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Automated image analysis of alveolar expansion patterns in immature newborn rabbits treated with natural or artificial surfactant

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Summary. Automated image analysis of histological lung sections was used to compare the efficacy of an artificial surfactant (dipalmitoylphosphatidylcholine + high-density lipoprotein, 10:1) and a natural surfactant (the phospholipid fraction of porcine surfactant, isolated by liquid-gel chromatography in ventilated immature newborn rabbits delivered after 27 days' gestation. Tidal volumes were significantly improved in each group treated with surfactant when compared with controls, but natural surfactant-treated rabbits had significantly higher tidal volumes than those receiving artificial surfactant. There were no statistically significant differences in alveolar expansion between the artificial surfactant group and the controls, but alveolar volume density and a shape factor (assessing the 'circularity' of terminal airspaces) were significantly higher in animals receiving natural surfactant. These animals also had a lower coefficient of variation of alveolar volume density and a lower alveolar average integral mean surface curvature, indicating a uniform pattern of alveoli with a smooth profile. We conclude that automated image analysis is useful for the quantitation of alveolar expansion patterns in immature neonatal lungs and that natural surfactant is superior to the artificial surfactant tested in the present study.

Keywords: automated image analysis, alveolar expansion, immature rabbits, tidal volume, natural and artificial surfactants

The expansion pattern of the neonatal lung depends upon the presence of surfactant phospholipids. These lipids facilitate the resorption of fetal lung liquid from the airspaces, and stabilize the aerated alveoli at end-expiration by generating a film with high resistance to surface compression. In the absence of surfactant, the neonatal lungs either remain liquid-filled or exhibit an irregular pattern of overexpanded and collapsed alveoli. These variations in lung expansion can be quantitated in histological sections by conventional morphometry (Weibel 1979) or by automated image

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analysis, using new computer programs developed in our laboratory (Rigaut *et al.* 1983; Rigaut & Robertson 1986).

In the present study we have applied automated image analysis in experiments on immature newborn rabbits, in order to compare the efficacy of two exogenous surfactant preparations: an artificial surfactant with modest effects in a clinical study (Halliday *et al.* 1984; 1986) and the phospholipid fraction of natural (porcine) surfactant, isolated by liquid-gel chromatography. The latter preparation is currently used to treat preterm babies in an international randomized multicenter trial, with promising preliminary results (Robertson 1986).

Materials and methods

Preparation of surfactants. The artificial surfactant is a mixture of dipalmitoylphosphatidylcholine and high-density lipoprotein (10:1, w/w), suspended by sonication in normal saline at a concentration of either 20 mg/ml or 100 mg/ml. The lower of these concentrations corresponds to the preparation used in the human trial (Halliday *et al.* 1984). The higher concentration, 100 mg/ml, was chosen to represent the maximum possible dose under the present experimental conditions; it exceeds the phospholipid concentration in our preparation of natural surfactant (see below).

In the present experiments the natural surfactant used was the phospholipid fraction from minced porcine lungs, isolated by chloroform-methanol extraction and liquid-gel chromatography. This preparation contains approximately 99% phospholipids and 1% proteins with low molecular weight ($\leqslant 15\,000$). It was suspended in normal saline in the same concentration as used in the current clinical trial, 80 mg/ml.

Experimental animals. In-vivo experiments were performed on 29 immature newborn rabbits of gestational age 27 days, obtained from four does by hysterotomy. Neonates were anaesthetized with intraperitoneal sodium pentobarbital (0·1 ml; 6 mg/ml). A tracheotomy was performed immediately after birth and the animals were randomly allocated to one of four groups. One group ($n=7$) received natural surfactant, two groups received artificial surfactant in concentration 20 mg/ml ($n=7$) or 100 mg/ml ($n=8$), and the fourth group ($n=7$) acted as controls. The surfactants were given via the tracheal cannula in a volume of 2 ml/kg and the animals were then transferred immediately to a system of multiple body plethysmographs (Lachmann *et al.* 1981) where pressure-controlled ventilation was carried out in a parallel fashion.

Artificial ventilation. For muscle relaxation, the animals were injected intraperitoneally with pancuronium bromide (0·1 ml; 0·2 mg/ml). They were then ventilated with 100% oxygen using a Servo-Ventilator 900B (Siemens-Elema, Solna, Sweden), set at a frequency of 40 per min and 50% inspiration time. The animals were ventilated with a standardized sequence of insufflation pressures, as previously described (Berggren *et al.* 1985). Tidal volumes were recorded at 5 min intervals by means of a specially designed Fleisch tube (Lachmann *et al.* 1979) connected to the body plethysmograph, a differential pressure transducer (EMT 32, Siemens-Elema), and an integrator unit (EMT 41, Siemens- Elema). The period of artificial ventilation lasted for 30 min, after which an electrocardiogram was recorded.

Preparation of lungs for histological examination. Animals were killed by an overdose of intraperitoneal sodium pentobarbital. The abdomen was opened and the diaphragm inspected for evidence of pneumothorax. Then the thorax was opened and a catheter tied into the pulmonary trunk. The lungs were inflated by raising the transpulmonary pressure to 22 kPa (30 cm H_2O); the pressure was then lowered to 7·4 kPa (10 cm H_2O) maintained for 15 min while the lungs were fixed by vascular perfusion. As fixative a

mixture of 1% glutaraldehyde and 3·5% formaldehyde was used, infused into the pulmonary arterial system at a pressure of 48 kPa (65 cm H_2O). The whole lungs were embedded in paraffin.

Automated image analysis of alveolar expansion. Large 5 μm thick sections from both lungs were stained with haematoxylin (15 min) and toluidine blue, pH 9·0 (2 min). The sections were examined with an IBAS image analyser (Kontron, Munich, FRG), using a program for automated evaluation of multiple stereological parameters of alveolar expansion (Rigaut & Robertson 1986). In the present study, we applied a slightly modified version of our original program, utilizing an autofocus device and a system for automatic, stepwise meandering of the histological section under the microscope. The fields were selected at random, but were rejected if they contained bronchi, bronchioles or large vessels, or if they exhibited obvious artifacts that would interfere with the subsequent measurements. The following parameters, all referring to the alveolar spaces, were determined automatically: volume density (V_V), a shape factor (SF), and average integral mean surface curvature ($\bar{K}$). In each

Table 1. Body weight (mean ± s.d.), tidal volume at 25 cm H_2O after 30 min of artificial ventilation, and heart rate (median values) in animals treated with natural or artificial surfactant, and in littermate controls.

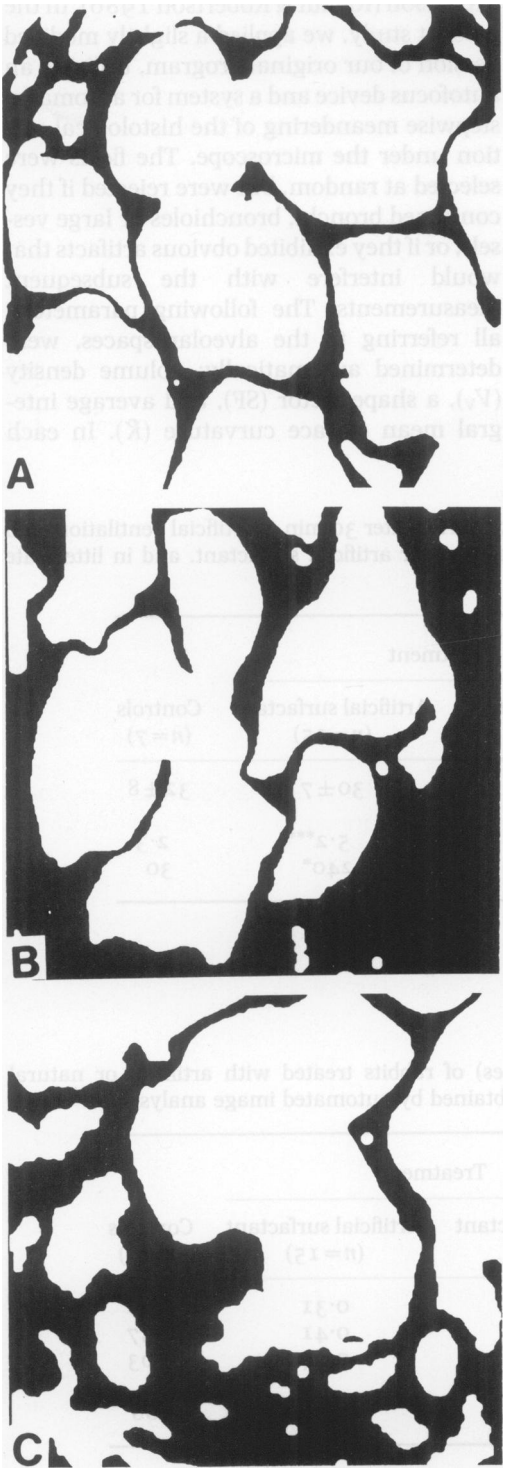
	Treatment		
Parameters	Natural surfactant ($n=7$)	Artificial surfactant ($n=15$)	Controls ($n=7$)
Body weight (g)	33 ± 8	30 ± 7	32 ± 8
Tidal volume at 18·4 kPa (25 cm H_2O) (ml/kg)	21·7***	5·2***	2·3
Heart rate (beats/min)	280**	240*	30

* P *vs* controls $<0·05$.
** P *vs* controls $<0·01$.
*** P *vs* controls $<0·001$.

Table 2. Alveolar expansion patterns (median values) of rabbits treated with artificial or natural surfactant and in littermate controls; the data were obtained by automated image analysis

	Treatment		
Parameters	Natural surfactant ($n=6$)	Artificial surfactant ($n=15$)	Controls ($n=7$)
Volume density (V_V)	0·67**	0·31	0·28
$CV(V_V)$	0·16**	0·41	0·37
Shape factor (SF)	0·09**	0·04	0·03
Average integral mean surface curvature ($\bar{K}$, cm^{-1})	53·5**	362	488

** P *vs* controls $<0·01$.



section, the field-to-field variability of alveolar V_V was assessed by calculating its coefficient of variation (*CV*). *SF* was defined according to the formula:

$$SF = \frac{4\pi\bar{A}}{(\bar{B})^2}$$

where: $\bar{A}$ is the mean alveolar profile area, and $\bar{B}$ the mean alveolar profile length (perimeter). This index is equal to 1 for an individual perfectly circular profile. $\bar{K}$ was obtained by averaging K_V, the integral mean alveolar surface curvature over surface density (Weibel 1979). Our original program has been modified for the correction of field-edge errors when measuring perimeters: the field-edge-intersection lengths are now measured directly by the image analyser. Thereby, the whole program is much faster than before, requiring only about 12 seconds per field.

Statistical analysis. Tidal volume, heart rate and measurements of alveolar expansion patterns were not distributed parametrically; median values are therefore described and differences analysed using the Kruskall–Wallis test. Only for measurements where *P* values $<0{\cdot}01$ were obtained with the Kruskall–Wallis test were multiple Mann–Whitney U tests used for intergroup comparisons. Body weight was distributed normally and is described as mean values $\pm$ s.d., with differences analysed using one-way analysis of variance.

Results

Physiological observations

When the two groups treated with artificial surfactant were compared there were no

Fig. 1. Binarized IBAS-images showing a uniform alveolar air expansion in an animal receiving natural surfactant (*A*), a less uniform expansion pattern following treatment with artificial surfactant (*B*), and an irregular expansion pattern in a control animal (*C*). $\times 225$.

significant differences in tidal volume or heart rate, and there was no trend to larger tidal volumes in the high dose group. For this reason we have analysed them together as a single group to simplify comparisons with the other two groups. Body weights of animals in each group are given in Table 1.

Each of the surfactant-treated groups had improved tidal volumes in comparison to controls ($P<0{\cdot}001$), but the values for animals treated with natural surfactant were significantly greater than those in the artificial surfactant group ($P<0{\cdot}01$) (Table 1).

Heart rate at the end of the experiment generally remained in the normal range for both groups of surfactant-treated animals and was significantly higher than that of controls (Table 1).

Image analysis of alveolar expansion

Differences between natural surfactant-treated animals and each of the two other groups existed for all measurements. V_V and *SF* were thus significantly higher ($P<0{\cdot}01$) and $CV(V_V)$ and *K* were significantly lower ($P<0{\cdot}01$) in the natural surfactant group and the controls. These data are shown in Table 2, and representative IBAS images are illustrated in Fig. 1*A–C*.

Discussion

Automated image analysis proved to be a rapid and, in comparison with conventional morphometry, less tedious method for quantification of alveolar expansion in rabbits treated with exogenous surfactant. Animals receiving natural surfactant had high values for V_V and *SF* and low values for $CV(V_V)$ and *K*, suggesting a uniform pattern of well expanded alveoli with comparatively smooth walls, stabilized during expiration. The morphometric findings are consistent with the higher tidal volumes found in these rabbits and confirm that the isolated phospholipid fraction of porcine surfactant is effective in the immature rabbit (Nohara *et al.* 1986).

The morphometric data from animals treated with artificial surfactant, do not differ from those of the controls. The comparatively high value for $CV(V_V)$ indicates a large field-to-field variability, or a very irregular expansion pattern. This artificial surfactant however, while not being as effective as natural surfactant, does increase tidal volume when compared with controls. It also lowers surface tension *in vitro* although both half adsorption times and equilibrium surface tension levels are higher than those of natural surfactant (Halliday *et al.* 1984). Our reported morphometric data suggest that the modest improvement in tidal volumes was not associated with uniform expansion of alveoli.

The present observations explain why artificial surfactant, made from dipalmitoylphosphatidylcholine and high-density lipoprotein, was ineffective in preventing respiratory distress syndrome in newborns (Halliday *et al.* 1984) and furthermore suggest that increasing the dose of phospholipid from 20 to 100 mg/kg would also be ineffective. We cannot exclude the possibility that the exogenous material might serve as a substrate for endogenous surfactant production after uptake and reutilization in the lung (Jacobs *et al.* 1983), but the potential benefits of such a mechanism could not be evaluated in the present short-term experiments.

Our findings are in agreement with clinical data indicating that natural surfactant is at present the best form of substitution for infants with respiratory distress syndrome (Robertson 1985). However, great care must be taken to ensure that the substances used are bacteriologically inactive and do not cause protein sensitization. Human studies with natural surfactant are currently being undertaken with these aims in mind.

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