In vitro and *in vivo* comparison between poractant alfa and the new generation synthetic surfactant CHF5633

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BACKGROUND: CHF5633 is a new generation synthetic surfactant containing both SP-B and SP-C analogues developed for the treatment of respiratory distress syndrome. Here, the optimal dose and its performance in comparison to the animal-derived surfactant poractant alfa were investigated.

METHODS: *In vitro* surfactant activity was determined by means of the Wilhelmy balance and the capillary surfactometer. The dose-finding study was performed in preterm rabbits with severe surfactant deficiency. CHF5633 doses ranging from 50 to 300 mg/kg were used. Untreated animals and animals treated with 200 mg/kg of poractant alfa were included for comparison.

RESULTS: *In vitro*, minimum surface tension (γ_{min}) was decreased from values above 70 to 0 mN/m by both surfactants, and they formed rapidly a film at the air–liquid interface. *In vivo* studies showed a clear dose-dependent improvement of lung function for CHF5633. The pulmonary effect of CHF5633 200 mg/kg dose was comparable to the pulmonary response elicited by 200 mg/kg of poractant alfa in preterm rabbits.

CONCLUSION: CHF5633 is as efficient as poractant alfa in our *in vitro* and *in vivo* settings. A clear dose-dependent improvement of lung function could be observed for CHF5633, with the dose of 200 mg/kg being the most efficient one.

Respiratory distress syndrome (RDS) is a condition of pulmonary insufficiency caused by a primary deficiency of alveolar surfactant. The lack of surfactant results in a high intra-alveolar surface tension that leads to alveolar collapse. Preterm neonates developing RDS have very low intrapulmonary surfactant pool (1–4) several folds lower than the pool size estimated for term neonates (5). Surfactant replacement therapy, which consists on instilling a dose of exogenous surfactant intratracheally, improves rapidly the overall lung function and reduces the mortality and the morbidity associated with RDS (6).

At the early development stages of the therapy, synthetic surfactants containing exclusively phospholipids were formulated (7,8). Further research clearly showed that the dynamic properties of R-dipalmitoylphosphatidylcholine (DPPC) alone are suboptimal (9), and that the presence of the hydrophobic surfactant proteins (SP), SP-B and SP-C, in natural surfactants significantly enhances the surface tension reduction action and provides stability to compressed films (10,11). Surfactant preparations containing both SP-B and SP-C show a combined effect of surfactant function (12). In fact, animal-derived surfactant preparations, commonly used in clinical practice, contain both SP-B and SP-C (13).

In view of the major role played by SP-B and SP-C for an optimal surfactant function, second-generation synthetic surfactants incorporated peptides that would further mimic the action of SP-B and SP-C (14). The rationale to develop synthetic surfactants has traditionally relied on the potential of improving the quality control of surfactant, reducing batch-to-batch variability, and avoiding the need of animal reservoir. Moreover, a very interesting feature of peptide-containing synthetic surfactants is their potential to resist inhibition in the setting of inflammation or edema (very likely to occur in the course of RDS) to a larger extent than animal-derived preparations (15,16).

The first peptide-containing synthetic surfactant preparations consisted of phospholipids and a peptide mimic of either SP-B or SPC. *Lucinactant* is an SP-B-based synthetic surfactant composed of a phospholipid mixture and *sinapultide*, a leucine- and lysine-reach peptide aimed at mimicking SP-B function (17). *Synsurf* is a surfactant consisting of a phospholipids mixture and two polypeptides: poly-l-lysine electrostatically complexed with poly-l-glutamic acid (18). *Lusupultide* is a mixture of phospholipids supplemented with 2% of human recombinant SP-C (19). These preparations showed promising results in preclinical models of lung disease (20,21) and some of them were tested in clinical trials (19,22–24). However, increased improvement of lung function by synthetic surfactants containing both SP-B and SP-C analogues, over that with a single surfactant protein, have previously been suggested (25,26).

Chiesi Farmaceutici has developed a new synthetic surfactant, CHF5633, which contains DPPC and 1-palmitoyl-2oleoyl-glycero-3-phospho-1-glycerol (POPG) (1:1) and incorporates analogues of both SP-B and SP-C, 1.5% of the peptide SP-C

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analogue and 0.2% of the peptide SP-B analogue, respectively. This composition yielded a drug product with optimal efficacy and stability behavior. Preliminary investigations with CHF5633 in preterm lambs with severe surfactant deficiency have shown superior arterial oxygenation and lung compliance compared to the animal-derived surfactant preparation *beractant* (14). CHF5633 has also shown a higher resistance to albumin inactivation and a lower mortality rate in ventilated preterm lambs, compared to the natural surfactant *poractant alfa* (16). In these studies, CHF5633 was administered at a dose of 200 mg/kg, emulating poractant alfa approved dosage, even if the two surfactants differ in phospholipid composition and contain SP with different length and amino acid composition.

In the present study, our aim was to determine the optimal dosage regimen for CHF5633. We conducted an *in vivo* dose–response study in a rabbit neonatal RDS model with a severe primary surfactant deficiency (27,28). Additionally, we also compared the performance, *in vitro* and *in vivo*, of CHF5633 to the well-tested and highly effective animal-derived surfactant *poractant alfa*.

METHODS

Surfactant Preparations

Poractant alfa (Curosurf, Chiesi Farmaceutici, Parma, Italy) is a natural surfactant, prepared from porcine lungs, containing almost exclusively polar lipids, in particular PC (about 70% of the total phospholipid content), and about 1% of specific low molecular weight hydrophobic proteins SP-B and SP-C, at a phospholipid concentration of 80 mg/ml.

CHF5633 (Chiesi Farmaceutici, Parma, Italy) is a synthetic surfactant that contains DPPC and POPG at 1:1 ratio (98.3%), SP-C analogue (1.5%), and SP-B analogue (0.2%), at a phospholipid concentration of 80 mg/ml.

In Vitro Surfactant Activity: Wilhelmy Balance

The surface tension of poractant alfa, CHF5633, and saline solution was measured at 37 °C with the Wilhelmy Surface Balance (Biegle, Vienna, Austria). The device uses a platinum plate connected to a strain gauge and inserted 1 mm into the hypophase, consisting of 20 ml of 150 mmol/l NaCl in a teflon through. Surfactant samples (312 µl, at a concentration of 80 mg/ml) or saline were added into the hyphophase, 4 cm away from the platinum plate and allowed to spread spontaneously for 3 min. Recordings were then made during cyclic 50% film compression, at a rate of 1/min, for minimum surface tension (γ min) value measurement. Four batches of CHF5633 were tested in parallel with poractant alfa. Saline solution was measured for comparison.

In Vitro Surfactant Activity: Capillary Surfactometer

The ability of CHF5633 and poractant alfa to form a film at the air–liquid interface was evaluated with the Capillary Surfactometer. This instrument simulates the morphology and function of a terminal conducting human airway with a glass capillary that in a short section is particularly narrow. A volume of 0.5 µl of the sample under test (CHF5633, poractant alfa, or saline solution) was deposited in the capillary and a flow of air was applied. The pressure inside the capillary was measured and recorded for 2 min.

Dose Finding of CHF5633 in Preterm Newborn Rabbits

Pregnant New Zeeland White rabbits (3.4–4.2 kg) were supplied by Charles River (Domaine des Oncins, France) and housed until the 27th day of gestation under standard conditions, according to the current procedures for animal housing and handling. The experimental procedure was approved by the Ethics Committee in animal experiments of Chiesi Farmaceutici and met the standard European regulations on animal research. Management and use of the animals complied with the EEC and national regulations for animal care.

On the 27th day of gestation does were given diazepam (5 mg/kg) and, 10 min later, were anesthetized with ketamine (25 mg/kg). As soon as the anesthesia was effective, the fetuses were delivered by cesarean section, immediately anesthetized with sodium pentobarbital (0.6 mg, i.p.) and tracheotomized, using a stainless steel cannula as tracheal tube (inner diameter: 2 mm). Fetuses, paralyzed with pancuronium bromide (0.06 mg, i.p.), were allocated to each treatment group and submitted to artificial ventilation with 100% oxygen, for 30 min using a servo-ventilator (Siemens 900 C, Erlangen, Germany) according to a fixed ventilation, as previously described (29). Briefly, fetuses were initially ventilated for 15 min with a positive inspiratory pressure (PIP) of 25 cmH_2O, followed by 5 min at a PIP of 20cmH_2O, another 5 min at a PIP of 15 cmH_2O, and a final step of 5 min in which the PIP was restored at the initial level of 25 cmH_2O. PEEP was not applied during the experiments. Tidal volume (V_T) and dynamic compliance (C_{dyn}) were recorded every 5 min with a computerized system (PowerLab, ADI instruments, Dunedin, New Zealand). Electrocardiogram (ECG, BioAmplifier, PowerLab, ADInstruments) was recorded immediately before the initiation of the ventilation and right after the end of the 30 min of experimental time. Animals with a normal P wave/QRS complex were counted as survivors. At the end of ventilation protocol, fetuses were sacrificed with Tanax (0.1 ml intracranial) and their abdomen inspected for pneumothorax (PTX). Animals with evidence of PTX were excluded from the study.

Data Analysis

Parametric data are given as mean ± SEM. Comparisons between groups for V_T and C_{dyn} were evaluated by Student's *t*-test with Levene's test for equality of variances (significance level $P < 0.05$). The correlation between the administered dose of CHF5633 and the observed V_T was assessed by linear regression analysis. Fisher's exact test was used to compare survival between groups ($P < 0.01$ was accepted as significant).

RESULTS

In Vitro Surfactant Activity: Wilhelmy Balance

Surface tension measurements with the Wilhelmy balance clearly demonstrated that poractant alfa and CHF5633 were very effective in reducing surface tension, compared to saline. Minimum surface tension (γ_{min}) was decreased from values above 70 to 0 mN/m by both surfactants (**Table 1**). The films formed by CHF5633, however, showed slightly higher maximum surface tension (γ_{max}) and surface area values. Both surfactant preparations showed homogeneous results irrespective of the measured batch, indicative of a consistent surfactant performance and reduced batch-to-batch variability.

In Vitro Surfactant Activity: Capillary Surfactometer

Poractant alfa and CHF5633 formed rapidly a film at the air–liquid interface within the capillary lumen so that, the air could continuously flow through the capillary and the pressure within the capillary was maintained near zero for the whole 120 s recording time. The capillary remained open 99.9% of the 120 s recording time either poractant alfa or CHF5633. Conversely, in the presence of saline, the airflow was continuously interrupted and capillary patency could be only maintained for less than 10 s, accounting for just 3.9% of the recording time. **Figure 1** shows representative recordings for (i) saline, (ii) poractant alfa, and (iii) CHF5633; the initial pressures for poractant alfa and CHF5633 were 6.6 and 3.8 cmH_2O, respectively, whereas the initial pressure with saline was 10.5 cmH_2O.

Table 1. Wilhelmy surface balance analysis of CHF5633 vs. Poractant alfa

Poractant alfa				CHF5633			
Batch #	**γ_{min} (mN/m)**	**γ_{max} (mN/m)**	**Area (mN)**	**Batch #**	**γ_{min} (mN/m)**	**γ_{max} (mN/m)**	**Area (mN)**
#1	0.0	32.8	1.13	**#1**	0.0	37.0	1.06
#2	0.0	30.2	0.97	**#2**	0.0	38.2	0.76
#3	0.0	30.5	1.06	**#3**	0.0	38.1	0.81
#4	0.0	30.4	1.06	**#4**	0.0	36.4	0.84
Saline	70.6	71.2	0.24	**Saline**	70.9	71.8	0.28

Maximum and minimum surface tension and surface area values obtained with the Wilhelmy Balance for poractant alfa and CHF5633.

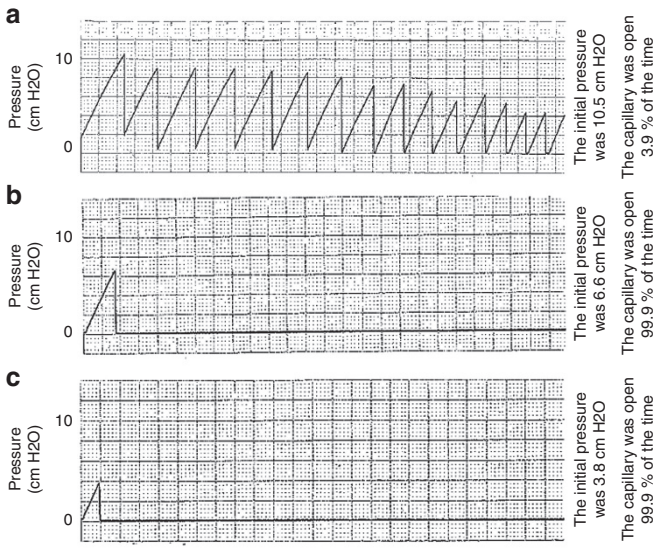

Figure 1. *In vitro* surfactant activity analysis by capillary surfactometer. Representative pressure tracings from the capillary surfactometer achieved after testing (**a**) saline solution, (**b**) poractant alfa (Curosurf), or (**c**) CHF5633.

Dose Finding of CHF5633 in Preterm Newborn Rabbits

The efficacy of different doses of CHF5633 for RDS treatment was then compared in premature newborn rabbits. The animals from each litter (15 litters in total) were allocated to five or six experimental groups, depending on the number of fetuses. The animals allocated in the control group received no material (negative control, $n = 21$). A group of animals received a 200 mg/kg dose of poractant alfa intratracheally (positive control, $n = 20$), and four further groups received CHF5633 at doses of 50 ($n = 23$), 100 ($n = 19$), 200 ($n = 22$), and 300 mg/kg ($n = 18$). All surfactant preparations were given at a phospholipid concentration of 80 mg/kg. Sample size, body weight, and the mean C_{dyn}, 5 min after the initiation of mechanical ventilation are presented in **Table 2**. No significant difference was found between groups in terms of bodyweight. Irrespective of the surfactant preparation (poractant alfa or CHF5633) or the dose (range 50–300 mg/kg), surfactant administration significantly improved lung function in comparison to untreated control animals ($P < 0.0001$).

The administration of CHF5633 showed a clear dose-dependent improvement of lung function (**Figure 2a**). Five minutes after surfactant administration, the dose of 100 mg/kg of CHF5633 was associated with a significantly higher mean C_{dyn} than with 50 mg/kg ($P < 0.01$), while lung function after administration of 200 mg/kg of CHF5633 was significantly better than with 100 mg/kg ($P < 0.01$). Further increase of the CHF5633 dose to 300 mg/kg did not produce any significant benefit in lung function, being the mean C_{dyn} values for this dose slightly but not significantly lower than with the 200 mg/kg dose. Within the CHF5633 dose-range of 50 to 200 mg/kg, the linear regression analysis showed a positive correlation between the phospholipid concentration and the V_T ($r = 0.791$, $P < 0.01$, **Figure 2b**).

Table 2. CHF5633 dose-finding

Surfactant/dose	*n*	Body weight (g)	$C_{dyn25cmH2O}$ 5′ (ml/cmH_2O/kg)
Control (no surfactant)	21	28 ± 1	0.06 ± 0.01
CHF5633/50 mg/kg	23	32 ± 1	0.22 ± 0.02*
CHF5633/100 mg/kg	19	30 ± 1	0.40 ± 0.04*
CHF5633/200 mg/kg	22	29 ± 1	0.67 ± 0.05*
CHF5633/300 mg/kg	18	28 ± 1	0.56 ± 0.05*
Poractant alfa/200 mg/kg	20	29 ± 1	0.60 ± 0.06*

Sample size, body weight, dynamic compliance 5 min after the start of mechanical ventilation, and survival ratio of the experimental groups.
*P vs. control (no surfactant) < 0.0001.

Throughout the whole experimental period, no significant differences in terms of V_T were observed between CHF5633 doses of 200 and 300 mg/kg and poractant alfa (200 mg/kg), regardless of the PIP level (**Figure 3**). At a PIP level of 25 cmH_2O, poractant alfa performed significantly better than CHF5633 50 mg/kg at any point ($P < 0.0001$). Significant differences of lung function favoring poractant alfa in comparison to CHF5633 administered at 100 mg/kg dose could also be detected 5 and 30 min after surfactant administration ($P < 0.01$). At high PIP, both CHF5633 200 and 300 mg/kg groups achieved significantly higher mean V_T than the CHF5633 100 mg/kg group ($P < 0.01$).

Lowering the PIP to 20 cmH_2O (at 20 min) produced a dramatic decrease of the V_T in all surfactant-treated groups. At this time point, significant differences between surfactant-treated groups in V_T occurred only between CHF5633 50 mg/kg group and CHF5633 200 mg/kg and poractant alfa groups ($P < 0.01$). Lowering the PIP to 15 cmH_2O decreased the V_T further to the level of the untreated control animals (alveolar collapse).

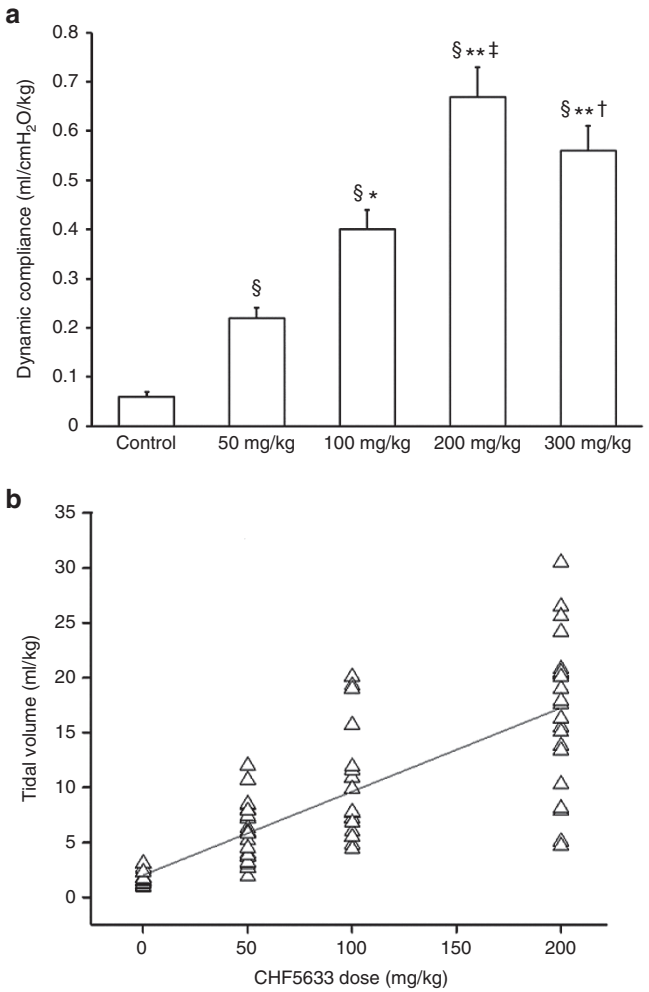

Figure 2. Dose-dependent improvement of lung function. Dynamic compliance after 5 min of mechanical ventilation (**a**) and linear regression analysis ($r^2 = 0.628$) of the tidal volume and the surfactant dose after 15 min of mechanical ventilation (**b**) in preterm rabbits with severe surfactant deficiency treated with different doses of the synthetic surfactant CHF5633. §*P* vs. Control (no surfactant) < 0.01; **P* vs. 50 mg/kg < 0.01; ***P* vs. 50 mg/kg < 0.0001; †*P* vs. 100 mg/kg < 0.01; ‡*P* vs. 100 mg/kg < 0.0001.

After restoring the PIP to 25 cmH_2O, at 30 min, the previously observed dose-dependent effect on lung function was again observed among the CHF5633-treated groups. At this time point, no significant differences between poractant alfa, CHF5633 200 mg/kg, and CHF5633 300 mg/kg were observed. However, the mean V_T of these three groups was significantly higher than those observed for CHF5633 100 mg/kg and CHF5633 50 mg/kg.

In Vivo Comparison Between Poractant Alfa and CHF5633 Administered at 200 mg/kg

In order to point out any potential difference in terms of lung mechanics between poractant alfa and CHF56333, the aforementioned experiment was replicated in a number of additional litters with a higher statistical power comparing the most effective CHF5633 dose (200 mg/kg) to a 200 mg/kg dose of poractant alfa, a dose indicated for the clinical treatment of RDS. In

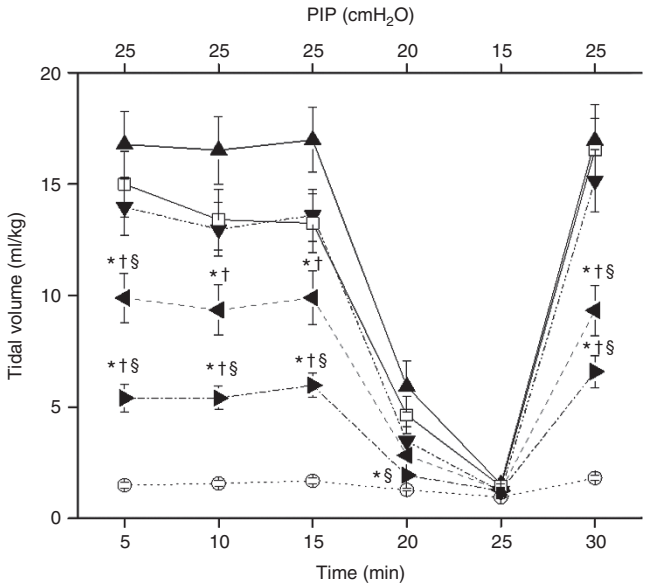

Figure 3. Dose-dependent response to PIP cycles. Tidal volumes (y-axis) in preterm rabbits with severe surfactant deficiency treated either with different CHF5633 doses (right-pointing solid triangles 50 mg/kg; left-pointing solid triangles 100 mg/kg; down-pointing solid triangles 200 mg/kg; and up-pointing solid triangles 300 mg/kg) or with a standard clinical dose of poractant alfa (200 mg/kg, empty squares). Untreated animals managed on mechanical ventilation (empty circles) are shown for comparison. Fetuses were initially ventilated for 15 min with a positive inspiratory pressure (PIP) of 25 cmH2O, followed by 5 min at a PIP of 20cmH2O, another 5 min at a PIP of 15 cmH2O, and a final step of 5 min in which the PIP was restored at the initial level of 25 cmH2O (x-axis, top). **P* vs. CHF5633-200 mg/kg < 0.01; †*P* vs. CHF5633-300 mg/kg < 0.01; §*P* vs. poractant alfa-200 mg/kg < 0.01.

this new set of experiments, the animals from each litter (16 new litters in total) were allocated to one of the three experimental groups: (i) Control group, which received no material ($n = 38$), (ii) poractant alfa group, which received 200 mg/kg of poractant alfa intratracheally ($n = 52$), or (iii) CHF5633 group, which received 200 mg/kg of CHF5633 intratracheally ($n = 53$). Sample size, body weight, and dynamic compliance values at 5 min (PIP 25 cmH_2O), 25 min (PIP 15 cmH_2O), and 30 min (PIP 25 cmH2O) are shown in **Table 3**. No significant differences between groups were found for the body weight. Compared to untreated control animals, both surfactant preparations significantly improved C_{dyn} already 5 min after surfactant treatment ($P < 0.0001$). C_{dyn} dropped in all groups as a consequence of turning the PIP down from 25 to 15 cmH_2O. C_{dyn} values with the PIP set at 15 cmH_2O were virtually the same in both surfactant-treated groups and significantly higher than those of the control group ($P < 0.01$). After restoring the PIP to 25 cmH_2O, C_{dyn} values in both surfactant-treated groups rapidly increased to the levels observed 5 min after surfactant administration.

The V_T of animals treated with 200 mg/kg of CHF5633 or poractant alfa were significantly higher compared to untreated control animals (**Figure 4**). The V_T of the CHF5633 200 mg/kg group remained stable for the first 15 min after surfactant administration, whereas in the poractant alfa group, V_T gradually decreased in the same time period compared to

Table 3. CHF5633 vs. Poractant alfa 200 mg/kg

Surfactant/dose	*n*	Body weight (g)	$C_{dyn25cmH2O}$ 5′ (ml/cmH_2O/kg)	$C_{dyn15cmH2O}$ 25′ (ml/cmH_2O/kg)	$C_{dyn25cmH2O}$ 30′ (ml/cmH_2O/kg)
Control/(no surfactant)	38	28 ± 1	0.10 ± 0.02	0.07 ± 0.01	0.10 ± 0.02
CHF5633/200 mg/kg	53	32 ± 1	0.60 ± 0.04**	0.12 ± 0.02*	0.60 ± 0.04**
Poractant alfa/200 mg/kg	52	32 ± 1	0.52 ± 0.04**	0.12 ± 0.02*	0.54 ± 0.04**

Sample size, body weight, and dynamic compliance (C_{dyn}) of the experimental groups. C_{dyn} values correspond to 5 min (PIP 25 cmH_2O), 25 min (PIP 15 cmH_2O), and 30 min (PIP 25 cmH2O) observation points after surfactant administration.
PIP, positive inspiratory pressure.
*P vs. control (no surfactant) < 0.01. **P vs. control < 0.0001.

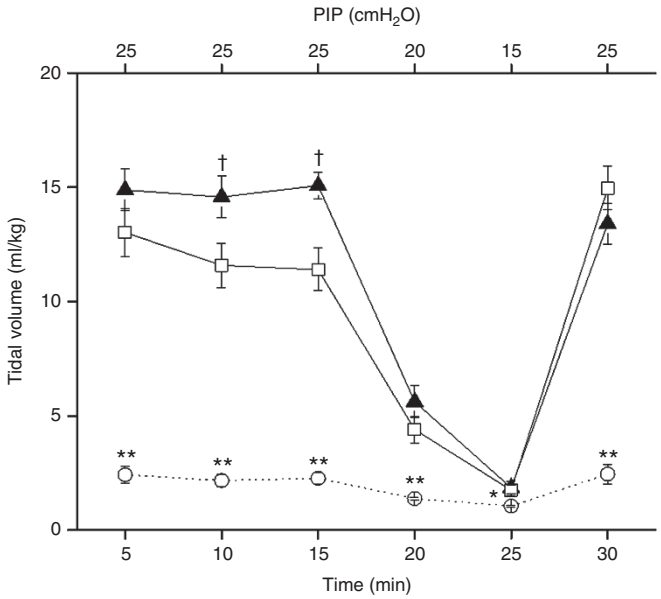

Figure 4. CHF5633 vs. Poractant alfa 200 mg/kg PIP cycles. Tidal volumes (y-axis) in preterm rabbits with severe surfactant deficiency treated either with CHF5633 (200 mg/kg, solid triangles) or with a standard clinical dose of poractant alfa (200 mg/kg, empty squares). Untreated animals managed on mechanical ventilation (empty circles) are shown for comparison. Fetuses were initially ventilated for 15 min with a positive inspiratory pressure (PIP) of 25 cmH2O, followed by 5 min at a PIP of 20cmH2O, another 5 min at a PIP of 15 cmH2O, and a final step of 5 min in which the PIP was restored at the initial level of 25 cmH2O (x-axis, top). *P vs. any surfactant treatment < 0.01; **P vs. any surfactant treatment < 0.0001; †P vs. poractant alfa-200 mg/kg < 0.05.

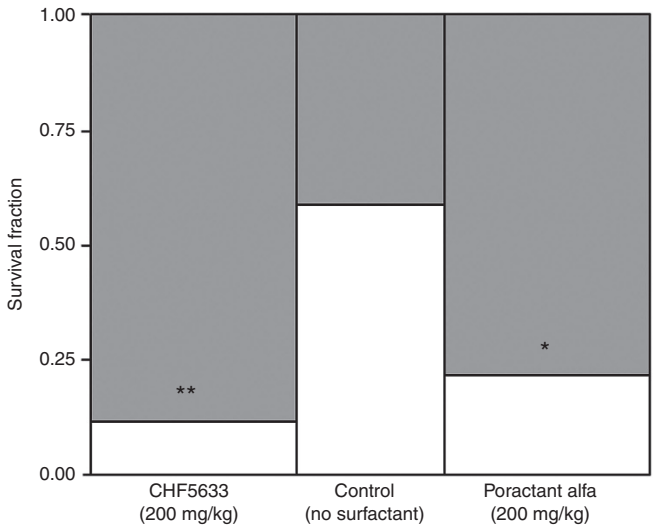

Figure 5. CHF5633 vs. Poractant alfa 200 mg/kg fraction of survivors. Mosaic plot comparing the survival fractions of CHF5633-treated, poractant alfa-treated, and untreated (control) preterm rabbits. The fraction of survivors corresponds to the solid grey area of each group, whereas the fraction of nonsurvivors is represented by the white area below. *P vs. control (no surfactant) < 0.01. **P vs. control < 0.0001.

the initial recorded value. This decrease in the poractant alfa group accounted for a marginal significant difference between surfactant-treated groups, 10 and 15 min after the initiation of mechanical ventilation ($P < 0.05$).

Surfactant-treated animals had a significantly higher fraction of survivors ($P < 0.01$) in comparison to untreated controls (**Figure 5**).

DISCUSSION

CHF5633 is the first synthetic surfactant in clinical development that includes, in addition to phospholipids, both SP-B and SP-C analogues. In the present study, we investigated the performance, *in vitro* and *in vivo*, of the synthetic surfactant CHF5633 and we compared it to the highly-effective clinically available animal-derived surfactant poractant alfa. In particular, we conducted a dose-finding study for CHF5633 in preterm rabbits with severe RDS to identify its optimal therapeutic dose.

In the first place, we compared the performance of CHF5633 and poractant alfa *in vitro*. The critical characteristics of a surfactant preparation intended for treatment of RDS are the efficiency to spread rapidly over the alveolar surface, and the ability to reduce the surface tension to values close to 0 mN/m upon compression, in order to avoid the alveolar collapse at the end of the expiratory phase. In this regard, both surfactant preparations formed rapidly a film at the air-liquid interface as determined by the Capillary Surfactometer, indicative of a rapid spreading of the surfactant films. Moreover, cycling compression with the Wilhelmy Balance yielded minimum surface tension (γ_{min}) values of 0 mN/m for both surfactant preparations, suggesting that both preparations may perform good even in the absence of positive end-expiratory pressure (PEEP). Poractant alfa films were characterized by a lower maximum surface tension (γ_{max}) and a bigger surface area than CHF5633. Nonetheless, the *in vitro* requirements for a "good" pulmonary surfactant were met by CHF5633.

The *in vivo* dose-finding study with CHF5633 showed a clear dose-dependent improvement of lung function. The dose-range

for this study was selected based in the dose-response study performed by Bongrani *et al.* for poractant alfa (29). The linear regression analysis revealed a positive linear correlation between the phospholipid concentration and the improvement of the respiratory parameters in the dose range between 50 and 200 mg/kg. Interestingly, even the lowest CHF5633 dose (50 mg/kg) administered to preterm rabbits elicited a significant improvement of lung function. Dose-dependent improvements of lung function have been described for animal-derived surfactant preparations, including, bovactant (Alveofact, Boehringer) (30), surfactant TA (Surfacten, Tokyo-Tanabe Co) (31), and poractant alfa (29). For the latter, dose-dependent improvements were noted up to a dose of 300 mg/kg (29). In the present study, a further increase of the CHF5633 dose to 300 mg/kg was of no further benefit, and was associated with a slightly lower pulmonary response than the 200 mg/kg dose, probably due to the excessive surfactant volume that had to be intratracheally instilled (3.75 ml/kg of CHF5633 to animals with an initial mean tidal volume of 1.5 ml/kg). However, it should be noted that 200 and 300 mg/kg doses of CHF5633 showed an equivalent pulmonary response as the animal-derived surfactant poractant alfa, administered at 200 mg/kg.

In the applied ventilation protocol, the PIP was stepwise turned down from 25 cmH_2O (0–15 min) to 15 cmH_2O (at 25 min), a fact that induced a dramatic decrease of C_{dyn}, and a drop of V_T in all groups (alveolar collapse). As soon as the pressure was again restored to 25 cmH_2O, all surfactant-treated groups, irrespective of the dose or the surfactant preparation, returned to the "pre-collapse" C_{dyn} and V_T values, which (in addition to the fact that no PEEP was used during the ventilation protocol) highlights the good *in vivo* efficacy of the novel synthetic surfactant CHF5633.

The CHF5633 dose of 200 mg/kg showed the highest efficacy. In order to identify any potential difference in performance between the synthetic and the animal-derived surfactants administered at 200 mg/kg, a new set of *in vivo* experiments with a higher statistical power (n = 52, poractant alfa; n = 53, CHF5633) was designed. During the first 15 min of mechanical ventilation, there was a downwards trend on V_T in the poractant alfa group, while V_T remained constant in the CHF5633 group. This difference in the V_T pattern resulted in transient significant differences between groups at 10 and 15 min. A similar downward trend after poractant alfa administration was also reported by Bongrani *et al.* in the same preterm animal model (29). The reason for this slight decrease of V_T is unknown but is not associated to ventilation management since ventilation settings remained unchanged for the initial 15 min of mechanical ventilation. It could be speculated that soon after the initiation of mechanical ventilation with a high PIP alveolar permeability might increase and the inflammatory cascade might be initiated, resulting in the inactivation of a fraction of the administered animal-derived surfactant by intra-alveolar proteins or inflammatory mediators; this phenomenon might have occurred to a lesser extent for the synthetic surfactant CHF5633 (16). It is noteworthy that the treatment of the preterm rabbits with any surfactant preparation significantly increased the fraction of survivors.

In summary, we found a clear dose-dependent improvement within the dose-range of 50 to 200 mg/kg for CHF5633, with the latter dose eliciting the most efficient pulmonary response in preterm rabbits with a severe surfactant deficiency. Furthermore, a comparison between CHF5633 and poractant alfa administered at 200 mg/kg yielded equivalent results. Therefore, the new generation synthetic surfactant CHF5633 at a dose of 200 mg/kg may perform as well as the animal-derived surfactant poractant alfa in preterm neonates with RDS.

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