JV, JD, JT have no conflict of interest to declare.

References:

1. **Alapati D**, **Rong M**, **Chen S**, **Hehre D**, **Rodriguez MM**, **Lipson KE**, **Wu S**. Connective tissue growth factor antibody therapy attenuates hyperoxia-induced lung injury in neonatal rats. *Am J Respir Cell Mol Biol* 45: 1169–1177, 2011.
2. **Balany J**, **Bhandari V**. Understanding the impact of infection, inflammation, and their persistence in the pathogenesis of bronchopulmonary dysplasia. *Front. Med.* 2: 1–10, 2015.
3. **Barrette AM**, **Roberts JK**, **Chapin C**, **Egan EA**, **Segal MR**, **Oses-Prieto JA**, **Chand S**, **Burlingame AL**, **Ballard PL**. Antiinflammatory effects of Budesonide in human fetal lung. *Am J Respir Cell Mol Biol* 55: 623–632, 2016.
4. **Bolton CE**, **Bush A**, **Hurst JR**, **Kotecha S**, **McGarvey L**. Lung consequences in adults born prematurely. *Postgrad. Med. J.* 91: 712–718, 2015.
5. **Chen C**, **Wang L**, **Chou H**, **Lang Y**, **Lai Y**. Up-regulation of Connective Tissue Growth Factor in Hyperoxia-Induced Lung Fibrosis. *Pediatr Res* 62: 128–133, 2007.
6. **Chen CM**, **Chang CH**, **Chao CH**, **Wang MH**, **Yeh TF**. Biophysical and chemical stability of surfactant/budesonide and the pulmonary distribution following intra-tracheal administration. *Drug Deliv* 26: 604–611, 2019.
7. **Chen S**, **Rong M**, **Platteau A**, **Hehre D**, **Smith H**, **Ruiz P**, **Whitsett J**, **Bancalari E**, **Wu S**. CTGF disrupts alveolarization and induces pulmonary hypertension in neonatal mice: Implication in the pathogenesis of severe bronchopulmonary dysplasia. *Am J Physiol - Lung Cell Mol Physiol* 300, 2011.
8. **Cooney TP**, **Thurlbeck WM**. The radial alveolar count method of Emery and Mithal: a reappraisal 2--intrauterine and early postnatal lung growth. *Thorax* 37: 580 LP – 583, 1982.
9. **Dolma K**, **Freeman AE**, **Rezonzew G**, **Payne GA**, **Xu X**, **Jilling T**, **Blalock JE**, **Gaggar A**, **Ambalavanan N**, **Lal CV**. Effects of Hyperoxia on Alveolar and Pulmonary Vascular Development in Germ Free Mice. *Am J Physiol*

Lung Cell Mol Physiol 318: 421–428, 2020.

10. **Doyle LW**, **Adams AM**, **Robertson C**, **Ranganathan S**, **Davis NM**, **Lee KJ**, **Cheong JL**, **Anderson P**, **Burnett A**, **Callanan C**, **Carse E**, **Charlton MP**, **Duff J**, **Hutchinson E**, **Hayes M**, **Kelly E**, **McDonald M**, **Opie G**, **Roberts G**, **Watkins A**, **Williamson A**, **Woods H**. Increasing airway obstruction from 8 to 18 years in extremely preterm/low-birthweight survivors born in the surfactant era. *Thorax* 72: 712–719, 2017.
11. **Doyle LW**, **Cheong JL**, **Ehrenkranz RA**, **Halliday HL**. Early (< 8 days) systemic postnatal corticosteroids for prevention of bronchopulmonary dysplasia in preterm infants. *Cochrane Database Syst Rev* 2017, 2017.
12. **Duijts L**, **van Meel ER**, **Moschino L**, **Baraldi E**, **Barnhoorn M**, **Bramer WM**, **Bolton CE**, **Boyd J**, **Buchvald F**, **del Cerro MJ**, **Colin AA**, **Ersu R**, **Greenough A**, **Gremmen C**, **Halvorsen T**, **Kamphuis J**, **Kotecha S**, **Rooney-Otero K**, **Schulzke S**, **Wilson A**, **Rigau D**, **Morgan RL**, **Tonia T**, **Roehr CC**, **Pijnenburg MW**. European Respiratory Society guideline on long-term management of children with bronchopulmonary dysplasia. *Eur Respir J* 55, 2020.
13. **Goldman MD**, **Saadeh C**, **Ross D**. Clinical applications of forced oscillation to assess peripheral airway function. *Respir Physiol Neurobiol* 148: 179–194, 2005.
14. **Hillman NH**, **Abugisisa L**, **Royse E**, **Fee E**, **Kemp MW**, **Hillman NH**, **Abugisisa L**, **Royse E**, **Fee E**, **Kemp MW**. Dose of budesonide with surfactant affects lung and systemic inflammation after normal and injurious ventilation in preterm lambs. *Pediatr. Res.* (2020). doi: 10.1038/s41390-020-0809-6.
15. **Hillman NH**, **Brett Kothe T**, **Schmidt AF**, **Kemp MW**, **Royse E**, **Fee E**, **Salomone F**, **Clarke MW**, **Musk GC**, **Jobe AH**. Surfactant plus budesonide decreases lung and systemic responses to injurious ventilation in preterm sheep. *Am J Physiol - Lung Cell Mol Physiol* 318: L41–L48, 2020.
16. **Husain AN**, **Siddiqui NH**, **Stocker JT**. Pathology of arrested acinar development in postsurfactant bronchopulmonary dysplasia. *Hum Pathol* 29: 710–717, 1998.
17. **Jobe AH**. Mechanisms of Lung Injury and Bronchopulmonary Dysplasia. *Am. J. Perinatol.* 33: 1076–1078, 2016.
18. **Klinger AP**, **Travers CP**, **Martin A**, **Kuo HC**, **Alishlash AS**, **Harris WT**, **Carlo WA**, **Ambalavanan N**. Non-invasive forced oscillometry to quantify respiratory mechanics in term neonates. *Pediatr Res* : 1–7, 2020.
19. **Kothe BT**, **Kemp MW**, **Schmidt A**, **Royse E**, **Salomone F**, **Clarke MW**, **Musk GC**, **Jobe AH**, **Hillman NH**. Surfactant plus budesonide decreases lung and systemic inflammation in mechanically ventilated preterm sheep. *Am J Physiol - Lung Cell Mol Physiol* 316: L888–L893, 2019.
20. **Kothe TB**, **Royse E**, **Kemp MW**, **Schmidt A**, **Salomone F**, **Saito M**, **Usuda H**, **Watanabe S**, **Musk GC**, **Jobe AH**, **Hillman NH**. Effects of budesonide and surfactant in preterm fetal sheep. *Am J Physiol - Lung Cell Mol Physiol* 315: L193–L201, 2018.
21. **Kothe TB**, **Sadiq FH**, **Burleyson N**, **Williams HL**, **Anderson C**, **Hillman NH**. Surfactant and budesonide for respiratory distress syndrome: an observational study. *Pediatr Res* : 1–6, 2019.
22. **Kuo HT**, **Lin HC**, **Tsai CH**, **Chouc IC**, **Yeh TF**. A Follow-up Study of Preterm

Infants Given Budesonide Using Surfactant as a Vehicle to Prevent Chronic Lung Disease in Preterm Infants. *J Pediatr* 156: 537–541, 2010.

23. **Lapcharoensap W**, **Gage SC**, **Kan P**, **Profit J**, **Shaw GM**, **Gould JB**, **Stevenson DK**, **O'Brodovich H**, **Lee HC**. Hospital Variation and Risk Factors for Bronchopulmonary Dysplasia in a Population-Based Cohort. *JAMA Pediatr* 169: e143676–e143676, 2015.
24. **Leroy S**, **Caumette E**, **Waddington C**, **Hébert A**, **Brant R**, **Lavoie PM**. A Time-Based Analysis of Inflammation in Infants at Risk of Bronchopulmonary Dysplasia. *J Pediatr* 192: 60-65.e1, 2018.
25. **Miller-Larsson A**, **Mattsson H**, **Hjertberg E**, **Dahlbäck M**, **Tunek A**, **Brattsand R**. Reversible fatty acid conjugation of budesonide: Novel mechanism for prolonged retention of topically applied steroid in airway tissue. *Drug Metab Dispos* 26: 623–630, 1998.
26. **Poets CF**, **Lorenz L**. Prevention of bronchopulmonary dysplasia in extremely low gestational age neonates: current evidence. *Arch. Dis. Child. Fetal Neonatal Ed.* 103: F285–F291, 2018.
27. **Ricci F**, **Catozzi C**, **Ravanetti F**, **Murgia X**, **D'Aló F**, **Macchidani N**, **Sgarbi E**, **Di Lallo V**, **Saccani F**, **Pertile M**, **Cacchioli A**, **Catinella S**, **Villetti G**, **Civelli M**, **Amadei F**, **Stellari FF**, **Pioselli B**, **Salomone F**. In vitro and in vivo characterization of poractant alfa supplemented with budesonide for safe and effective intratracheal administration. *Pediatr Res* 82: 1056–1063, 2017.
28. **Richter J**, **Toelen J**, **Vanoirbeek J**, **Kakigano A**, **Dekoninck P**, **Verbeken E**, **Deprest J**. Functional assessment of hyperoxia-induced lung injury after preterm birth in the rabbit. *Am J Physiol Lung Cell Mol Physiol* 306: L277-83, 2014.
29. **Roubliova XI**, **Deprest JA**, **Biard JM**, **Ophalvens L**, **Gallot D**, **Jani JC**, **Ven De Van CP**, **Tibboel D**, **Verbeken EK**. Morphologic changes and methodological issues in the rabbit experimental model for diaphragmatic hernia. *Histol Histopathol* 25: 1105–1116, 2010.
30. **Salaets T**, **Gie A**, **Jimenez J**, **Aertgeerts M**, **Gheysens O**, **Velde G Vande**, **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.
31. **Salaets T**, **Richter J**, **Brady P**, **Jimenez J**, **Nagatomo T**, **Deprest J**, **Toelen J**. Transcriptome analysis of the preterm rabbit lung after seven days of hyperoxic exposure. *PLoS One* 10: 1–15, 2015.
32. **Salaets T**, **Tack B**, **Jimenez J**, **Gie A**, **Lesage F**, **de Winter D**, **Berghen N**, **Allegaert K**, **Deprest J**, **Toelen J**. Simvastatin attenuates lung functional and vascular effects of hyperoxia in preterm rabbits. *Pediatr. Res.* (December 9, 2019). doi: 10.1038/s41390-019-0711-2.
33. **Thunqvist P**, **Gustafsson P**, **Norman M**, **Wickman M**, **Hallberg J**. Lung function at 6 and 18 months after preterm birth in relation to severity of bronchopulmonary dysplasia. *Pediatr Pulmonol* 50: 978–986, 2015.
34. **van der Merwe J**, **van der Veeken L**, **Ferraris S**, **Gsell W**, **Himmelreich U**, **Toelen J**, **Ourselin S**, **Melbourne A**, **Vercauteren T**, **Deprest J**. Early neuropathological and neurobehavioral consequences of preterm birth in a rabbit model. *Sci Rep* 9: 1–11, 2019.

35. **Vento G**, **Capoluongo E**, **Matassa PG**, **Concolino P**, **Vendettuoli V**, **Vaccarella C**, **Frezza S**, **Zuppi C**, **Romagnoli C**, **Ameglio F**. Serum levels of seven cytokines in premature ventilated newborns: Correlations with old and new forms of bronchopulmonary dysplasia. *Intensive Care Med* 32: 723–730, 2006.
36. **Wu S**, **Platteau A**, **Chen S**, **McNamara G**, **Whitsett J**, **Bancalari E**. Conditional overexpression of connective tissue growth factor disrupts postnatal lung development. *Am J Respir Cell Mol Biol* 42: 552–563, 2010.
37. **Yeh TF**, **Chen CM**, **Wu SY**, **Husan Z**, **Li TC**, **Hsieh WS**, **Tsai CH**, **Lin HC**. Intratracheal administration of budesonide/surfactant to prevent bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 193: 86–95, 2016.
38. **Zhou T**, **Yu Q**, **Lin H**, **Wang Z**, **Fu G**, **Lei L**, **Shi Y**, **Zhang L**, **Qin L**, **Liu Y**. The Role of CTGF in Inflammatory Responses Induced by Silica Particles in Human Bronchial Epithelial Cells. *Lung* 197: 783–791, 2019.

Figure legends:

Figure 1: Study design. A C-section was performed on day 28 of gestation and pups were randomized to normoxia, hyperoxia, hyperoxia plus intratracheal budesonide-surfactant. Hyperoxia-exposed rabbits were either treated with intratracheal Budesonide/Surfactant (Hyperoxia-ITBS) or left untreated (hyperoxia). After seven days, pups were sacrificed and pulmonary function testing was performed. Organs were harvested for histological and molecular analysis

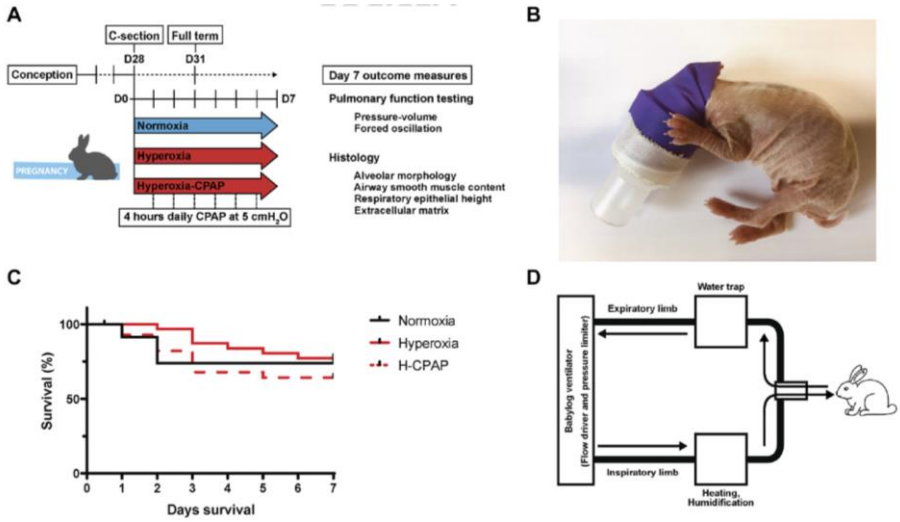


Figure 2: Intratracheal Budesonide/Surfactant mitigates hyperoxia-induced lung function changes in premature rabbit pups exposed to hyperoxia particularly at the level of the small airways on day 7. a) Respiratory impedance measured using the forced oscillation technique with pseudo-random oscillations over a range of 0.5 to 19.5 Hz depicting the real (i.e. resistance) and imaginary (i.e. reactance) part of the impedance; b) Small airway resistance and reactance determined by subtraction of highest-frequency impedance (Z (19.5 Hz)) from lowest-frequency impedance (Z (0.5 Hz)) measurement. Data are presented as mean ± SD, n=8–9 per group. N: normoxia; H: hyperoxia; H-BS: Hyperoxia + Budesonide/Surfactant. ****: p<0.0001.

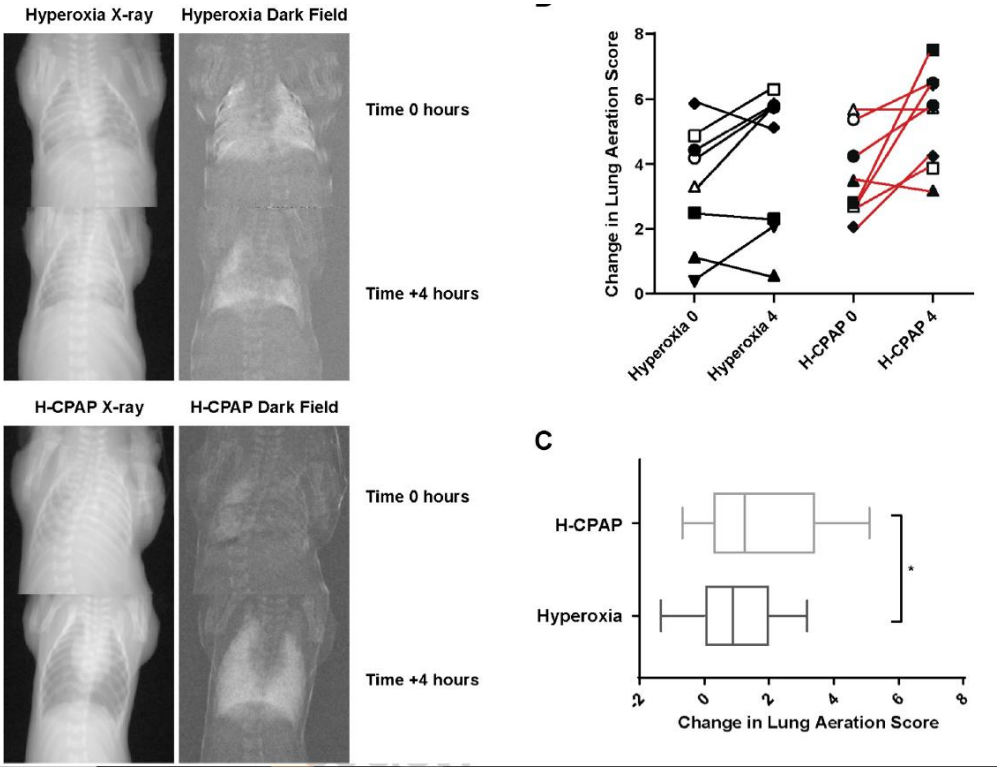


Figure 3: Intratracheal Budesonide/Surfactant ameliorates pressure-volume-based parameters after seven days of hyperoxia in preterm rabbits. a) Pressure-volume loops; b) weight-corrected static compliance; c) weight-corrected inspiratory capacity. Data are presented as mean ± SD, n=8–9 per group. N:

normoxia; H: hyperoxia; H-BS: hyperoxia + Budesonide/Surfactant; V_{Tr}: tracheal volume; P_{Tr}: tracheal pressure; Cst: Static compliance (weight-corrected). *: p<0.05; ****: p<0.0001

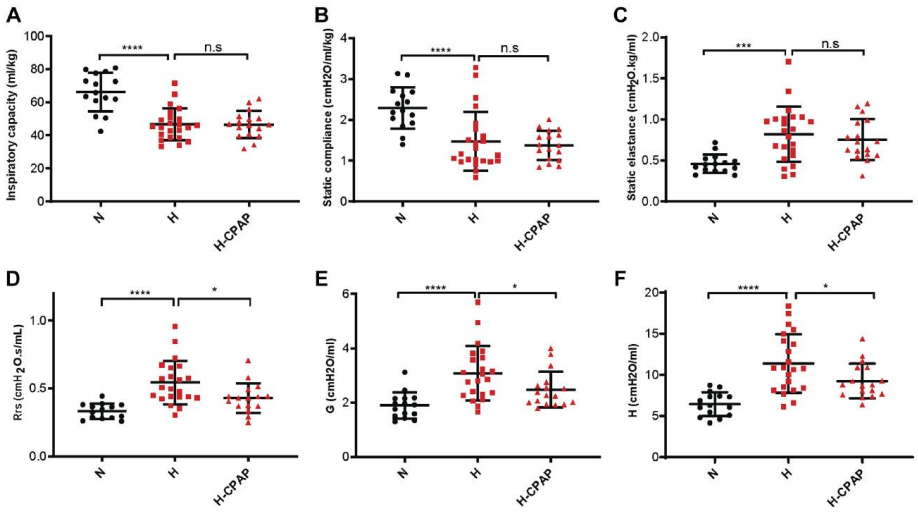
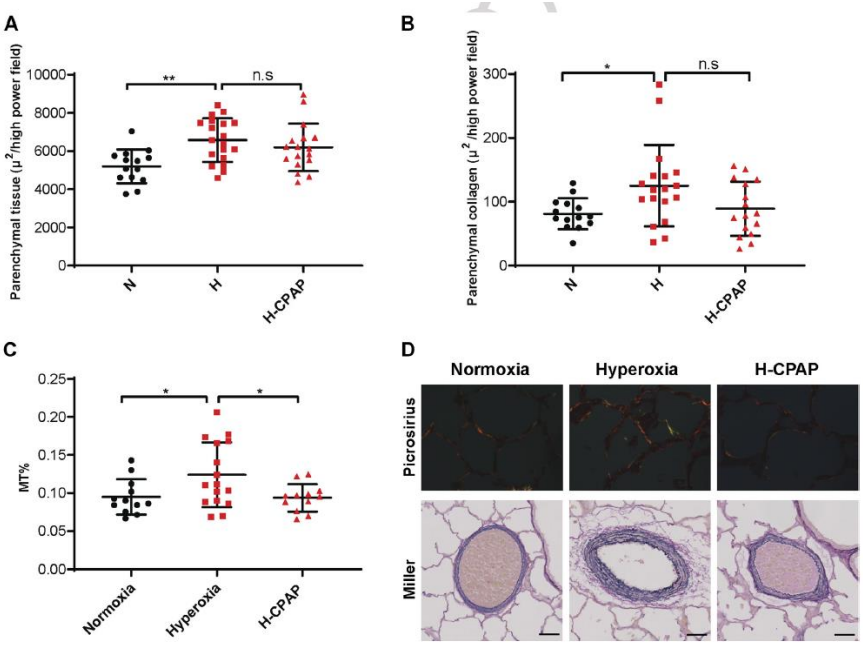


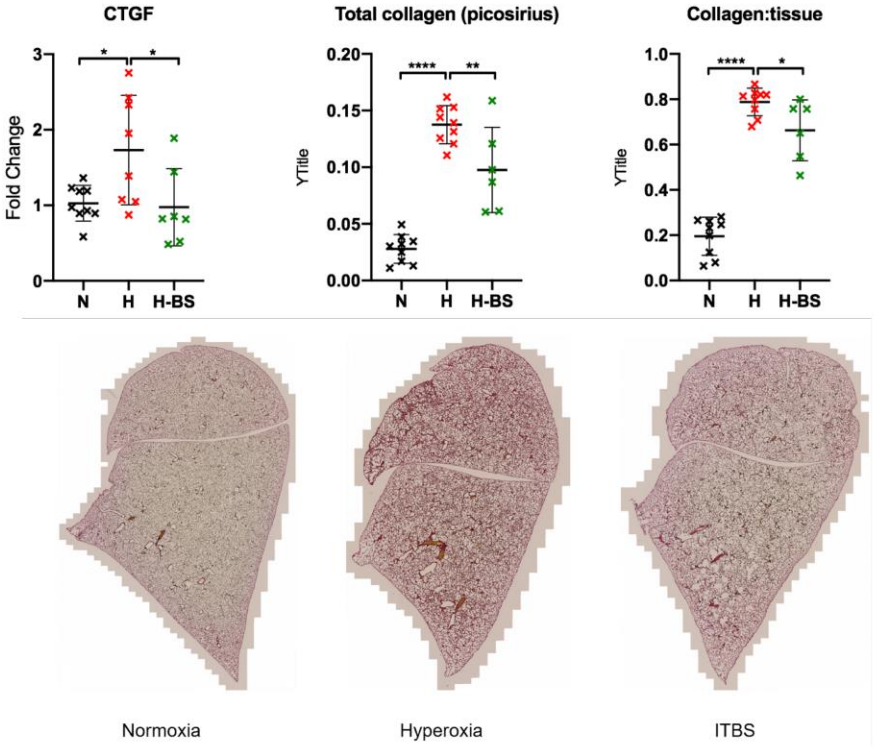
Figure 4: Intratracheal delivery of Budesonide/Surfactant mitigates hyperoxia-induced alveolar septal thickening and is associated with downregulation of inflammatory cytokines. a) Mean linear intercept (Lm); b) Mean transsectional wall length (Lmw); c) structure-function correlation (Pearson) between Lmw and weight-corrected static compliance; d) representative images of H&E-stained lung slides; e-f) Fold change in mRNA expression of e) CCL-2 and f) IL-8. Data are presented as mean ± SD, n=**7**–9 per group. N: normoxia; H: hyperoxia; H-BS: hyperoxia + Budesonide/Surfactant; Lm: mean linear intercept; Lmw: mean transsectional wall length; Cst: static compliance (weight-corrected). Scale bar = 50 µm. *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001.



561

562 **Figure 5:** Intratracheal Budesonide/Surfactant limits hyperoxia-induced
563 parenchymal remodeling. a) Fold change of connective tissue growth factor is
564 tempered gene expression b) Total lung collagen content c) Lung collagen:tissue
565 ratio d) Representative images of Sirius Red stained lungs Data are presented as
566 mean ± SD, n=6-9 per group. N: normoxia; H: hyperoxia; H-BS: Hyperoxia +
567 Budesonide/Surfactant. *p<0.05 **p<0.01 ****: p<0.0001.

568



569

570

571 **Table 1:** Intratracheal Budesonide/Surfactant attenuates hyperoxia-induced lung

572 injury in preterm rabbits; Overview of lung function parameters. Data are

573 presented as mean ± SD, n=8–9 per group. Statistical analysis one-way ANOVA

574 with correction for multiple comparison (Dunnett's test) N: normoxia; H:

575 hyperoxia; ITBS: hyperoxia +budesonide/surfactant.

576

Table 1. *Effect of hyperoxia and CPAP on pulmonary function tests in a hyperoxia preterm rabbit model of BPD*

	Normoxia	Hyperoxia	H vs. N *P* Value	H-CPAP	H-CPAP vs. H *P* Value
	Pressure-volume-based pulmonary function tests				
Inspiratory capacity, mL/kg	66.15 ± 11.62	46.7 ± 9.58	$P < 0.001$	46.53 ± 8.16	$P = 0.99$
Static compliance, ~~$cmH_2O \cdot mL^{-1} \cdot kg^{-1}$~~	2.29 ± 0.51	1.47 ± 0.72	$P < 0.001$	1.37 ± 0.36	$P = 0.83$
Static elastance, $cmH_2O \cdot mL^{-1} \cdot kg$	0.46 ± 0.11	0.82 ± 0.33	$P < 0.001$	0.75 ± 0.25	$P = 0.68$
	Forced oscillation-based pulmonary function tests				
Tissue damping, cmH_2O/mL	1.90 ± 0.48	3.08 ± 1.01	$P < 0.0001$	2.48 ± 0.66	$P = 0.04$
Tissue elastance, cmH_2O/mL	6.45 ± 1.44	11.4 ± 3.54	$P < 0.0001$	9.25 ± 2.11	$P = 0.03$
Central airway resistance, $cmH_2O \cdot mL^{-1} \cdot s$	0.12 ± 0.05	0.15 ± 0.05	$P = 0.08$	0.12 ± 0.04	$P = 0.10$
Respiratory system resistance, $cmH_2O \cdot mL^{-1} \cdot s$	0.33 ± 0,06	0.54 ± 0.16	$P < 0.0001$	0.43 ± 0.11	$P = 0.02$
Dynamic elastance, cmH_2O/mL	6.34 ± 1.75	12.99 ± 4.57	$P < 0.0001$	10.48 ± 2.78	$P = 0.06$
Dynamic compliance, mL/cmH_2O	0.16 ± 0.04	0.088 ± 0.03	$P < 0.0001$	0.10 ± 0.02	$P = 0.3$

Values are means ± SD. Pressure-volume [normoxia (N), $n = 15$ pups; hyperoxia (H), $n = 23$ pups; hyperoxia plus continuous positive airway pressure (H-CPAP), $n = 17$ pups] and forced oscillation (normoxia, $n = 15$ pups; hyperoxia, $n = 22$ pups; H-CPAP, $n = 17$ pups) tests were performed on *day 7* of life following preterm delivery. BPD, bronchopulmonary dysplasia. *P* values were adjusted for multiple comparisons (Sidak).

577

578