1 **Intermittent CPAP limits hyperoxia induced lung damage in a rabbit model**
2 **of bronchopulmonary dysplasia**

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17 Running head: Intermittent CPAP limits hyperoxia induced lung damage

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29 experiment. AG, TS, JV performed the dark field experiments and collected,
30 analyzed and interpreted the data. AG, TS, JV₁, YR, JV₂, JD, JT all contributed to the
31 writing of the manuscript.

32

33 **Statement of financial support:** AG is funded by the Erasmus+ Program of the
34 European Commission (2013-0040), TS is partially funded by the SAFEPEDRUG
35 project (supported by the agency for innovation by Science and Technology in
36 Flanders IWT SBO 130033), YR is holder of an FWO-SB fellowship (Research
37 Foundation - Flanders, 1S71619N), JD is partially funded by the Charity Fund of
38 Great Ormond Street Hospital

39

40 The authors have no conflicts of interest to declare

41

42 **Abstract**

43 A significant proportion of preterm infants develop bronchopulmonary dysplasia
44 (BPD) leading to poor lifelong respiratory health. Limited treatment options
45 exist with continuous positive airway pressure ventilation (CPAP) being one of
46 the few associated with diminished BPD. However, little is known on the effect of
47 the distending pressure of CPAP on the developing lung exposed to hyperoxia.
48 We aimed to identify the functional and structural effects of CPAP in the preterm
49 hyperoxia rabbit model of BPD.

50
51 Premature rabbit pups were randomized to normoxia, hyperoxia ($\geq 95\% \text{ O}_2$) or
52 hyperoxia plus 4 hours daily CPAP ($\text{FiO}_2 0.95$, 5 cm H_2O). On day 7 post-delivery
53 we performed invasive pressure-volume and forced oscillation based pulmonary
54 function tests, prior to lung harvest for histological evaluation. Alveolar and
55 vascular morphology, airway smooth muscle, respiratory epithelium height,
56 extracellular matrix components and inflammatory cytokine expression were
57 quantified.

58
59 Hyperoxia-reared pups had restrictive lungs: alveolar walls were thickened, with
60 the lung parenchymal tissue, collagen content and airway smooth muscle
61 content increased. In addition, peripheral pulmonary artery wall thickness was
62 increased. CPAP increased alveolar recruitment and limited the structural effect
63 of hyperoxia to the respiratory epithelium and pulmonary arteries. Additionally,
64 CPAP improved lung function, mitigating hyperoxia-associated changes to
65 respiratory system resistance, tissue damping and tissue elastance.

66

67 Hyperoxia disrupted functional and structural lung development. Daily
68 intermittent CPAP limited hyperoxia-associated decreased lung function and
69 attenuated structural changes to pulmonary arteries and respiratory epithelium
70 while having no structural alveolar consequences. The mechanism by which
71 CPAP has these beneficial effects needs further investigation.

72 **Key words:** respiratory therapy, hyperoxia, bronchopulmonary dysplasia,
73 continuous positive airway pressure, lung function test

74

75 **Introduction**

76 Despite significant advances in modern neonatal care, preterm infants continue
77 to develop respiratory distress syndrome requiring respiratory support and
78 oxygen supplementation. However, both of these life-saving interventions can
79 injure the neonatal lung, perturb lung development and lead to
80 bronchopulmonary dysplasia (BPD).

81

82 Infants with BPD have prolonged oxygen dependence, increased wheezing
83 throughout childhood and are more frequently admitted to a hospital with lung
84 disease. BPD survivors have diminished pulmonary function throughout life with
85 airway obstruction increasing with age (13). Of greater concern is that
86 surviving BPD infants do not attain their full lung function potential, possibly
87 developing COPD earlier in life (2, 7).

88

89 Following the introduction of antenatal steroids, surfactant therapy and
90 improved neonatal intensive care the survival of premature infants has greatly
91 improved. Significant neurological, ophthalmological and gastrointestinal
92 complications have decreased with modern neonatal care. In contrast, the
93 incidence of BPD is increasing as more extremely premature infants survive the
94 neonatal period (38).

95

96 Preventative and therapeutic options for BPD are scarce. The combination of
97 intratracheal surfactant and steroid therapy may decrease the risk of BPD while
98 avoiding the neurological side effects of systemic corticosteroids (48). However,
99 a significant number of extremely premature infants continue to develop BPD.

100 Limiting disruption of lung development remains a key strategy to minimize the
101 risk of BPD. Non-invasive ventilation has shown promise to decrease lung injury
102 and has been enthusiastically adopted into clinical practice. Compared to
103 invasive ventilation, CPAP is associated with decreased BPD and less childhood
104 wheezing in preterm infants (36, 37).

105

106 Despite the widespread clinical use of CPAP, surprisingly little is known about
107 the effect that the distending pressure of CPAP has on the preterm lung and
108 airways. *Ex vivo* experiments have demonstrated physical force, such as
109 mechanical stretch, can alter function, structure and inflammation in lung tissue
110 and airways (9, 10, 18, 30). Clinical studies have examined the influence of CPAP
111 on the incidence of death or BPD, however few examine lung function and lung
112 structure. Of particular concern are recent findings demonstrating an association
113 of CPAP with increased airflow obstruction in childhood (8). With the paucity of
114 human data, animal studies are required to evaluate the functional and
115 structural consequences of CPAP in the context of prematurity and hyperoxia
116 exposure.

117

118 We tested the hypothesis that the application of a distending pressure to the lung
119 by CPAP is biomechanically transduced to have biological effects. In this article
120 we characterize the functional and structural consequences of intermittent CPAP
121 in the hyperoxia preterm rabbit model of BPD.

122

123

124

125 **Materials and Methods:**

126

127 Experiments were approved by the Ethics committee for Animal
128 Experimentation of KU Leuven (P081/2017) and performed according to current
129 animal welfare guidelines.

130 Animal protocols: Time-mated rabbits (New Zealand White-Dendermonde
131 hybrid) were provided by the Biomedical Sciences animal facility of KU Leuven.
132 Dams were individually housed in a temperature-controlled environment with
133 12 hourly light-dark cycles and free access to chow and water.

134 Pups were prematurely delivered on day 28 (term of 31 days) as previously
135 described (15). Briefly, dams were sedated with a combination of intramuscular
136 ketamine (35 mg/kg bodyweight, Ketamine 1000 CEVA; CEVA Santé Animal,
137 Brussels, Belgium) and xylazine (6 mg/kg bodyweight, Vexylan®; CEVA Santé
138 Animal). Once sedated, pups were delivered via hysterotomy and dams
139 euthanized with a 1ml intravenous dose of T61 (T61®; Intervet Belgium,
140 Mechelen, combination of 200 mg embutramide, 50 mg mebezonium, and 5 mg
141 tetracain hydrochloride per millilitre). Pups were dried and stimulated prior to
142 being placed in an incubator (custom incubator, Okolab, Pozzuoli, Italy) with an
143 ambient temperature of 32 degrees Celsius, 50% relative humidity and oxygen
144 concentration of 95%. All pups that survived the first hour of birth were
145 randomized to normoxia, hyperoxia ($\geq 95\%$ O₂), or hyperoxia plus CPAP (H-
146 CPAP) groups (Figure 1A). Pups were fed a commercial milk substitute (Day
147 One®, Protein 30%, Fat 50%; FoxValley, Illinois, US) twice daily via an orogastric
148 tube. Feeding volume was incrementally increased from 80ml/kg/day to

200ml/kg/day. Additional probiotics (Bio-Lapis®; Probiotics International Ltd, Somerset, UK) and immunoglobulins (Col-o-Cat®, SanoBest, Hertogenbosch, Netherlands) were added to the feeds. On day 2 of life, an intramuscular dose of vitamin K1 (0.25 mg/kg BW, Konakion pediatrique®; Roche, Basel, Switzerland) was administered. Pups were stimulated to urinate following feeds.

Continuous positive airway pressure: on days 1-6, H-CPAP pups received 4 hours of CPAP (5cm H₂O, 6l/min humidified air flow, inhaled oxygen content of ≥95%), delivered with a neonatal ventilator (Babylog 8000, Dräger, Lübeck, Germany). Pups had individually made masks connecting them to the CPAP circuit. Similar to a previously described murine CPAP model (22), the mask consisted of a finger of a latex examination glove, cut to fit over the head of the pup and form a seal around the neck (Figure 1B,D).

Dark field imaging (DF): on day 1, phase-contrast x-ray imaging was performed to study lung aeration in hyperoxia and H-CPAP. Imaging was performed at 2 time points (T-0 and T+4) separated by 4 hours of hyperoxia or H-CPAP (5cm H₂O, flow of 6l/min humidified air, inhaled oxygen content of 95%). Pups were anaesthetized for the imaging with Isoflurane (2.5 MAC, 2.5%, 2l/min, ISO-VET®, EuroVet, Heusden-Zolder, Belgium) delivered via a nose cone. Dark field imaging was performed using a Talbot-Lau interferometer operating at 40 kVp with a 2 µm G2 grating period and system visibility of 22% (44). The radiation dose per round was 0.51 mGy expressed in entrance air Kerma. DF and conventional attenuation images were reconstructed from the X-ray acquisitions (31, 44). DF signal strength was scored in 4 lung quadrants relative to control images. Each quadrant was scored from 1 (no signal), to 8 (maximum signal), by

173 4 blinded observers. The average of the 4 quadrants, and change from T-0 and
174 T+4 was calculated and considered representative each animal.

175 Pulmonary function testing (PFT): pressure-volume and forced oscillation
176 maneuvers were performed via a tracheostomy on day 7 of life, 18-20 hours
177 after the final application of intermittent CPAP, using the FlexiVent system
178 (SciReq, FlexiVent, Montreal, Canada). FlexiVent module 1 was used for animals
179 <40gram body weight, module 2 for animals >40g body weight. Animals were
180 sedated with intramuscular ketamine and xylazine (35mg/kg and 6mg/kg
181 respectively) and a tracheostomy performed. An 18 gauge metal cannula was
182 inserted to the trachea and secured with an airtight suture. Pups were ventilated
183 with a tidal volume of 10ml/kg and positive end expiratory pressure of 3 cmH₂O
184 at a rate of 120 breaths/minute. Prior to initiating lung function tests, two deep
185 inflation maneuvers were performed where the lungs were inflated to a pressure
186 of 30 cmH₂O to maximally inflate the lungs and standardize lung volume. Both
187 pressure volume (inspiratory capacity, static compliance, static elastance) and
188 forced oscillation maneuvers (tissue damping, tissue elastance, central airway
189 resistance, respiratory system resistance, dynamic compliance, dynamic
190 elastance) were performed as previously described by our group (32). The mean
191 of 3 separate measurements for each maneuver, with a coefficient of
192 determination >95%, was calculated and used as a single data point for analysis.

193 Histological lung assessment: following PFT, lungs were harvested and pressure
194 fixed as previously described (32). Briefly, following pulmonary function testing,
195 deeply anaesthetized animals were euthanized and the lungs removed via a
196 thoracotomy. The trachea was cannulated with a 20 gauge catheter and the left

197 lung pressure fixed for 24 hours at a constant hydrostatic pressure of 25 cmH₂O
198 in 4% paraformaldehyde, while the right lung was snap frozen for molecular
199 analysis. Following pressure fixation, *ex vivo* left lung volume was calculated
200 using water displacement prior to paraffin embedding. 5 µm sections were made
201 and stained as described below. Stained sections were scanned (Axio Scan®
202 Slide Scanner, Zen Zeiss, Oberkochen, Germany) to allow digital image analysis.

203 Alveolar morphology was performed on haematoxylin and eosin (H&E) stained
204 lung slides. An ImageJ-algorithm randomly selected 20 fields (500x500µm) per
205 lung for alveolar morphology analysis, performed according to stereological
206 principals. Additionally, radial alveolar count (RAC) was performed 20 times per
207 lung (5, 26). Calculations of mean linear intercept (Lm), alveolar airspace (Lma)
208 and inter-alveolar septal thickness (Lmw) were made as previously described by
209 our group (33). Blood vessels, airways and debris were manually excluded.
210 Vascular morphology analysis was performed on Miller stained slides, examining
211 a minimum of 10 pulmonary arteries with an external diameter of 30-100µm per
212 lung slide, measuring both internal and external diameter of the media to
213 calculate the vascular medial thickness (32).

214 Airway smooth muscle content was evaluated using immunohistochemistry. A
215 primary α smooth muscle actin (αSMA) antibody (mouse anti-human; M0851,
216 DakoCytomation, Glostrup, Denmark) was used in combination with a
217 horseradish peroxidase conjugated secondary antibody (goat anti-mouse; 115-
218 035-044, Jackson ImmunoResearch, Ely, UK). Aminoethyl carbazole was used as
219 a chromogen.

220 Airway smooth muscle content was calculated on 10 randomly selected airways
221 per slide. Airways with a diameter of 75-350 μm were included if cut in cross-
222 section to their long axis. Airways with a long to short axis ratio $>2:1$ were
223 excluded. Airway smooth muscle content was analyzed using digital image
224 analysis and standardized color thresholding in ImageJ (Fiji, software, version
225 1.0). The total airway smooth muscle content was corrected to the perimeter of
226 the airway (28).

227 The respiratory epithelial height was measured on 10 randomly identified
228 airways on a single H&E stained slide per animal. The respiratory epithelial
229 height was measured perpendicular to the basement membrane on 15 points
230 around the airway lumen using a pre-determined grid (41).

231 Parenchymal collagen content was assessed on 10 random, non-consecutive
232 400X lung fields, without airways or blood vessels. Each field was photographed
233 with polarized- (collagen quantification), and non-polarized light (tissue
234 quantification). Digital image analysis was performed using ImageJ with
235 standardized thresholding to calculate collagen from polarized-light images and
236 tissue content from corresponding non-polarized light images. Parenchymal
237 elastin content was assessed on Miller's elastin stained slides (45, 46). A
238 minimum of 10 randomly selected fields (500x500 μm) without airways or blood
239 vessels were analyzed with digital image analysis (ImageJ) to calculate elastin
240 and total tissue content.

241 A single blinded observer performed all histological evaluation.

242

243 Gene expression: Total RNA was extracted from lung homogenates and cDNA
244 synthesized using TaqMan™ reverse transcription reagents (Thermo Fischer
245 Scientific). The expression of IL-8 and CCL2 was detected using Platinum SYBR
246 Green qPCR Supermix-UDG with ROX (Thermo Fischer Scientific). Specimens
247 were run in triplicate and normalized to the housekeeping gene HPRT (primers
248 in supplementary data, <https://doi.org/10.6084/m9.figshare.11815785.v1>).
249 Quantification was performed using the comparative Ct method. Statistical
250 analysis was performed on $\Delta\Delta C_t$ and fold change used for visualization.

251 Statistical analysis: Statistical analysis was performed with Prism 7.0c
252 (GraphPad, San Diego, USA). One-way ANOVA with Sidak's multiple comparison
253 test was used to compare hyperoxia to normoxia, and H-CPAP to hyperoxia.
254 Survival was compared using a log-rank (Mantel-Cox) test. Change in DF lung
255 aeration in hyperoxia and H-CPAP, T-0 and T+4 hours, was analyzed using a t-
256 test with Welch's correction. The mean of multiple measurements for a
257 parameter was calculated and presented as a single data point. Values are
258 expressed as mean $\pm$ standard deviation

259

260 We determined a sample size of 14 pups/group, based on previous studies in the
261 model, would provide a power $\geq 90\%$ with a two-sided type 1 error of 10% to
262 detect a 30% decrease in tissue damping. Higher-than-expected survival in
263 hyperoxia and H-CPAP groups increased the group sizes. A value of $p < 0.05$ was
264 considered significant.

265

266

267 **Results:**

268

269 **Survival and biometrics**

270

271 The DF experiment included 16 pups from 6 dams, which were excluded from
272 other readouts. 84 pups from 12 dams were examined to evaluate the functional
273 and structural effects of hyperoxia and H-CPAP. Birth weight, weight gain,
274 harvest weight and survival were similar in all groups (Figure 1B,
275 supplementary table 1, <https://doi.org/10.6084/m9.figshare.11815161.v1>).
276 Two pups died due to CPAP complications (abdominal distension n=1,
277 pneumothorax n=1).

278

279

280 **Alveolar recruitment by CPAP does not prevent hyperoxia-associated loss**
281 **of lung volume and inspiratory capacity**

282

283 DF imaging was performed to examine changes in lung and alveolar recruitment
284 following either hyperoxia or H-CPAP. After 4 hours, H-CPAP treated pups
285 showed improved alveolar recruitment (p=0.03) (Figure 2A). The mean lung
286 aeration score of hyperoxia and H-CPAP were 3.36 and 3.65 respectively at T-0,
287 while at T+4 the mean aeration scores were 4.23 and 5.41 demonstrating an
288 improvement of aeration score of 1.77 in H-CPAP compared to 0.89 in hyperoxia
289 (Figure 2B,C).

290

291 Increased lung aeration and alveolar recruitment following H-CPAP did not lead
292 to increased *in vivo* measurements of inspiratory capacity. Inspiratory capacity
293 was significantly reduced by hyperoxia ($p<0.001$) and was not altered by H-
294 CPAP ($p>0.99$) (Table 1, Figure 3A).

295

296 The decrease of *in vivo* inspiratory capacity by hyperoxia was reflected by *ex vivo*
297 lung volume measurement. *Ex vivo* lung volume was diminished by 14% in
298 hyperoxia ($p=0.02$) and was not affected by H-CPAP ($p=0.97$) (Table 2).

299

300 **CPAP reduces pulmonary function decline in hyperoxia**

301

302 We evaluated the consequence of hyperoxia exposure on lung function by
303 performing both pressure-volume and forced oscillation based pulmonary
304 function testing (PFT). Pressure volume PFT was used to examine static lung
305 function, while forced oscillation PFT examined the dynamic properties of the
306 respiratory system. Pulmonary function was negatively influenced by hyperoxia,
307 while H-CPAP improved forced oscillation based PFT but not pressure-volume
308 based tests (Table 2).

309

310 Hyperoxia significantly decreased static compliance ($p<0.001$), and increased
311 static elastance ($p<0.001$), resulting in stiffer lungs more resistant to expansion.
312 H-CPAP did not alter either static compliance ($p=0.83$) or static elastance
313 ($p=0.68$) (Figure 3B,C).

314

Single wave forced oscillation examines the response of the lung to a sinusoidal waveform, allowing the measurement of resistance, elastance and compliance of the complete respiratory system. Similar to pressure-volume based measurements dynamic compliance was decreased ($p<0.0001$) and dynamic elastance increased ($p<0.0001$). Additionally, hyperoxia increased respiratory system resistance ($p<0.0001$). Broadband forced oscillation examines the respiratory system response to a wide range of frequencies and, using a multi-compartment model, examines tissue and airway compartments. Tissue damping, reflecting alveolar energy dissipation, was raised in hyperoxia ($p<0.0001$). Additionally tissue elastance, reflecting alveolar energy preservation, was increased ($p<0.0001$). Central airway resistance tended to be higher in hyperoxia (Normoxia 0.12 ± 0.05 cmH₂O.s/ml versus Hyperoxia 0.15 ± 0.05 cmH₂O.s/ml) though the difference was not statistically significant ($p=0.08$).

H-CPAP significantly decreased respiratory system resistance ($p=0.02$) (Figure 3D). While dynamic elastance was lower in the CPAP group (Hyperoxia 12.99 ± 4.57 cmH₂O/ml versus CPAP 10.48 ± 2.78 cmH₂O/ml), the difference was not statistically significant ($p=0.06$). Dynamic compliance was unaffected by CPAP. Furthermore, H-CPAP attenuated both the increase in tissue damping ($p=0.04$) (Figure 3E), and tissue elastance ($p=0.03$) (Figure 3F). Central airway resistance was unaffected by H-CPAP ($p=0.1$).

Lung parenchyma is altered by hyperoxia exposure but unaffected by CPAP

340 We examined alveolar morphology and lung parenchymal tissue, collagen and
341 elastin content to determine whether hyperoxia and H-CPAP influenced alveolar
342 development and the lung parenchyma structure following preterm delivery in
343 our model.

344

345 Alveolar structure was significantly altered by hyperoxia (Table 2). Compared to
346 normoxia, hyperoxia-reared pups had larger alveoli (Lm) ($p < 0.01$) with thicker
347 alveolar septa (Lmw) ($p < 0.0001$) and a decreased RAC. The alveolar airspace
348 (Lma) was similar in hyperoxia and normoxia ($p = 0.1$). Despite the increase in
349 alveolar recruitment, H-CPAP did not influence alveolar size, wall thickness,
350 airspace size or RAC compared to hyperoxia (Table 2).

351

352 Consistent with the increased alveolar septal thickness, hyperoxia remodeled the
353 extracellular matrix (ECM). Hyperoxia exposure increased both the total
354 parenchymal tissue ($p < 0.05$) and collagen content ($p = 0.03$) of the lung. While H-
355 CPAP did not alter total parenchymal tissue ($p = 0.53$), lung collagen content was
356 28% lower in the CPAP group (Hyperoxia $125.2 \pm 63.88 \mu\text{m}^2$ versus CPAP
357 $89.04 \pm 42.48 \mu\text{m}^2$), however the difference was not statistically significant
358 ($p = 0.07$) (Figure 4A,B,D). Similarly investigation of lung elastin content revealed
359 an increase in total lung tissue content ($p = 0.02$) following hyperoxia exposure.
360 However, neither lung elastin nor the elastin to tissue ratio was altered by
361 hyperoxia or H-CPAP (supplementary figure,
362 <https://doi.org/10.6084/m9.figshare.11816838.v4>).

363

364 **Hyperoxia-associated change to airway structure diminished by CPAP**

365

366 To evaluate the effect of hyperoxia on airway structure, we quantified both the
367 airway small muscle content and the respiratory epithelial height.

368

369 Hyperoxia altered the structure of the conducting airways increasing both
370 respiratory epithelial height and airway smooth muscle (ASM) content (Figure
371 5A-D). However, the size of airways as measured by airway perimeter and
372 diameter was not altered by hyperoxia (Supplementary figures,
373 <https://doi.org/10.6084/m9.figshare.11816838.v4>). Unlike the lung
374 parenchyma, airway structure was altered by H-CPAP (Figure 5A-D). H-CPAP
375 prevented the hyperoxia-associated increase in respiratory epithelial height
376 ($p<0.05$). CPAP did not alter the ASM content ($p=0.12$).

377

378 **Hyperoxia-associated pulmonary vascular changes reduced by CPAP**

379 To assess the impact of hyperoxia on the pulmonary vasculature we examined
380 the medial layer of peripheral pulmonary arteries.

381

382 Hyperoxia exposure resulted in an increase in the medial thickness of the
383 peripheral pulmonary arteries ($p<0.01$) indicating muscularization of the
384 pulmonary arteries. This muscularization of the peripheral pulmonary arteries
385 was attenuated by the application of H-CPAP ($p=0.03$) (Figure 4 C,D).

386

387 **Hyperoxia induced inflammatory gene expression not altered by CPAP**

388 To investigate the mechanism of hyperoxia induced lung injury we examined the
389 gene expression of inflammatory cytokines IL-8 and CCL-2. Hyperoxia exposure

390 caused significant up-regulation of the expression of both IL-8 ($p < 0.001$) and
391 CCL-2 ($p < 0.001$). H-CPAP did not alter the expression of IL-8 or CCL-2
392 (Supplementary data 3, <https://doi.org/10.6084/m9.figshare.11815785.v1>).
393

394 **Discussion:**

395
396 In this study we evaluated the effect of intermittent daily CPAP on the lung
397 parenchyma, airways, and pulmonary vasculature using the hyperoxia preterm
398 rabbit model of BPD. We tested the hypothesis that by applying a distending
399 pressure to the airways and alveoli, CPAP would have biological effects beyond
400 that of alveolar recruitment. We found CPAP to benefit aspects of lung function
401 and structure (Graphically summarized in Figure 6).
402

403 To prove that CPAP provided lung recruitment in our model we used dark field
404 (DF) imaging. This technique has been shown to be more sensitive to acute lung
405 changes than conventional transmission X-ray imaging and could be a potential
406 tool to identify neonatal lung injury (11, 47). We demonstrated increased DF
407 signal after 4 hours H-CPAP (indicating increased air-tissue interfaces) showing
408 5 cmH₂O CPAP is sufficient to reach and recruit the alveoli in our model. Alveolar
409 recruitment and lung expansion by H-CPAP did not increase lung volume. Both
410 *in vivo* inspiratory capacity and *ex vivo* total lung volume was not increased by H-
411 CPAP. This contrasts a study where CPAP was shown to increase *ex vivo* lung
412 volume in immature ferrets (49). However, in the ferret study continuous CPAP
413 was applied for 2 weeks without lung injury suggesting that either prolonged

414 CPAP is required to influence lung volume, or lung growth is blunted by
415 hyperoxia in our model.

416

417 Hyperoxia disrupted normal lung function development in the preterm rabbit.
418 Hyperoxia-reared pups had stiffer lungs with decreased static compliance and
419 increased static elastance. Similar to the lack of effect on lung volume, H-CPAP
420 did not alter the static pressure-volume-based PFT. Neither static compliance
421 nor static elastance was altered by H-CPAP. A limitation of the static pressure
422 volume lung function maneuvers is the supra-physiological volumes used to
423 perform the tests, limiting the insight into lung function at tidal volumes. Forced
424 oscillation tests (FOT) do not require large volume changes and may provide
425 more insight into lung physiology at tidal volumes. FOT has the additional
426 advantage of using a multi-compartment model to discriminate between
427 peripheral and central lung compartments. FOT examining the respiratory
428 system compliance and elastance were consistent with pressure-volume tests,
429 demonstrating decreased dynamic compliance and increased dynamic elastance
430 following hyperoxia exposure. Peripheral tissue mechanics were disrupted by
431 hyperoxia, with increased tissue damping and tissue elastance. Respiratory
432 system resistance, a combination of central and peripheral lung compartments,
433 was significantly increased following hyperoxia. While central airway resistance
434 was increased, statistical significance was not reached. These findings indicate
435 that hyperoxia exposure altered the function and mechanics of both the airway
436 and peripheral lung. Unlike pressure-volume tests, where H-CPAP had little
437 effect, H-CPAP attenuated the functional changes of hyperoxia in FOT tests. The
438 increase in tissue damping, tissue elastance, and respiratory system resistance

439 improved following H-CPAP, suggesting functional remodeling of the peripheral
440 lung by the distending pressure of CPAP. However, we could not correlate these
441 functional changes to structural changes in the lung. These findings are
442 particularly noteworthy as FOT is increasingly used to evaluate neonatal lung
443 function and have demonstrated functional differences between preterm infants
444 with and without BPD (43). The influence of CPAP on forced oscillation
445 pulmonary function tests in neonates has not been examined.

446

447 Hyperoxia exposure remodeled the alveolar structure, resulting in larger alveoli
448 with thicker alveolar septa and a decreased RAC. These alveolar findings are
449 consistent with those of murine BPD models where hyperoxia exposure
450 increased mean linear intercept and alveolar septal thickness, and decreased the
451 RAC (6, 27). Similarly, human tissue studies have demonstrated infants with BPD
452 to have increased alveolar size and fibrosis of the alveolar septa (12). In line with
453 the baboon model, alveolar morphology was not further disrupted by CPAP (40).
454 The lack of effect on alveolar structure by CPAP, described in our study and the
455 baboon study, could provide insight as to why CPAP has not significantly
456 changed the incidence of BPD. However, the effect of CPAP on human alveolar
457 development remains unknown.

458

459 In keeping with the remodeling of the alveolar septa, the lung parenchyma was
460 altered by hyperoxia exposure. Both total parenchymal tissue and collagen
461 content are increased following hyperoxia exposure. This corresponds with the
462 increased alveolar septal thickness found in alveolar morphology. In our model
463 lung elastin content was unaffected by hyperoxia or H-CPAP. While the quantity

464 of elastin was not altered by hyperoxia we were unable to evaluate the quality of
465 elastin or the crosslinking of collagen and elastin which is disrupted by
466 hyperoxia (24). Lung development and function depends on the appropriate
467 development of the ECM and disruption of either the quantity or quality of the
468 ECM adversely effects lung structure and function (14, 23). While H-CPAP did not
469 alter total lung parenchymal tissue, a trend towards decreased collagen content
470 was present. This contrasts findings in mechanical ventilation where positive
471 pressure ventilation has been shown to increase interstitial tissue content of
472 infants who demised with BPD (39). Our findings suggest that CPAP has a less
473 disruptive effect on the lung parenchymal than mechanical ventilation.
474 However, the effect of CPAP on the ECM of the infant lung is uncertain.

475

476 Furthermore, we studied the effect of hyperoxia and CPAP on the structure of the
477 airway. We found an increase in respiratory epithelial height following
478 hyperoxia exposure indicating respiratory epithelial injury. Additionally, the
479 airway smooth muscle content (ASM) was significantly increased by hyperoxia.
480 H-CPAP mitigated the increase in respiratory epithelial height, suggesting less
481 epithelial injury. The increase in respiratory epithelial height was prevented by
482 H-CPAP, however no changes were seen in the smooth muscle. The ASM content,
483 while lower following H-CPAP, was not significantly decreased compared to
484 hyperoxia. The structural changes to the airway following H-CPAP did not result
485 in a significant functional improvement in central airway resistance, although a
486 trend towards lower central airway resistance was present. The hyperoxia-
487 associated changes in airway structure we describe are consistent with changes
488 described in both human and animal studies. Thickened respiratory epithelium

489 and increased ASM has been found in infants who died from BPD, suggesting that
490 structural changes to the respiratory epithelium persists beyond exposure to
491 ventilation or oxygen supplementation (42). ASM has been shown to increase
492 following neonatal hyperoxia exposure in rodent models (4, 28). Unlike our
493 findings with H-CPAP, mechanical ventilation has been associated with increased
494 airway injury. Brief mechanical ventilation was shown to result in thickening of
495 the respiratory epithelium and increased airway smooth muscle in fetal lambs
496 (29). With airway dysfunction being the key finding in children surviving with
497 modern BPD, interventions limiting injury to the airway are of particular
498 interest. Our findings suggest that the distending pressure of CPAP could have
499 long-term benefits by preventing hyperoxia-associated changes to the airway.

500

501 In addition to the lung parenchyma and airways, the pulmonary vasculature
502 plays a key role in the pathophysiology of BPD. In our model, hyperoxia exposure
503 thickened the peripheral pulmonary artery medial layer indicating more
504 muscular pulmonary arteries. We have previously shown increased pulmonary
505 artery medial thickness to be associated with pulmonary hypertension (15).
506 CPAP prevented the muscularization of the peripheral pulmonary arteries in
507 hyperoxia. The findings indicate that CPAP could play a role in limiting the
508 development of pulmonary hypertension in the context of prematurity and
509 hyperoxia exposure. Clinically this could have a large impact, as pulmonary
510 hypertension occurs in 20% of infants with BPD, and is associated with
511 increased mortality and in combination with mechanical ventilation is a
512 predictor of respiratory disease later in life (1, 25).

513

514 To further examine the effect of CPAP on the lung we evaluated the gene
515 expression of inflammatory cytokines CCL-2 and IL-8. CCL-2 and IL-8 mRNA was
516 identified by previous transcriptome analysis in our model to be significantly up
517 regulated in the lung by hyperoxia (35). Hyperoxia exposure significantly
518 increased the inflammatory response in the lung. Unlike mechanical ventilation,
519 H-CPAP did not further increase inflammation. These findings support the use of
520 CPAP to prevent lung injury but do not explain the mechanism by which CPAP
521 benefits the lung in hyperoxia. We hypothesize that the benefits of CPAP result
522 from the biological transduction of physical stretch of the lung. *Ex vivo* studies
523 have demonstrated lung stretch and the activation of the YAP/TAZ pathway to
524 have multiple effects on the lung and lung development (18, 20, 21, 30). To date
525 the influence of CPAP on the induction of stretch and the biological effect of
526 stretch induction remain unknown.

527

528 We chose the preterm rabbit to evaluate CPAP in the context of hyperoxia as the
529 model has several advantages. Firstly, the rabbit has similar lung development to
530 humans with alveolar development beginning in utero. Secondly, pups are able
531 to survive preterm delivery in the late saccular/early alveolar phase of lung
532 development (34). Preterm birth is essential to develop BPD, with increased
533 prematurity associated with an increased incidence of BPD (17). Term rodent
534 models, such as mice and rats, are often used to model BPD as they are born
535 prior to alveolar development, despite the lungs of these animals being
536 functionally mature. Larger animal models, such as sheep and non-human
537 primates, allow premature delivery and have lung development closer to that of
538 humans. However cost, gestational time, and ethical concerns limit their use.

539

540 Hyperoxia was used together with preterm birth as it is clinically relevant in the
541 multi-factorial development of BPD (3). We acknowledge the extreme hyperoxia
542 is a limitation in our current study as such high levels of inhaled oxygen are not
543 used in clinical neonatology. The phenotype of BPD has changed as modern
544 neonatology now limits oxygen exposure and ventilation associated lung injury.
545 Previously the “old” BPD was associated with high levels of inhaled oxygen and
546 mechanical ventilation in moderately premature infants, resulting in a fibrotic
547 lung disease. With the advances of modern neonatal care the “new” BPD is a
548 more subtle disease with disruption of alveolar development the key pathology
549 in extremely premature infants (16). The majority of animal studies use levels of
550 hyperoxia in excess of that used in modern clinical settings (19). Future animal
551 models should evolve to more accurately reflect the modern “new” BPD.

552

553 Intermittent CPAP was used to investigate the hypothesis that the distending
554 pressure of CPAP is biologically transduced to have lasting functional and
555 structural effect. Lung function testing and lung harvest was performed 18-20
556 hours following CPAP to evaluate whether changes to the lung were maintained
557 after the application of CPAP. We chose to use 4 hours of CPAP as extended
558 periods of CPAP (> 4 hours) in our model led to facial edema and pressure
559 necrosis of the ears. Future experiments should examine whether increased
560 duration of CPAP offers further benefit to the lung. While the functional and
561 structural effect of continuous CPAP as used in modern neonatology is not clear,
562 our findings demonstrate that CPAP can have a lasting effect on the lung.

563

564 This study has numerous strengths and specific weaknesses. We were able to
565 examine extensive *in vivo* lung function testing and examine alveolar,
566 extracellular matrix, airway, and vascular structure in a premature animal model
567 of BPD.

568

569 Weaknesses include the inability to perform serial PFT, and lack of forced
570 expiratory maneuvers and airway reactivity challenges which are planned for
571 future experiments.

572

573 Neonatal care increasingly uses non-invasive ventilation to treat respiratory
574 distress in preterm infants. Whereas there is good evidence for the use of CPAP
575 in the management of acute respiratory distress, the effect of long term CPAP on
576 the developing lung is not clear. Functional and structural data of the effect of
577 CPAP on preterm lung function and long-term lung health are lacking. While new
578 forced oscillation lung function testing techniques will allow lung function
579 testing in neonates, animal studies using preterm animals are required to
580 examine both the structural and functional consequences of CPAP on the
581 developing lung.

582

583 **Conclusion:**

584

585 In this study of a well characterized premature rabbit pup model of BPD we
586 establish that intermittent CPAP has advantageous functional and structural
587 effects on preterm lungs exposed to hyperoxia. Intermittent CPAP limited the
588 consequence of preterm hyperoxia exposure however hyperoxia-induced

589 alveolar injury was unaffected. Future experiments investigating CPAP should
590 examine the mechanisms underlying the biological effects of applying a
591 distending pressure to the lung.

592

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781 Figure Legends:

782

783 Figure 1: (A) study design for intermittent CPAP in the hyperoxia preterm rabbit
784 model of bronchopulmonary dysplasia (B) image of rabbit with custom latex
785 sleeve and connector to attach the rabbit to the Babylog 800 respiratory circuit
786 (C) Kaplan-Meier survival curve (D) Schematic diagram of the circuit used to
787 deliver CPAP to the preterm rabbit.

788

789

790 Figure 2: CPAP increases lung aeration (4 hours, 5cm H₂O) on day 1 of life in
791 preterm rabbits reared in hyperoxia (A) Representative dark field and
792 conventional X-ray images pre- and post-CPAP (B) Dark field lung aeration
793 scores, T-0 and T+4 hours (C) Mean change in dark field scores between T-0 and
794 T+4 hours, n=8 for all groups, *p<0.05

795

796 Figure 3: The functional consequence of hyperoxia and CPAP in the hyperoxia
797 preterm rabbit model of bronchopulmonary dysplasia. (A-C) pressure-volume
798 maneuvers (normoxia n=15, hyperoxia=23, H-CPAP n=17) (A) Inspiratory
799 capacity corrected for weight (B) static compliance corrected for weight (C)
800 static elastance corrected for weight. (D-F) forced oscillation maneuvers
801 (normoxia n=15, hyperoxia n=22, H-CPAP n=17) (D) single wave respiratory

802 system resistance (E) broadband tissue damping (F) broadband tissue elastance.

803 Values expressed as mean $\pm$ SD, *p-values adjusted for multiple comparisons

804 (Sidak) *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001 n.s.=non-significant

805

806

807 Figure 4: Lung parenchyma, lung collagen content (normoxia n=13-14, hyperoxia

808 n=18-19, H-CPAP n=16-17) and peripheral pulmonary artery morphology

809 (normoxia n=12, hyperoxia n=16, H-CPAP n=12) (A) Total lung parenchymal

810 tissue volume (B) lung parenchymal collagen content (C) Peripheral pulmonary

811 artery morphology (D) Representative images of picrosirius red staining viewed

812 under polarizing light and pulmonary arteries stained with Miller's elastin stain.

813 Scale bar represents 50 μ m. Values expressed as mean $\pm$ SD, *p-values adjusted

814 for multiple comparisons (Sidak)*p<0.05 **p<0.01 ***p<0.001 n.s.=non-

815 significant

816

817

818 Figure 5: Airway morphology (A) Respiratory epithelial height (normoxia n=13,

819 hyperoxia n=19, H-CPAP n=18) (B) representative H&E images of respiratory

820 epithelial height (C) Airway smooth muscle content (normoxia n=12, hyperoxia

821 n=20, H-CPAP n=15), (D) representative alpha smooth muscle actin images. Scale

822 bars of 50 μ m in representative airway images, scale bar of 25 μ m in zoomed in

823 airway wall images. Values expressed as mean $\pm$ SD, *p-values adjusted for

824 multiple comparisons (Sidak) *p<0.05 **p<0.01 ***p<0.001 n.s.=non-significant

825

826 Figure 6: The functional and structural effects of continuous positive airway
827 pressure in the hyperoxia preterm rabbit model of bronchopulmonary dysplasia

A

B

C

D

A

B

C

BRONCHOPULMONARY DYSPLASIA

CONTINUOUS POSITIVE AIRWAY PRESSURE

	Normoxia (N)	Hyperoxia (H)	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 ₂ O/ml/kg	2.29±0.51	1.47±0.72	p<0.001	1.37±0.36	p=0.83
Static elastance cmH ₂ O.kg/ml	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 ₂ O/ml	1.90±0.48	3.08±1.01	p<0.0001	2.48±0.66	p=0.04
Tissue Elastance cmH ₂ O/ml	6.45±1.44	11.4±3.54	p<0.0001	9.25±2.11	p=0.03
Central airway resistance cmH ₂ O.s/ml	0.12±0.05	0.15±0.05	p=0.08	0.12±0.04	p=0.10
Respiratory system resistance cmH ₂ O.s/ml	0.33±0.06	0.54±0.16	p<0.0001	0.43±0.11	p=0.02
Dynamic Elastance cmH ₂ O/ml	6.34±1.75	12.99±4.57	p<0.0001	10.48±2.78	p=0.06
Dynamic Compliance mL/cmH ₂ O	0.16±0.04	0.088±0.03	p<0.0001	0.10±0.02	p=0.3

Table 1: The effect of hyperoxia and CPAP on pulmonary function tests in the hyperoxia preterm rabbit model of BPD. Pressure volume (normoxia n=15, hyperoxia=23, H-CPAP n=17) and forced oscillation (normoxia n=15, hyperoxia n=22, H-CPAP n=17) tests performed on day 7 of life following preterm delivery. Values expressed as mean ± SD, *p-values adjusted for multiple comparisons (Sidak)

	Normoxia (N)	Hyperoxia (H)	H vs N *p-value	H-CPAP	H-CPAP vs H *p-value
Lm (μm)	71.4 $\pm$ 5.4	79.9 $\pm$ 6.2	p<0.001	79.9 $\pm$ 6.6	p=0.99
Lma (μm)	58.8 $\pm$ 4.7	62.7 $\pm$ 6.4	p=0.1	62.5 $\pm$ 5.9	p>0.99
Lmw (μm)	12.6 $\pm$ 1.6	17.3 $\pm$ 2.5	p<0.0001	16.8 $\pm$ 1.8	p=0.77
Radial alveolar count	6.8 $\pm$ 0.5	5.0 $\pm$ 0.3	p<0.0001	5.2 $\pm$ 0.4	p=0.31
Left lung volume (ml/kg)	34.7 $\pm$ 4.8	29.9 $\pm$ 5.5	p=0.02	30.2 $\pm$ 5.0	p=0.97

Table 2: The effect of hyperoxia and CPAP on alveolar morphology in the hyperoxia preterm rabbit model of BPD. Normoxia n=17, Hyperoxia n=18, H-CPAP n=17. Values expressed as mean $\pm$ SD, * p-values adjusted for multiple comparisons (Sidak), Mean linear intercept (Lm), Mean transactional wall length (Lmw), Mean airspace size (Lma)