Title:

A COMPARISON OF SYNTHETIC SURFACTANTS

Subtitle:

Evaluation of a novel surfactant (1,2-dipalmitoyl-*sn*-phosphatidylcholine and trehalose [$C_{12}H_{22}O_{11}$]) and comparison with other synthetic formulations

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree, accept where assistance is acknowledged.

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Abstract

The aim of this study was to test a synthetic protein-free surfactant preparation, LPM-1, with the same chemical composition as commercially available Exosurf (Glaxo Wellcome), but containing in addition, a sugar, trehalose (TRE). Towards this end, **a study was designed** to firstly test the hypothesis that the true difference in acute physiological effects between a mixture of DPPC, tyloxapol, hexadecanol and trehalose (LPM-1), and Exosurf, (DPPC, tyloxapol and hexadecanol) is zero, in a surfactant-deficient animal model. **A second study** addressed the physiological effects of DPPC, hexadecanol, tyloxapol and trehalose (LPM-1) compared to treatment with trehalose (TRE) or saline, in order to determine (1) the contribution of TRE to the mixture of DPPC, hexadecanol and tyloxapol, and (2) to assess the effect of the LPM-1 surfactant replacement on the epithelial lining fluid composition by means of analysing broncho-alveolar lavage fluid. **Thirdly**, the effects of TRE and / or calcium were studied on the surface properties of DPPC suspensions, by *in vitro* analysis using the ring detachment method of Du Noüy

The *in vivo* research comprised of two studies, performed in randomised controlled fashion. In the first study, 24 New Zealand White adult rabbits were randomised into 4 groups, while in the second study, 15 animals were randomised into 3 groups. In the first *in vivo* study, three synthetic surfactants, LPM-1, Exosurf and LPM-2, and a saline group were tested. LPM-1 is a new formulation that consists of a mixture of DPPC, TRE, hexadecanol and tyloxapol. LPM-2 is a formulation with a composition equivalent to that of commercially available Exosurf, prepared on site.

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In both studies animals were subjected to repeated lavage with large volumes of warm saline (25 ml/kg) in order to establish surfactant deficiency and acute lung injury. Five minutes after the last lavage, vehicle, i.e. surfactants LPM-1, Exosurf, or LPM-2, or saline, in the first *in vivo* study, and LPM-1, TRE or saline in the second *in vivo* study, was instilled, and the course of the animals followed over the next 3 hours. Ventilator settings were standardized before and after lavage. The effects of surfactant treatment on gas exchange (arterial PaO_2, oxygenation index (OI), arterial-alveolar oxygen (a/A) ratio), percentage calculated shunt, and total dynamic respiratory compliance (CRS_{dyn}), and histopathological changes were compared with changes in saline treated controls. Arterial blood gases in 100% oxygen and CRS_{dyn} were measured before and after lavage, at 15 minute intervals for the first 30 min, then at 60, 90, 120, and 180 min after vehicle instillation.

Oxygenation improved to a similar extent after LPM-1 and Exosurf instillation, surpassing that of LPM-2 or saline. Overall, intratracheal instillation of both Exosurf and LPM-1, rapidly improved the gas exchange and reduced the intrapulmonary shunt, but did not restore the lung to its pre-lavage condition. From the 2nd *in vivo* study it was evident that trehalose-only, was inefficient as a lung surfactant, failing to improve oxygenation indices or the calculated percentage shunt, or influencing respiratory compliance. The addition of the sugar, trehalose (TRE), to the on-site 'Exosurf' mixture (LPM-2) brought the activity of the resultant LPM-1 to the same level as that of commercial Exosurf, but failed to raise the activity above that of Exosurf. These physiological improvements were sustained for up to 3 hours. Saline-treated animals had

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no improvement in gas exchange despite management with variable PIP (to maintain a tidal volume of ~10 ml / kg) and constant PEEP of 5 cm H_2O.

In-vitro results, obtained by the Du Noüy tensiometer, showed higher mean ordinate surface tension values for the DPPC-only and DPPC + TRE mixtures, and the slopes of their respective graphs smaller in magnitude than those of the other formulations, suggesting that these formulations had less surface tension-lowering capability than the other surfactants. At 20°C (20 mg / ml DPPC-surfactants) the mean ordinate values of DPPC and DPPC + TRE, 70.13 and 69.47 dyne / cm, respectively, were not significantly different from each other. The mean ordinate values of LPM-1 and the formulation containing DPPC + TRE + tyloxapol + $CaCl_2$ were lower, but similar, as were the values of LPM-2 (on-site Exosurf) and LPM-2 + $CaCl_2$. Thus, three internally homogeneous subgroups could be identified which differed significantly, namely: DPPC and DPPC + TRE, LPM-2 and LPM-2 + $CaCl_2$, and DPPC + TRE + tyloxapol + $CaCl_2$ and LPM-1. Similar conclusions apply to the ordinate values of the surfactants at 37°C, and to the mean slope values at 20°C, with the exception that the subgroups, LPM-2 and LPM-2 + $CaCl_2$, and LPM-1 and DPPC + TRE + tyloxapol + $CaCl_2$ are not so clearly separated. A similar analysis of mean slope values was performed. Here too a significant difference between substances was found, DPPC alone or in combination with TRE, again being significantly different from the other surfactants.

The most prominent light microscopy findings of the lungs of animals included general lymphatic dilatation, congestion and lung polymorphonuclear infiltration, with no difference between study groups. Hyaline membranes were present in all surfactant groups, but significantly more so in the saline treated group. In the first *in vivo* study, the

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presence of neutrophils in the lung interstitium as well as alveoli, was a common finding in all of the study groups towards the end of the study protocol. A significant increase in the BAL-fluid neutrophil count occurred in all animals, concurrent with a significant decrease in the BAL macrophage count. No significant change occurred in the peripheral neutrophil count during the 3-hour study, suggesting recruitment of neutrophils from storage pools. Treatment with synthetic surfactant (LPM-1) did not have a significant effect on modifying the inflammatory response, since there was no significant difference in the BAL-derived cell counts between the LPM-1 and -saline groups. Epithelial damage was a consistent finding in all groups. The damage was more evident by electron microscopy examination and included hydropic changes, most readily observed in the mitochondria. The airspaces of study subjects showed the presence of oedema fluid. This luminal oedema appeared to be more prominent in the control group and LPM-2 (on site 'Exosurf') group. Organellar debris, probably originating from lysis of epithelial cells, was present, despite treatment with synthetic surfactant. The electron microscopical appearance of the epithelial-lined substance ("hyaline membranes") in the present study showed a marked variability within groups as well as within the same case. The majority of cases showed a mix of membrane types with both granular and fibrillar materials present within the same membrane. In some cases there were layering of the membranes into distinct bands. The instillation of LPM-1 resulted in the formation of a slightly different type of epithelial lining fluid after lavage, when compared to the prelavage composition. The most pronounced changes occurred within the fatty acids, whilst the phosphatidylcholine values remained unchanged. Palmitic acid concentrations (C16:0) increased significantly, suggesting enrichment of the epithelial lining fluid after

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instillation of LPM-1. This increase in C16:0 was concurrent with significant decreases in the percentage C16:1, C18:0, and C18:2. In contrast to previous studies, we describe higher levels for phosphatidyldimethylethanolamine (PEA). An explanation may be that the lipid identified as PEA, was in fact partly phosphatidylglycerol (PG)-a lipid whose accurate identification was precluded for technical reasons.

After surfactant instillation, the PC/SM ratio, a reflection of the lecithin / sphingomyelin (L/S), decreased significantly in the TRE-group between the first and final lavage, but remained statistically unchanged in the animals treated with LPM-1 or saline. The change in ratio was mainly accounted for by a decrease in BAL-fluid PC content together with a rise in SM content. A poor correlation existed between the BAL-derived PC/SM ratio and indices reflecting oxygenation status (a/A ratio, OI), as well as the CRS_{dyn} at the time of the final lavage.

In conclusion, the primary hypothesis was accepted, LPM-1 performed similarly to Exosurf *in vivo*, improving oxygenation, but not CRS_{dyn}. None was clearly superior to the other.

Some questions remain. The reason why LPM-1 (LPM-2 + TRE) did not behave in a superior manner, *in vivo,* to Exosurf, is partly unclear. This finding was somewhat surprising since the chemical composition of Exosurf and LPM-2 did not differ, and the addition of TRE to LPM-2 (on-site Exosurf), did improve the *in vivo* activity of the resultant LPM-1, above that of LPM-2. A possible explanation for observed differences in performance include methodological issues, i.e. the preparation of the on-site

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formulations, especially that of LPM-2 (on-site Exosurf), may differ from the way in which true commercial Exosurf is prepared.

Abstrak

Die doel van die studie was om 'n sintetiese proteïn vrye surfaktant te ontwikkel en die produk te vergelyk met 'n kunsmatige surfaktant reeds in kliniese gebruik.

Die bekende uit die literatuur en die onbekende van die produk wat evalueer sou word, lei op tot die samestelling van die **nul hipotese** van die PhD naamlik dat geen verskil in longfunksie sou gewys word tussen die toetsproduk en reeds gebruikte kommersiële surfaktant nie. Die **hipotese** was dat 'n suiker (trehalose), in kombinasie met Dipalmitoiel fosfatidielcholine (DPPC), gaswisseling en longfunksies sal verbeter vir 'n long met 'n lae surfaktant konsentrasie.

Vir die studie is jong volwasse wit New Zealand konyne gebruik en is hulle met 'n gestandaardiseerde en menslike manier gebruik in eksperimentele werk. Die diere is onder intraveneuse narkose geplaas en verskillende kardiovaskulêre en pulmonologiese aspekte is gemeet. Die long surfaktant is uitgewas deur middel van fisiologiese soutoplossing wat tot liggaam temperatuur verhit is en daarna is die diere prospektief gerandomiseer tot eksperimentele groepe.

Met vooraf bepaalde tydsintervalle is die fisiologiese metings herhaal en was die metings toegespits daarop om longmeganiese funksie en gasoordrag vermoë te evalueer. Lig

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mikroskopiese en elektron mikroskopiese studies is ook op die longe gedoen en verder is brongoalveolêre vloeistof ook ontleed.

Die groepe met ondersoek was:

1. DPPC, heksadekanol, tyloxapol en trehalose (LPM-1).
2. DPPC, heksadekanol, tyloxapol (LPM-2 ∴ LPM-1 sonder trehalose). Hierdie is 'n proteïnvrye surfaktant plaaslik berei (dieselfde samestelling as Exosurf).
3. Exosurf®. (Kommersiële preperaat reeds in gebruik). Hierdie is 'n proteïnvrye sintetiese surfaktant.
4. Trehalose, 'n non-reduserende disakklaried van glukose.

Addisioneel is daar ook *in vitro* studies gedoen waarin die oppervlakte spannings aktiwiteite van die verskillende surfaktant oplossings vergelyk is.

Die statistiese analise is gedoen in samewerking met Prof. J. Maritz wat 'n unieke metode ontwikkel en gepubliseer het om herhalende veranderlikes op 'n statisties verantwoordbare manier te ontleed.

In die **eerste** van die studies, is LPM-1, Exosurf®, fisiologiese soutoplossing en 'n plaaslik bereide "Exosurf" (LPM-2), met 'n chemiese samestelling identies aan dié van kommersiële Exosurf®, evalueer.

In 'n **tweede** studie is die fisologiese effekte van LPM-1 vergelyk met trehalose of fisiologiese soutoplossing om die volgende te ondersoek:

1) Die bydrae van trehalose tot 'n mengsel van DPPC, heksadekanol en tyloxapol (LPM-2).
2) Die gevolg van LPM-1 surfaktant toediening op die konyn se brongo-alveolêre vloeistof samestelling.

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'n **Derde**, *in vitro* studie, het die oppervlaktespannings-effekte van trehalose en of kalsiumbyvoegings tot DPPC-oplossings gemeet deur middel van die ring metode van Du Noüy.

In die eerste *in vivo* studie verbeter oksigenasie en persentasie longaftakking tot dieselfde mate na LPM-1 en Exosurf® toediening en word die **hipotese van die proefskrif bevestig**. In die breë gesien, is die tydsprofiele van LPM-1 en Exosurf® ten opsigte van oksigenasie en persentasie longaftakking statisties betekenisvol beter en van 'n sneller aard, as die tydsprofiele van dieselfde indekse na die toediening van fisiologiese soutoplossing of LPM-2. Die tydsprofiel van dinamiese longvervormbaarheid, na die toediening van LPM-1 of Exosurf®, is dieselfde, maar betekenisvol beter as die vervormbaarheid na toediening van LPM-2 of fisiologiese soutoplossing. Alhoewel die oksigenasie indekse in die geval van LPM-1 en Exosurf® betekenisvol verbeter oor die studietydperk, vind volkome herstel tot die basislynwaardes (voor spoeling) nie plaas nie. Bykomend, geen van die surfaktante het na toediening enige noemenswaardige verbetering in longvervormbaarheid tot gevolg gehad nie.

Die rede vir die swakker vertoning van LPM-2 en Exosurf is onbekend en sal in opvolg studie ondersoek word.

In die tweede *in vivo* studie is dit duidelik dat trehalose op sy eie, 'n oneffektiewe surfaktant is aangesien die preperaat na toediening geen verbetering teweegbring ten opsigte van oksigenasie indekse, persentasie longaftakking, of long-dinamiese vervormbaarheid nie. Die toevoeging van trehalose tot LPM-2, om LPM-1 te lewer, neem wel die aktiwiteit van LPM-1 tot dieselfde *in vivo* vlak as dié van kommersiële

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Exosurf®, maar slaag nie daarim om 'n hoër fisiologiese *in vivo* aktiwiteit as dié produk te bereik nie. Die diere wat met fisiologiese soutoplossing behandel is toon geen verbetering in enige fisiologiese parameter nie.

Die *in vitro* resultate wat verkry is deur die Du Noüy tensiometer toon hoër gemiddelde ordinaat oppervlaktespannings waardes vir 'n formule wat slegs uit DPPC bestaan, asook vir 'n mengsel van DPPC + trehalose. Die helling van die grafieke van hierdie oplossings is ook kleiner as die van die ander formulas wat daarop dui dat DPPC op sigself, en DPPC + trehalose, weinig vermoë het om oppervlaktespanning te verminder. Daarteenoor verlaag die volgende oplossings die oppervlaktespanning ten opsigte van gedistilleerde water betekenisvol en wel in 'n konsentrasie afhanklike manier by beide 21°C en 37°C: LMP-1-, LPM-2-, DPPC + trehalose + tyloxapol + $CaC\ell_2$-, en LPM-2 + $CaC\ell_2$.

Die prominentste ligmikroskopiese bevindinge van die longe van die diere sluit in: Algemene limfvat dilatasie, stuwing, en long neutrofiel infiltrasie. Betreffende hierdie histologiese bevindinge is daar geen verskille aangetoon tussen die groepe nie. Hialienmembrane was teenwoordig in al die groepe, maar betekenisvol meer in die groep wat fisiologiese soutoplossing ontvang as vervangingsterapie. In die tweede *in vivo* studie is daar 'n betekenisvolle styging in die neutrofiel- en daling in makrofaagtelling, van die brongoalveolêre vloeistof spoeling in al drie die groep aangetoon. Terselfdertyd vind geen noemenswaardige daling in die perifêre (sistematiese) neutrofieltelling plaas nie. Hierdie bevindinge dui daarop dat die brongoalveolêre selveranderinge toegeskryf kan word aan verwerwing van neutrofiele vanuit 'n longstoringspoel eerder as rekrutering

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vanuit die sistemiese sirkulatoriese poel. Surfaktant (LPM-1), behandeling het geen betekenisvolle vermindering in long inflammasie teweeggebring nie. Epiteelskade was 'n algemene ligmikroskopiese bevinding in al die groepe.

Die samestelling van die brongoalveolêre vloeistof verander na installering van LPM-1. Die prominentste verandering word waargeneem in die vetsuur samestelling terwyl die DPPC waardes onveranderd bly. Die vetsuur, palmitiensuur (palmitic acid), (C16:0), verhoog betekenisvol na toediening van LPM-1. Daarteenoor verminder die konsentrasie van C16:1, C18:0 en C18:2. In kontras met vorige studies, beskryf die huidige studie hoër konsentrasies van fosfatidieletanolamien, moontlik as gevolg van tegniese verskille in die metingsmetodes. 'n Betekenisvolle verlaging in die fosfatidielcholine:sfingomiëlien (PC/SM) verhouding word waargeneem tussen die eerste en die finale longspoeling van die trehalose-groep, terwyl dit onveranderd bly in die diere wat LPM-1 of fisiologiese soutoplossing ontvang.

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On research:

"Do not ask whether these ideas are new or old, but ask primarily, whether they are in accordance with reality"

Malphigi

On the effect of proper gas exchange:

"Air possesses a certain occult virtue. In this virtue the secret food of life lies hidden"

Hermann Boerhaave

On the recipient of the research product:

"I, that am curtail'd of this fair proportion,

Cheated of feature by dissembling nature,

Deform'd, unfinish'd, sent before my time

Into this breathing world scarce half made up-"

Shakespeare: Richard III

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1. A comparison of synthetic surfactants for replacement therapy

1.1 Introduction

The living human being must constantly take up oxygen and eliminate carbon dioxide. In prematures, gas exchange is often seriously hampered by the absence of lung surfactant, manifesting as hyaline membrane disease (HMD), alternatively known as respiratory distress syndrome (RDS).

In developing countries RDS remains the most frequent cause of death in these infants (Luke et al 1993, Kliegman 1995, Department of Health 1994, De Vonderweid et al 1997, Stolz 1998).

In 1929 Von Neergaard noted that the retractile forces of the lung were not only dependent on lung elastic properties, but also on the surface tension in the alveoli. Subsequently, Mead et al (1957) confirmed surface tension as a factor in pulmonary pressure-volume hysteresis. The initial evidence for the existence of a relationship between a disorder of the surfactant system and HMD/RDS was provided by Avery and Mead in 1959, who showed that lung extracts from babies with HMD/RDS were deficient in surface-active substance (Avery and Mead 1959). Pattle (1958) extracted the surface-active material from lungs and described it as a 'substance of waxy consistency producing a film thickness of 58 Angstroms.' This "surface-active substance" subsequently became known as surfactant (Notter and Shapiro 1987).

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The poor expansion of the premature infant's air saccules or alveoli after birth is related to a reduced amount of total phospholipids, especially dipalmitoylphosphatidyl choline (DPPC). The discovery of the association between disorders of surfactant and the pathogenesis of neonatal RDS stimulated research and development of replacement therapy. Initial results with a lecithin (DPPC) aerosol preparation were disheartening (Chu et al 1967). The first properly performed human trial with animal-origin (natural) surfactant was reported in 1980 (Fujiwara 1980) and the treatment appeared to be effective in RDS. Since then, more than 30 randomised, placebo-controlled trials, involving more than 6000 babies, confirmed that both natural surfactants and synthetic formulations, improved acute physiological parameters and mortality from RDS (Halliday 1995, Soll and McQueen 1992). Both types of surfactant reduce the odds of neonatal mortality by about 40% and the odds of pneumothorax by 35-70% (Halliday 1995, Soll 1996). Eighty percent of the decline in the US infant mortality between 1989 and 1990 could directly be attributed to the use of surfactant (Schwartz et al 1994). It has been stated that the story of lung surfactant is, in many ways, one of the most successful triumphs of medical science (Gross 1995).

In developed/industrialised countries, the postnatal treatment of neonatal RDS with surfactant replacement therapy is considered routine practice. In developing countries, such as South Africa, this is not the case. The reasons for the latter are the large number of potential candidates as well as the cost of the commercially available products.

In South Africa, the incidence of low birth weight ranges between 8 and 20% (Smith et al 1996, Deeny et al 1987, van Rijswijk and Ingle 1996, Stein and Ellis 1974). In the Western Cape, the average rate is 17%. This figure is 2-3 times higher than the

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reported rates for industrialised countries (Luke et al 1993). Low birth weight infants are frequently delivered prematurely and have neonatal RDS as one of their commonest clinical problems (Rush and Segall 1978, Cooper et al 1994).

Studies in South Africa have shown that the prevalence of RDS in premature infants differs according to ethnic group (Malan et al 1974, Cooper et al 1994). The recorded incidence of RDS among infants below 34 weeks of gestational age varies between 13% in coloured (Department of Paediatrics and Child Health, Tygerberg Hospital Statistics, 1994), to 36% and 62% (Cooper et al 1994), in black and white infants, respectively. The author estimates that, in South Africa, approximately 8000 newborn infants annually require surfactant replacement for neonatal RDS.

In industrialised countries, the economic impact of surfactant therapy is favourable and the cost per quality-adjusted life year (QALY) for surviving infants is low (Schwartz et al 1994).

Concerns about the sustained high cost of commercial surfactant formulations and possible sensitisation to animal proteins contained in natural surfactant preparations (Strayer and Robertson 1992, Bambang Oetomo 1993), and the search for a surfactant which resembles natural endogenous lung surfactant, have stimulated research efforts directed at the development of alternative synthetic surfactant compounds for treatment of RDS. The optimal form of surfactant in replacement therapy has yet to be identified, be inexpensively produced, and be made available for widespread use (Gross 1995).

Since the discovery of surfactant deficiency as a cause of neonatal RDS, surfactant research focus has shifted from placebo-controlled trials to randomised controlled trials,

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development of new synthetic preparations, methodology of drug administration and the use of surfactant in conditions other than RDS.

Experimental mixtures of surfactant, composed of dipalmitoylphosphatidylcholine (DPPC) as surface-tension lowering agent, peptides, cholesterol, fatty acids (to aid adsorption and spreading), and more recently, non-ionic polymers (to reverse inactivation of natural surfactant), have been studied (Tanaka et al 1986, Taeusch et al 1999). Different clinical surfactant preparations vary with regard to their in vitro biophysical properties and in vivo physiological efficacy in animal models of neonatal RDS (Cummings et al 1992). Protein-free, synthetic surfactants were less effective in lowering surface tension and restoring gas exchange than natural surfactants. However, these findings were at odds with initial placebo-controlled human trial results which recorded similar survival rates for infants for all of the surfactant preparations, regardless of their chemical composition (Soll and McQueen 1992, Holm 1993). As the number of clinical trials increased, the apparent discrepancies between animal and human studies were again highlighted by the results of meta-analyses of head-to-head, randomised controlled trials, comparing synthetic surfactants with natural surfactants in infants with neonatal RDS. Meta-analysis of 6 trials demonstrated a 19% reduction in the odds of death, favouring natural surfactants (Halliday 1995).

The role of surfactant replacement in diseases other than neonatal RDS also requires further study. The dramatic efficacy in neonatal RDS stimulated researchers to consider surfactant administration for acute adult respiratory distress syndrome (ARDS) (Warren 1998). The pathophysiology of ARDS is characterised by multi-organ

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impairment, injury to the alveolar-capillary structure and lung surfactant dysfunction (Lewis and Jobe 1993, Tierney and Johnson 1965, Ashbaugh et al 1967, Petty et al 1979).

In contrast to neonatal RDS, acute ARDS is initially characterised by surfactant inactivation rather than by insufficient production (Lewis and Jobe 1993, Ashbaugh et al 1967, Petty et al 1977, Petty et al 1979, Ueda et al 1994). The initial surfactant inactivation during the course of the disease process is the result of excessive micro-vascular leakage of fluid and proteins (Ueda et al 1994, Fuchimukai et al 1987). Surfactant replacement in acute ARDS would thus have to overcome the inhibitory effects of the alveolar exudates and be administered for longer periods and in sufficient quantities to cover the larger surface area of the adult lung. Larger, and more than the "usual" doses of exogenous surfactant, imply high cost for the initial treatment of these patients.

1.2 Hypothesis for this thesis:

Null hypothesis

The true difference in the acute physiological effects between a mixture of DPPC and Trehalose (a non-reducing disaccharide, unrelated to the known surfactant chemical structure) and commercially available synthetic surfactant (Exosurf, Glaxo Wellcome) is zero.

1.3 The aim of this study

The aim of this thesis is therefore to test the hypothesis that there would be no difference between acute physiological effects resulting from treatment with dipalmitoylphos-

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phatidylcholine (DPPC) supplemented with a non-reducing disaccharide (Trehalose), when compared to Exosurf.

1.4 Literature survey

In 1959, Avery and Mead demonstrated for the first time the absence of the material responsible for lowering surface tension in extracts of lungs of infants who died of HMD, now generally referred to as respiratory distress syndrome (RDS). The authors found that, without exception, the surface behaviour of lung extracts of 9 infants with HMD was different from that of infants who died of other causes but similar to those of infants who weighed less than 1200gram. They hypothesised that a lack of surface-active material was the cause for the raised alveolar surface tension. Early descriptions of the histological examination of the lungs of infants who died due to HMD revealed widespread resorption of air and collapse of the walls of many alveolar ducts and alveoli (resorption atelectasis). The inner surfaces of alveolar ducts that remained open contained a covering layer of homogeneous acidophilic material, described as hyaline membranes (Potter 1957). During this early period some researchers emphasized the abnormal "stickiness" of terminal airways that resisted the entry of air. However, the continued focus placed on the presence of hyaline membranes in autopsy samples, delayed the discovery of the absence of surfactant (Comroe 1977) as the primary cause of neonatal RDS. It was later realised that the pathological hallmark of RDS was diffuse atelectasis, and not the formation of 'membranes', which had a relative non-specific association with RDS. These membranes are mostly found in the dilated terminal

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bronchioles (Finley-Jones et al 1974) and overlay areas from which the airway epithelium is missing (Gandy et al 1970).

The mechanical stability of the lung alveolar structure is largely dependent on the presence of intrinsic surface-active material (Clements et al 1961, Schurch et al 1976). Compared to infants without HMD who have high concentrations of total pulmonary phospholipids (22.3 mg per gram lung tissue), infants who have died with HMD have low concentrations of pulmonary phospholipids (12.6 mg per gram lung tissue) (Brumley et al 1967). The low concentrations of total phospholipid reported in infants dying with HMD are similar to the values obtained from lung extracts of infants who died due to extreme immaturity without HMD (Adams et al 1965).

Although a lung surfactant deficiency initiates neonatal RDS, additional and multifaceted lung pathophysiologic events, including pulmonary arterial hypertension (Evans and Archer 1991); the tendency to develop pulmonary edema (Jobe 1990, DeSa 1969, Sundell et al 1987, Berry et al 1991) and delayed closure of a ductus arteriosus, followed (Archer 1993). The abnormal anatomy, function and dysfunction of the diaphragm, as well as chest wall distortion (Devlieger 1987), usually become evident during the postnatal course of the illness. All of these factors have the potential to interfere with lung mechanics and gas exchange.

In 1929 when von Neergaard noted the effects of surface tension on the pressure-volume characteristics of the excised lung (von Neergaard 1929). He found that the pressure to inflate such a lung with gum arabic in Tyrode solution was about one-quarter to one-third of the pressure needed to achieve the same volume change using air (Hills 1988). Since

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then, comprehensive research has been performed and the relevant data summarized in many reviews (Farrell 1982, Wright and Clements 1987, Hills 1988, Merritt et al 1989, Haagsman and van Golde 1991, Jobe 1993, van Golde et al 1994, Kresch et al 1996, Halliday 1997, Notter and Wang 1997, Clements and Avery 1998).

Two major static forces in the lung cause an inflated lung to recoil inward:

1) Elastic properties of the lung tissue itself
2) Surface tension produced by the layer of fluid that lines the inside of the alveoli (Von Neergaard 1929, Clements et al 1958, King 1974). This layer of fluid (liquid molecules) can profoundly resist lung expansion. When liquid molecules are completely surrounded by identical molecules, molecules move freely in all directions due to their mutual attraction toward one another. However, in the case of a liquid-air interface, liquid molecules are attracted to the liquid molecules contained within that liquid. This molecular, cohesive force at the liquid-air interface is called surface tension. In the lung the alveolus with its fluid lining resembles a sphere, i.e., a type of liquid bubble. The 'bubble' model forms the cornerstone of understanding the way in which surfactant functions (Hills 1988). In order to explain the physical behaviour of soap- or liquid bubbles, Laplace's equation is used (Kotas 1982). This mathematical expression relates the effects of surface tension to the collapsing pressure of the alveolus and, hence to lung recoil (Hills 1988). The lung and soap bubble are not exactly analogous, since soap bubbles have a constant low surface tension, while the alveoli have a changing surface tension. The pressure from surface tension (ST), at a spheric surface, is estimated by the Laplace equation (Hills 1988):

$$P = 2\,ST / r$$

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P is the pressure difference (dyne/cm), ST is surface tension (dyne), and r is the radius of the liquid sphere (cm). The factor 2 is required when the law applies to a liquid bubble with one liquid-gas interface. The arrangement of this model shows that the distending pressure of a liquid sphere is directly proportional to the surface tension of the liquid and inversely related to the radius (size) of the sphere (Jardins 1993). Therefore, when the size of the "bubble" increases, the distending pressure necessary to hold the bubble open, decreases. The opposite is true in the absence of surfactant. According to Laplace, for a decrease in size, a high transpulmonary pressure has to be generated to keep the small alveoli open. In the normal lung, this is largely offset by endogenous surface-active material. This material, now known as lung surfactant, was initially known as a 'bubble-stabilizing' factor (Buckingham and Avery 1962). The term was initially chosen to describe the rapid surface-tension-lowering capacities of the material at the air-liquid interface and not its composition (Sanders 1982). The term "surfactant" is a contraction of "surface-active agent", and refers to any compound with the ability to reduce surface tension (Notter and Shapiro 1987).

The surfactant molecule has both hydrophobic and hydrophilic characteristics, which allows it to be positioned at the surface of the alveolar gas-liquid interface so that the hydrophilic end is in the liquid phase and the hydrophobic end is in the gas phase. This unique positioning or "packing" of the surfactant contributes to its surface tension-lowering capabilities at the alveolar-gas interface in proportion to the ratio of surfactant to surface area (Weibel et al 1966, Williams 1977, Jardins 1993).

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A finding similar to what is actually occurring at the air-liquid interface of the distal airways in the lung has also been described by early researchers, who extracted phospholipids from human erythrocytes and floated them on the surface of a water solution. The phospholipids formed a unimolecular film (monolayer) with the hydrophilic head groups facing the water and the hydrophobic tails pointing up into the air. This stacking resulted in an increase in the area covered by the monolayer, to twice the surface area of the original cell (Lodish et al 1995).

Quantitative measurements of surface tension are expressed in milliNewtons per meter or dyne per centimeter (1 dyne / cm is 1mN/m). A minimum level of 5-12 dyne / cm is considered as evidence of effectively "packed" and functional surfactant (when the alveolus is very small). During exhalation, in the presence of normally functional surfactant, surface tension is lowered from 50mN / m at total lung capacity to minimum levels below 10 mN / m at functional residual capacity (Clements et al 1970, Goerke 1974, Jardins 1993). Lamellar bodies secreted from type 2 cells, containing the essential components of the surfactant system, swell in the alveolar lining and form the characteristic lattice structure known as tubular myelin (discussed below) (Van Golde et al 1994). From this extra-cellular, surface-associated reservoir (Schurch et al 1995), the surface film spread as a fluid mixture of saturated and unsaturated phospholipids with an equilibrium surface tension of ~25 mN / m (Van Golde et al 1994). DPPC is solid at body temperature and resists surface compression. After the fluid molecules (unsaturated molecules / non-DPPC components) have been squeezed out at the end of expiration, the contractile forces at the air-liquid interface decreases to nearly zero, thereby stabilizing the distal air spaces (Rider 1995). At this stage the stability of

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air spaces becomes independent of alveolar size (Van Golde et al 1994). In this context, and since surfactant can assume the characteristics of either a bi-layer or a multi-layer, it fulfils an anti-stickiness function (Morgenroth 1988).

Repeated cyclic compression and selective squeezing-out of unsaturated film components (as occurring during normal breathing), gradually increases the content of DPPC and reduces the percentage surface area required to reduce surface tension to minimum values. For instance, to attain near-zero surface tension during the first compression of a purified rabbit surfactant, the surface area had to be reduced by 45% (Van Golde et al 1994). After 20 cycles only 27% area-reduction was required to reduce surface tension to minimum values.

In the absence of surfactant, the infant is unable to sustain an adequate end-expiratory volume of gas (functional residual capacity) due to alveolar collapse. As a consequence there is considerable resistance to re-inflation; gas exchange is seriously impeded, and progressive atelectasis and respiratory failure ensues (Clements et al 1961, Schurch et al 1976, Boston et al 1966, Sinclair 1966, Clements 1997). Hysteresis of the lung, in the absence of surfactant, indicates that recruitment of lung units from a collapsed state is associated with large shear forces (Mead et al 1970). Shear forces contribute to the development of terminal bronchiolar epithelial lesions after short periods of spontaneous breathing in both newborn rabbits (within 15 to 150 min) (Nilsson and Robertson 1985) and low birth weight newborn infants with RDS (Finlay-Jones et al 1973, Gandy et al 1970). After conventional (artificial) positive pressure ventilation of 5 min or more, the lungs of premature newborn rabbits constantly show necrosis and desquamation of bronchiolar epithelium (Nilsson 1982). The above-mentioned findings were also noted in

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the lungs of mechanically ventilated newborn infants dying from HMD (Taghizadeh and Reynolds 1976).

The disruption of the bronchiolar epithelium results in open surfaces through which fluid and plasma proteins (Egan et al 1975, Ikegami et al 1986, Jobe et al 1983) and cellular components (Holm and Notter 1987; Gandy et al 1970) leak into the interstitium and into the airway lumen. Surfactant keeps interstitial and intrapleural pressures less subatmospheric than would otherwise be the case, thereby reducing the driving force for pulmonary oedema (Notter and Wang 1997). However, excessive mechanical ventilation is not the only cause for protein and fluid leak in preterm lungs. This bi-directional flux of macromolecules across the alveolar epithelium is also a function of both the immaturity of the preterm lung and the result of the postnatal increase in blood flow to the lungs (Berry et al 1991).

Both in-vitro and in-vivo studies have shown that surfactant interfacial properties are hampered by, or inhibited (i.e. surface tension rises) in the presence of plasma proteins, such as albumin and fibrinogen, haemoglobin, red blood cell membrane lipids and proteases (Ueda et al 1994, Wang and Notter 1998, Fuchimukai et al 1987, Seeger et al 1985, Ikegami et al 1983, Ikegami et al 1984, Jobe et al 1983, Jobe 1989, Bummer et al 1994, Holm and Notter 1987, Manalo et al 1996). In infants with RDS, airway samples obtained by suction immediately after tracheal intubations show both a high surface tension (27.3 +/- 3 dynes / cm) and the presence of proteins (Ikegami et al 1983). These findings correlated with the severity of respiratory failure (Ikegami et al 1986), suggesting that infants with RDS have surfactant inactivation as a component of their disease.

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During the early management of neonatal RDS, infants receive assisted ventilatory support and are often exposed to high inspiratory concentrations of oxygen. Prolonged exposure to 100% oxygen generates oxygen metabolites that may result in hyperoxic lung injury (Matalon and Nickerson 1986, Repine 1986). Early (within 24-48h) during the course of hyperoxia-induced lung injury in adult rabbits, there is progressive alveolar epithelial injury and increased permeability to solute resulting in a loss of FRC, hypoxemia and pulmonary oedema (Matalon and Nickerson 1986). The damaged airway epithelial cells and various other cells release potent pro-inflammatory cytokines which causes changes in vascular permeability, increased adhesion molecule expression by endothelial cells, and subsequent movement of leucocytes from the pulmonary vascular compartment to the lung interstitium and alveolar spaces (Ozdemir et al 1997). Increased capillary permeability, together with the formation of interstitial (increasing lung water content) and alveolar edema and an influx of plasma protein significantly affect and change the biophysical functions of surfactant at the alveolar surface (Holm et al 1985, Holm and Notter 1986, Ikegami et al 1983). These abnormalities include decreased lung surfactant phospholipid, decreased lung surfactant activity in bronchoalveolar lavage and altered pressure-volume relations (Holm and Notter 1986).

Oxygen-induced changes in the type 2 pneumocyte surfactant biosynthesis may account, at least in part, for observed changes in lung phospholipid levels (Holm et al 1988). There is also evidence linking the severity of neonatal RDS with the magnitude of the deficiency of surfactant protein A (SP-A) rather than that of disaturated phosphatidylcholine (PC) (Floros et al 1989).

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Suffice to say that the negative effects of positive pressure ventilation and the exposure to high concentrations of oxygen in the inspired gas, resulting in the formation of reactive oxygen radicals, significantly impact on the already functionally immature surfactant-deficient lung of the preterm neonate and contributes significantly to the ultimate degree of clinical RDS.

Pulmonary surfactant is described as a lipoprotein complex produced by type 2 pneumocytes (Abrams 1966, Bourbon 1995, Haagsman and Van Golde 1991). Scarpelli et al (1967) challenged the initial description of surfactant as a lipoprotein complex, claiming that the evidence for this was only indirect. They presented evidence that the lung surfactant system is a complex mixture of dipalmitoylphosphatidylcholine (DPPC), other phospholipids (secondary phospholipids), neutral lipids and complex polysaccharides. Subsequently, researchers demonstrated that one of the lung surfactant components, surfactant protein (SP) 26-36 (now known as SP-A), is a glycoprotein, containing carbohydrate components which include galactose, mannose, sialic acid, fucose, and galactosamine (Wright and Clements 1987, Bhattacharyya 1981, Bhattacharyya et al 1984, Lynn 1984). Radioautographic studies in the mouse lung confirmed the incorporation of modest concentrations of galactose-3H into lamellar bodies via the Golgi complex. This indicates that the secretory product produced by type 2 pneumocytes include phospholipid, protein and polysaccharide precursors (Chevalier and Collet 1972).

Because its components are many, varied, and not always well characterized, surfactant is more readily defined functionally than structurally (Wright and Clements 1987). According to Kotas, surfactant is that material which functions at the alveolar lining layer

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which reversibly adjust surface tension in order to permit the lung to retain air at low lung volumes. In addition, surfactant "keeps" the alveoli dry, protects cell surfaces from inspired gas and maintains pulmonary toilet (Kotas 1982). Wright and Clements (1987) suggested that a surface film should have the following characteristics: it should be a monolayer; should be able to reduce surface tension to 12 mN/m or less and it should be stable at low lung volumes (Clements 1977). Suzuki and co-workers emphasized that for a surfactant to be functional, the combination of low film compressibility and effective spread should be present during in vivo ventilation of the lungs (Suzuki et al 1986).

Endogenous surfactant is a heterogeneous substance that exists in many different forms. Surfactant is synthesized in alveolar type 2 cells (Macklin 1954), probably in the endoplasmic reticulum (ER) (Batenburg and Hallman 1990), and stored in lamellar bodies (Buckingham and Avery 1962, Buckingham et al 1966, Gil and Reiss 1973). A possible scenario for the intracellar processing of newly synthesized surfactant phospholipids has been suggested (Wright and Clements 1987, Ikegami and Jobe 1993). Subsequent to surfactant phospholipid synthesis from phospholipid precursors (fatty acids, glycerol, choline) in the ER, they are transferred (translocated) to the Golgi apparatus, then to the lamellar body, and finally into the alveolar lumen. Rose et al (1999), have recently shown that the state of the actin microfilament system beneath the apical surface of the type 2 cell is intimately linked to the exocytosis of lung surfactant. Its depolymerization allows lamellar body membrane fusion and exocytosis (Rose et al 1999). Once secreted, the composition of the alveolar monolayer is refined with each respiratory cycle. Unsaturated phospholipids, fatty acids and proteins are squeezed out to leave a purified, saturated PC surface monolayer, DPPC (McCormack 1995). The

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squeezed out components and collapsed monolayer yield small phospholipid aggregates with less surfactant protein, a phospholipid composition similar to whole surfactant. This has poor surface-active properties when administered to surfactant deficient rabbits (Yamada et al 1990).

Surfactant material consists mainly of a complex mixture of lipids and at least 4 surfactant-associated proteins (SP) (Whitsett et al 1986, King et al 1973, King 1982, King 1974, Klass 1972, Wright and Clements 1987, Clements and Avery 1998, Whitsett and Stahlman 1998, Kuroki and Voelker 1994, Batenburg and Hallman 1990). The phospholipids contributes approximately 85-90% of the lung surfactant by weight, about a third is 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC) (Brown 1963, Johansson et al 1995, Batenburg and Hallman 1990), while the remaining two-thirds is made up by secondary phospholipids, a mixture of zwitterionic and anionic phospholipids (Wang et al 1997, Van Golde et al 1988, Sanders 1982). Phospholipids are classified according to whether their headgroups are zwitterionic or anionic at neutral pH. In the lung surfactant of vertebrate species, zwitterionic phospholipids are made up by phosphatidylcholines (PC), of which DPPC is the most prevalent, and phosphatidylethanolamine (PEA) and sphingomyelin (Wang et al 1997). Phosphatidylcholine is a zwitterion, which means that it bears a negative and positive charge and is thus electrically neutral. Smaller quantities of other phosphtidylcholines, and anionic and acidic phospholipids, including phosphatidylglycerol (PG) and phosphatidylinositol (PI), make up about 10-15% of the total phospholipids in mammalian lungs (Van Golde et al 1988). Approximately 30-60% of the phoshatidylcholine (also known as lecithin) in surfactant is disaturated, largely DPPC (Wright and Clements 1987, Batenburg and Hallman 1990, Holm et al 1996). The

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'true' value for the DPPC content of lung surfactant appears to be in the order of ~40% (Holm et al 1996). The nitrogen compound is choline, an alcohol. It is polar and hydrophilic, while the palmitic acid residues (C-18 fatty acid chains) are nonpolar and hydrophobic (Sackheim and Lehman 1985, Ikegami and Jobe 1993). The choline part of the molecule associates with the liquid phase in the alveoli and the palmitic acids orient toward air (project into the alveolar lumen). The dipalmitoyl molecule is a solid at body temperature and it does not undergo a solid-to-fluid phase transition until 41°C (Sanders et al 1982). Thus, lipids and / or proteins other than DPPC are required in 'surfactant' to increase its ability to spread. During compression, the disaturated phospholipid forms tightly packed, solid films in the rigid gel phase below its liquid crystal transition temperature of 41°C, and gives minimum surface tensions < 1 dyn / cm in compressed films at body temperature (Notter and Wang 1997).

It has previously been stated that PEA does not posses normal surface activity (Scarpelli et al 1967). Recently Wang et al (1997) showed that zwitterionic phospholipids have a major role over anionic phospholipids in interacting with hydrophobic surfactant protein in the adsorption, surface tension-lowering, spreading of the film and pulmonary mechanical activity of the hydrophobic components of calf lung surfactant.

Synthesis and secretion of surfactant lipids and proteins are stimulated by several secretagogues (Bourbon 1995, Di Renzo et al 1989, Wright and Dobbs 1991, Ikegami and Jobe 1993, Batenburg and Hallman 1990, Xu et al 1990, Mendelson and Boggaram 1991) as well as mechanical stress which is brought about by physiological stimuli such as stretching of the alveolar type 2 epithelial cells (Wirtz and Dobbs 1990, Nicholas et al 1982, Rooney et al 1994). Nicholas et al (1982) described an increase in

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both the rate of release and total amount of surfactant phospholipids in the alveolar compartment of the isolated perfused rat lung within minutes of elevating the tidal volume. Glucocorticoids (GC) have been shown to accelerate fetal lung maturation as evidenced by an increased rate of lung phosphatidylcholine biosynthesis, the stimulation of the activity of the rate-regulatory enzyme (Rooney et al 1994), and increased levels of newly synthesized SP-A and SP-A mRNA (Floros et al 1989). In the developing rabbit lung in vivo, the SP-B mRNA levels rose significantly whilst the SP-C mRNA lowered somewhat after treatment with betamethasone (Connelly et al 1991). In human fetal lung explants between 16 and 24 weeks of gestation, dexamethasone enhances the synthesis of proteolipids associated with surfactant phospholipids (Whitsett et al 1987). However, paradoxical effects of glucocorticoids have been described. For instance, in the fetal rabbit lung, glucocorticoids appear to have both an initial inhibitory effect, and after 48 –72 hours, a stimulatory effect on SP-A gene expression. It has been suggested that this finding could be related to the differentiation state of the fetal lung tissue (Mendelson et al 1993).

Surfactant is readily obtained by bronchoalveolar lavage and can be purified by differential centrifugation or on sucrose density gradients (Rooney et al 1994, Oulton 1979, Magoon et al 1983, Gross and Narine 1989, Gil and Reiss 1973). Lung surfactant, as isolated by centrifugation of endobronchial lavage fluid of dogs (Benson et al 1984) and adult mice (Gross and Narine 1989), and by sucrose density gradient of rat lung homogenates (Gil and Reiss 1973), is composed of at least two morphologically distinct macromolecular forms. One form mentioned above, is the lamellar body (LB). It is a globular (vesicular) structure consisting of numerous

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lamellae, which afford it the appearance of onion peels. Lamellar bodies are present as intracellular organelles, the intracellular storage form of surfactant, as well as extracellular. They act as the precursors of the next form namely, tubular myelin (TM) (Williams 1977, Weibel et al 1966). In its extracellular form, both LB's and TM are categorized as large aggregate surfactant. Large aggregate surfactant contains PC, SP-A, SP-B, and SP-C and rapidly lowers surface tension (Magoon et al 1983, Gross and Narine 1989).

Tubular myelin is a lattice of parallel bi-layers and is thought to be the immediate precursor of the surfactant monolayer at the air-liquid interface (Wright and Clements 1987, van Golde et al 1994). The latticework-like tubular myelin is dependent on the presence of SP-A and SP-B (Suzuki et al 1989).

Researchers have described extracellular LB with unravelling membranes continuous with those of TM (Williams 1977). Labelling studies in mice suggest that surfactant is secreted from type 2 cells as different subtypes. The 'ultraheavy' subtype labels first (newly secreted surfactant) and appears to be of the same density as purified LB. It represents large aggregates (LA) of TM and is considered to be the transitional form of secretion between lamellar bodies and heavy subtype (small amounts of TM) (Gross and Narine 1989, Magoon et al 1983). The heavy TM fraction contains surfactant- associated apoproteins as well as phospholipids and adsorbs rapidly to an air-water interface (highly surface active), creating a surface film (Magoon et al 1983, Gross and Narine 1989, Poulain et al 1992). Studies suggest a cycle from LA to small aggregates (SA) in the alveolar space (Gross and Narine 1989). The LA fraction serves to provide the monolayer and the SA is subjected to reuptake and recycling and phagocytosis (Gunther 1998).

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Because of the extensive recycling of surfactant subfractions it remains difficult to explain by way of a simplistic model the precursor-product relationship between lamellar bodies and alveolar phospholipids (Fisher et al 1991). As previously mentioned, at any given time one may find several morphologic forms in the alveolar space. The subfractions are heterogeneous and the compartments separated by in vitro techniques may in actual fact overlap in vivo (Magoon et al 1983). The conversion from ultra heavy and heavy subtypes (LA) to light subtypes (SA) occurs in vitro by surface-area cycling at 37°C, suggesting that mechanical distortion of the alveoli is integral to the conversion process (Rider 1995, Gunther et al 1999). A study performed in surfactant-deficient preterm rabbits, treated with either heavy or light subtype surfactant, showed that the heavy subtypes had superior in vivo function compared with light subtypes (Ueda et al 1994). When serum protein was added to the heavy subtype surfactant (active form of surfactant, containing SP-A, SP-B, and SP-C), the conversion to the light subtype surfactant (inactive form of surfactant, lacking in surfactant proteins) was accelerated (Ueda et al 1994). A significant reduction in SP-B content of the LA fraction occurs during cycling and this is closely related with a proportional loss of activity of this fraction (Gunther et al 1999).

Although DPPC is the single most prevalent component which contributes to the surfactant's surface-active properties, the in-vitro adsorption rates, when suspended in buffer, appears much slower than those observed for the whole surface-active complex (Notter and Wang 1997). Nor does it respread well in a pure surface film after dynamic compression to film collapse (Notter and Wang 1997). Other surface components affecting the mono-molecular film structure, such as PG, proteins, alterations in surface

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pH, and inorganic ions interact with DPPC to 'speed up' the in vivo biophysical behaviour of lung surfactant (Whitsett and Stahlman 1998). There is a definite increase in adsorption rates when a suspension of the total mixture of surfactant lipids is added to DPPC. Furthermore, the adsorption rates increases to that which is required under physiological conditions when surfactant protein (SP), SP-A and SP-B, are present (King 1974, Shiffer et al 1988). It is interesting to note that the adsorption behaviour of exogenous surfactants, with relatively low DPPC contents (40%) combined with hydrophobic SP-A and-B, were found to be closer to a mammalian surfactant than those of mixtures with 60-80% DPPC (Holm et al 1996).

Surfactant-associated proteins, produced and secreted by the respiratory epithelial cells, are critical to surfactant composition and to its surface tension-lowering behaviour (Shiffer et al 1988, Weaver and Whitsett 1989). The four specific surfactant-associated proteins include the hydrophilic glycoproteins, SP-A and SP-D, and the small hydrophobic proteolipids, SP-B and-C (Van Golde et al 1994, Rooney et al 1994). Of the four surfactant proteins, SP-A is the most abundant on a weight basis (McCormack 1995). All 4 surfactant apoproteins are synthesized in type 2 cells (Hawgood and Shiffer 1991, Samuels et al 1999), but Clara cells lining respiratory bronchioles may also secrete SP-A and SP-B (Wong et al 1996). These proteins seem to regulate a number of important processes including surfactant secretion, transformation of secreted lamellar bodies into tubular myelin, adsorption of the surface film, and uptake and recycling of surfactant material by the alveolar epithelium. It also stimulates phagocytosis of bacteria and viruses (Van Golde et al 1994, Rider 1995). Following its synthesis, the intracellular surfactant apoproteins are transported through the

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multivesicular body (MVB). The MVB transports the apoprotein from the Golgi system to the lamellar bodies (Chevalier and Collet 1972, Batenburg and Hallman 1990, Rooney et al 1994). These MVB are reportedly not involved with PC transport (Chevalier and Collet 1972). Multivesicular bodies represent vesicles within vesicles. They occur as 3 different types within the alveolar type 2 cell and have prominent roles to play in cellular function, including the formation of lamellar bodies and receptor-mediated uptake of secreted surfactant material (surfactant apoprotein), i.e., surfactant recycling (Williams 1987).

Together with DPPC, other phosholipids (including PG) and calcium ions, it was shown that SP-A and SP-B enhances the in vitro formation of the tubular myelin structure of surfactant (Benson et al 1984, King and MacBeth 1981, Suzuki et al 1989, Hallman et al 1991, Van Golde et al 1994). Both, SP-B and SP-C play important roles in the ordering of surfactant lipids, enhancing the transport rates of surfactant lipids to the hypophase surface in the alveoli (Takahashi et al 1990, Hawgood and Shiffer 1991). Although SP-A is the major surfactant protein, its removal from lung surfactant does not appear to result in major loss of function when tested in vitro or in vivo (Shiffer et al 1988, Whitsett et al 1986, Takahashi et al 1990). Hallman et al (1991) proposed that SP-A protects or 'shields' the hydrophobic SP-B, allowing rapid surface adsorption of saturated phospholipids.

Surfactant protein-A (previously described as SP 26-36) is synthesized as a precursor protein and the addition of a carbohydrate moity results in the production of larger molecular weight proteins which shares sequence homology with several C-type lectins, i.e., C1q of complement and acetylcholinesterase (Wright and Clements 1987,

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White et al 1985). The function of the carboxy-terminal carbohydrate is partly to direct the protein to various subcellular organelles and the lamellar bodies to be secreted. SP-A is specific to the respiratory system and has been found in the lungs of all air-breathing creatures studied to date (McCormack 1995).

In vivo studies have shown that exogenous surfactant mixtures that contain SP-A are taken up by type 2 cells into lamellar bodies to a greater extent than mixtures that do not contain this protein (Wright et al 1987, Young et al 1993). SP-A also appears to act as a ligand to direct surfactant lipids to type 2 cells, thus regulating re-uptake (recycling) and surfactant homeostasis, i.e., directing lipids to different intracellular sites (Wright and Clements 1987, Wright et al 1987). SP-A may regulate transcript levels of the other surfactant proteins, especially SP-B and SP-C (Strayer and Korutla 1997). Of clinical interest is the modulatory effect on SP-A production of both the prenatal and postnatal treatment with dexamethasone (Floros et al 1989) and the data suggesting a relationship between SP-A and SP-B loci and the development of RDS in certain subgroups of infants (Kala et al 1998). Extrapolating from these findings, it would appear possible to identify certain subgroups of patients who are at higher risk of developing RDS by genetic analyses in the future.

Surfactant protein B has been isolated from whole surfactant by a number of methods (Beers et al 1992, Rooney et al 1994). It is synthesized as a precursor polypeptide. The proteolipid derived from the SP-B precursor contain several cysteine residues which allows the molecule to form a dimer with another SP-B molecule (Rooney et al 1994). The further partitioning of the SP-B peptides within the phospholipid layer increases resistance to surface tension by increasing lateral stability of the phospholipid layer

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(Cochrane and Revak 1991). Complicated and extensive research has illucidated SP-B gene expression (type 2 cell and bronchiolar epithelial cell-specific), as well as the molecular pathways involved in its synthesis, secretion and recycling (Rooney et al 1994). SP-B, together with SP-C, markedly enhances the surface tension lowering-properties of surfactant phospholipids (Hawgood and Shiffer 1991). It has been suggested that SP-B is more effective in 'squeezing out' unsaturated phospholipids (such as phosphatidylglycerol) from the monolayer (Mathialagan and Possmayer 1990). It has to be added, however, that there appears to be little evidence to support the aforementioned "squeezing-out" hypothesis of lung surfactant (Rana et al 1993). The hypothesis suggests that upon repetitive compression-expansion cycles, a surfactant surface film will reorganize to exclude all components, except DPPC, thus resulting in a stable, low surface tension film.

Hydrophobic SP-C is the least understood surfactant protein (Ikegami and Jobe 1998). It is synthesized (only by the type 2 cells) and processed from a precursor to the mature peptide which is localized to lamellar bodies with SP-B and saturated phosphatidylcholine (Beers and Fisher 1992). The metabolic pathways for SP-C production and recycling is difficult to study (contamination by phospholipids) and is distinct from other surfactant proteins and phospholipids (Beers and Fisher 1992, Glasser et al 1991). Like SP-B, treatment with glucocorticoids has been shown to modulate SP-C message levels, accelerating the appearance of SP-C mRNA in human fetal lung explants (Liley et al 1989, Whitsett et al 1987). However, dexamethasone administered in the mid-trimester decreased levels of SP-C mRNA in fetal rabbits. This supports the view that the genes for the surfactant-associated proteins are independently

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regulated (Connelly et al 1991). SP-C is known as a true lipoprotein, i.e., it contains covalently linked lipid residues. The 'lipid modification' of the SP-C molecule is brought about by palmitoylation (two palmitic acid residues linked to cysteins) and increases the molecule' hydrophobic nature (Curstedt et al 1990, Beers and Fisher 1992, Rooney et al 1994). It has been suggested that the palmitate in SP-C could potentially mediate membrane anchorage, site-targeting and protein-lipid interactions (Beers and Fisher 1992). SP-C facilitates the rate of adsorption of phospholipids for film formation, is present in the film, and can cause packing changes of the phospholipids in the surface film (Perezgil et al 1992, Qanbar et al 1996). Natural surfactant is thought to contain 1% SP-C by weight (Ikegami and Jobe 1998).

SP-D is a hydrophilic collagenous glycoprotein (Mendelson et al 1993), present in secretory granules of the type 2 cell and non-ciliated bronchiolar epithelial cells (Clara-like cells) (Wong et al 1996). It is similar in structure to SP-A, and to other members of the lectin family (Mendelson et al 1993). There is evidence that SP-D may function in lung host defence mechanisms.

Following its secretion and after functioning as a surface tension-lowering agent, surfactant must be removed from the alveolus. Although there is ongoing secretion, the surfactant pool appears to remain constant and therefore removal is necessary to balance production (Rooney et al 1994). There is abundant evidence for the recycling of surfactant (Wright and Dobbs 1991, Wright and Clements 1987, Fisher et al 1991, Rider 1995). Almost 50% of the alveolar surfactant PC is lost from the lung with each turnover time. Small amounts of surfactant material are ingested and degraded by alveolar macrophages and some material exit via the airways (Wright and Dobbs 1991,

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Rooney et al 1994). Under steady-state circumstances, surfactactant secretion into the alveoli and its removal are in balance. The turnover time (the time required to fill the alveolar pool if it were empty) of alveolar surfactant lipids (phosphatidylcholine), ranges from 5-10 hours (Van Golde et al 1994, Rider 1995) and the calculated recycling efficiency ranges from 23-57% in adult animals (Rider 1995). The turnover time in newborn rabbits is somewhat longer at 10 hours and the recycling efficacy (reutilization of PC) is greater than 90% (Jacobs et al 1983). Some of the internalized phospholipid is recycled intact into the lamellar bodies. In addition to phospholipids, SP-A, SP-B, and SP-C also re-enter the type 2 cell (Young et al 1993, Breslin et al 1992, Baritussio et al 1992). After reuptake into the type 2 cell, PC, SP-A, and SP-B are found initially in multi-vesicular bodies and, within minutes, in the lamellar bodies (LB) (Young et al 1993). Phosphatidylcholine may be recycled directly to LB's or degraded for reutilization.

Surfactant component clearance is most extensively studied for PC. Possible routes of clearance from the intact lung include movement up the muco-ciliary escalator, transfer across the epithelium/endothelium barrier into the blood or lymph, or degradation within the lung and transport of the products to other organs such as the kidneys and liver where it is utilized to synthesize new lipid (Wright and Dobbs 1991). Differences in clearance and degradation of PC within the lung exists according to development i.e., considerable amounts of PC cleared from the lungs of adult animals compared to the loss measured in the newborn lung. For instance, in the adult rabbit, 24 hours after intratracheal instillation of a trace dose of labelled surfactant, 90% of the tracer disappears from the alveolar space and 70% of the total labelled surfactant is lost from the entire lung

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compartment (Rider 1995). This is compared to a 15% loss from the lungs of newborn rabbits treated with intratracheally administered labelled surfactant (Pettenazzo et al 1986). Direct measurements of intratracheally-administered surfactant in animal models indicate that only about 7% of treatment doses of surfactant are lost via the upper airways over 24 hours (Pettenazzo et al 1988). Alveolar macrophages clear approximately 10-15% of alveolar PC per day (Rider 1995). Recently, Cogo et al (1999) provided new data on endogenous surfactant metabolism in infants requiring mechanical ventilation. The authors demonstrated significant incorporation of labelled plasma free fatty acids, albumin-bound palmitic acid and linoleic acid into human surfactant PC, after intravenous administration, at 8.7 hours and 10 hours, respectively.

Surfactant related material is secreted into amniotic fluid as intact lamellar bodies (Jimenez and Johnston 1976) and its measurement provide insight into fetal lung surfactant maturity (Oulton et al 1980). The characterization of a pellet fraction obtained from centrifuging human amniotic fluid at different gestational ages reveals a correlation between the biochemical development and morphological maturation (electron microscopic analysis of the pellet fraction) of the lung surfactant system (Oulton et al 1980). At 14-18 weeks gestation, the pellet fraction consists of membrane-bound vesicles devoid of internal lamellae (nonlamellated structures). The phospholipid composition does not resemble that of mature surfactant and consists mainly of sphingomyelin (39% of total phospholipid content), palmitic acid, phosphatidylethanolamine (PEA) and DPPC (28% of total phospholipid). This finding persists until 30-32 weeks gestation, where after the total phospholipid concentration increases (rose from 4% at 14-18 weeks to 52% at 36 weeks) and the composition changes.

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Lecithin (DPPC) and PI concentrations rose between 30 and 35 weeks gestation. At 36 weeks gestation, the pellet fraction contains characteristic lamellar bodies. Of the total phospholipid composition, DPPC contributed 73%, and sphingomyelin 4%, of the phospholipid weight percentage. The lamellar bodies at this stage lack PG. From 36 weeks gestation to post term, the lamellar bodies increase markedly. Concommitently, the DPPC concentration increases, and lamellar bodies now contain phosphatidylglycerol. The appearance of PG in the amniotic fluid indicates that the fetal lung surfactant system is in its final stage of maturation. The accumulation of PG occurs rapidly between 36 and 37 weeks of gestation. Once PG exceeds 1% of the total phospholipids, the risk of developing RDS is negligible. In preparation for birth, the fetal lung therefore synthesizes and secretes surfactant in the form of lamellar bodies (Williams 1977). The regulatory mechanism by which it occurs involves the type 2 cell's response to calcium-phosphatidylserine-dependent protein kinase C activation (Samuels et al 1999).

The mRNA for SP-A is first detectable in the lungs of human fetuses early in the second trimester and localizes to pre-type 2 cells and bronchiolar cells at 19-20 weeks (Khoor et al 1993). At 21 weeks of gestation, bronchial cells in the main and segmental bronchi stain positive for SP-A (Endo and Oka 1991). The number of these cells increases until the 32nd week of gestation. Surfactant apoproteins are present in low concentrations in human amniotic fluid at 18-20 weeks of gestation, their expression increases significantly during the early third trimester, very similar too, and following the increase of surfactant phospholipids (Batenburg and Hallman 1990, Bourbon 1995, Farrell et al 1990, King et al 1975, Kuroki et al 1985, Shelley et al 1982). SP-C

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proprotein is detectable in epithelial cells of the early second (16 weeks) trimester in the human fetal lung and its expression can be enhanced by dexamethasone (Solarin et al 1997). This finding suggests that antenatal steroids given for preterm labour could enhance the type 2 cell to produce SP-C, thereby assisting the immature lung in the 'preparation' for breathing air.

Premature infants are hampered in their rate of surfactant synthesis and excretion. Although accurate data are lacking, there is evidence that a significant time delay occurs before the surfactant pool size increases after birth in infants with RDS. The adult pattern of pool sizes and surface activity of alveolar surfactant is not present at birth in newborn rabbits, but evolves slowly over the first day of life (Stevens et al 1987, Cogo et al 1999). In human infants with RDS the concentration of saturated PC does not increase much until after the 2nd day of life (Hallman et al1991). Measurements in preterm monkeys with RDS show that alveolar surfactant PC increases by 0.5 mg/kg per hour over the first 4 to 5 days of age (Jackson et al 1984, Ikegami and Jobe 1993).

The demonstration that the lungs of infants who died with HMD had a lack of surfactant prompted attempts to treat the condition by the administration of agents that contained surface activity. The concept of treating infants suffering from RDS with an airway instillation appeared logic. The problem however was that DPPC on its own could not be used as supplement for natural surfactant. DPPC required too much time to form a mono-layer at the air-liquid interface and when it formed it was 'stiff' (Enhorning 1997). Initial results with an aerosol preparation containing dipalmitoyl lecithin (DPL), now known as DPPC, in Singaporean infants with RDS, were discouraging (Chu et al 1967). Although the investigators demonstrated deposition of DPL in the peripheral lung tissue,

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improvement in lung compliance was inconsistent. Replacing surface-active preparations through direct endotracheal instillation in animal models with RDS proved more successful (Enhorning et al 1973, Enhorning et al 1972). Surfactant not only increased pressure-volume relationships of the lung, but also improved the survival time of treated animals over controls. Early tracheal instillation (before the onset of breathing) of natural surfactant into a prematurely delivered animal model prevented the development of many features characteristic of the RDS (Adams et al 1978, Nilsson et al 1978). Striking differences in lung histology between the treated and control animals were demonstrated. Lung sections from control animals revealed poor alveolar expansion, more hyaline membranes and peripheral epithelial damage than surfactant-treated animals. In addition, premature lambs, treated with natural surfactant at the time of delivery have diminished transvascular movement of fluid across the lung microcirculation, a reduction in vascular protein permeability, and a reduction in lung edema when compared to controls (Carlton et al 1995).

Notter and Shapiro (1987) outlined the biophysical requirements for an effective lung surfactant for replacement as follows:

1) Adsorption of surfactant from liquid subphase to the liquid-gas interface
2) Lowering surface tension with dynamic compression
3) Respreading after monolayer collapse
4) Varying surface tension of the surfactant during alveolar recruitment and derecruitment

In 1980, Fujiwara et al, treated 10 infants with a single instillation of a modified natural surfactant (bovine lung mince extraction, supplemented with DPPC and PG) by way of

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an endotracheal tube and demonstrated marked improvements in gas exchange and lung expansion. Since then numerous controlled trials have been performed documenting the efficacy and safety of synthetic and natural surfactant replacement therapy in neonates suffering from HMD / RDS (OSIRIS study 1992, Horbar et al 1993, Schwartz et al 1994, Long et al 1991, Soll 1997). Overviews of the outcome of numerous randomised trials have been assessed using meta-analysis (Soll and McQueen 1992, Soll 1997, Halliday 1995a). Both types of surfactant, used either as prophylaxis (within minutes of delivery) or treatment (within hours of delivery)-, reduce the odds of pneumothorax by 35-70% and the odds of neonatal mortality by about 40% (Halliday 1995b, Halliday 1997, Soll 1996). More than 80% of babies treated with surfactant show a satisfactory acute response which is more rapid for natural than for synthetic surfactants (Horbar et al 1993, Da Costa et al 1999).

Currently, surfactants that are in clinical use fall into three general groups:

1) Natural surfactant, i.e., those obtained from mammalian lung minces (Tanaka et al 1986), including Survanta (Abbott Laboratories) and Curosurf (Chiesi Pharmaceuticals),
2) Those derived from mammalian lung lavage (Infasurf, Forrest Laboratories; Alveofact, Boehringer),
3) Those that are synthetically manufactured (Exosurf, GlaxoSmithKline; Pneumactant, Brittania Pharmaceuticals).

Two synthetic surfactants have been tested and approved for clinical use. The one most widely used is Exosurf, a synthetic surfactant containing predominantly DPPC with tyloxapol and hexadecanol to aid in surface adsorption (Durand et al 1985, Soll 1997,

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Halliday 1995). The other one is artificial lung expanding compound (ALEC), a synthetic formulation consisting of DPPC and unsaturated phosphatidylglycerol in a ratio of 7:3 (Morley 1989). These surfactants are protein-free, i.e., lack hydrophobic surfactant proteins, SP-B and SP-C, which are present in the natural preparations (Whitsett et al 1986). The differences between the biochemical features of surfactant preparations and the mechanical methods of preparing them may result in different physiological and clinical effects (Ikegami et al 1979, Whitsett et al 1986, Benson et al 1984). With regard to the protein-free synthetic products, it is known that DPPC does not in itself adsorb effectively to an air-liquid interface at 37°C and that "fluidizers" such as unsaturated phospholipids, promote its surface adsorption (Suzuki et al 1986). The majority of *in-vitro* studies, for instance, demonstrate that preparations manufactured from mammalian lung-lavage perform significantly better than lung-mince formulations and also demonstrated that natural surfactants are superior to the protein-free synthetic products (Ikegami et al 1979, Kattwinkel 1998). The class of natural surfactants tend to lower surface tension both in vitro and in vivo more rapidly than synthetic preparations, improves lung-thorax compliance to a greater extent, and reduces airway epithelial damage in immature rabbits (Alfred and Auten 1995, Corcoran et al 1994). In order to prevent disruption and necrosis of the airway epithelial cells, it would probably require very early (prophylactic) replacement treatment. Airway epithelial lesions occurring in immature rabbits after a period of spontaneous breathing, followed by assisted ventilation and treatment with either a natural or synthetic surfactant, have previously been shown not to be significantly ameliorated through the 'delayed' approach of instilling surfactant (Morley et al 1980).

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The general impression that surface properties of animal source surfactants are superior to those of a synthetic nature has led to the assumption that all surfactants, to be effective, should have these properties. The in-vitro superiority of natural surfactant over synthetic surfactant has not been confirmed by clinical trials involving small numbers of infants (Da Costa 1999, Smith et al., unpublished observations, Soll 1997, Arnold et al 1996). The issue was further complicated when Scarpelli and co-workers demonstrated that three commercially available surfactants (Exosurf, Infasurf, and Survanta) failed *in vitro* to achieve two of four generally accepted physical criteria of lung surfactant (Scarpelli et al 1993). They concluded that either the surfactant criteria or the generally accepted *in vitro* models (surfactometer, surface balance techniques) require re-evaluation or that the formulations do not function according to the concept of mono-molecular films in situ.

Nevertheless, results obtained from larger head to head randomised controlled trials (n=6) in infants with neonatal RDS (Halliday 1995) now support the notion that natural surfactants perform better in clinical settings. Meta-analysis of these trials, involving 3536 infants shows a 19% reduction in the odds of death favouring natural surfactants (Halliday 1995a, Halliday 1995b, Soll 1997). Defining the extent of the clinical benefit of natural surfactant over synthetic, protein-free surfactant is however, not so clear. Meta-analysis indicates that, in order to save the life of one additional infant, 42-45 infants would have to be treated with a natural surfactant (Halliday 1997, Halliday 1995a). This, in terms of neonatal mortality from a global perspective point of view, amounts to significant numbers of lives saved. However, mortality in terms of individual units would not be dramatically improved.

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Some of the discrepancy observed between the results derived from in-vitro assessments, in vivo short-term measurements in animal models, and those obtained from studies performed in human preterm infants could be specie-related. Although there are disadvantages in testing the early effects of different surfactant preparations and ventilation patterns in animal models (especially the adult lung lavage model), several studies have reaffirmed its usefulness (Lachmann et al 1980, Kresch et al 1996, Sandhar et al 1988, Lachmann 1989). However, the most relevant animal model for surfactant deficiency in HMD appears to be the use of the premature animal in vivo (Kresch et al 1996). Another reason for observed differences between studies could be linked to the presence of some, or all, of the apoproteins for optimal in-vitro, and even in-vivo, biophysical activity of surfactant (Hall et al 1992, Whitsett 1986). For instance, SP-B appears to be a critical factor for optimum immediate surfactant function (Mizuno et al 1995). Synthetic preparations may function in-vivo by a mechanism that is not only a reflection of their surface-active properties, it may also be explained by changes to the surfactant which occur after the introduction of the surfactant into the biological system (Ikegami et al 1987, Hall et al 1992, Jobe 1993). The synthetic surfactants either combine with endogenous apoproteins after administration, or their components are incorporated in other pathways (Ikegami et al 1993, Holm 1993). In order to test the hypothesis that exogenously administered surfactant could maintain or improve its function within the preterm lung, Ikegami et al (1993) instilled a natural surfactant (Survanta, Abbott Lab), a synthetic surfactant (Exosurf, Glaxo Wellcome), and two non-commercial protein-free, lipid mixtures into the lungs of preterm lambs and ventilated them for 5 hours. At the end of the experiment the authors recovered (by

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lavage) the surfactant material, which now consisted of a mixture of the exogenous surfactant formulation and the lamb's surfactant. The BAL fluid from Survanta-treated lambs contained more SP-A than did BAL fluid from the lambs treated with the other surfactants. In all cases, the recovered product was more effective than the original exogenous surfactant formulation when they were compared for physiological activity in a preterm rabbit model, suggesting endogenous activation phenomena. In-vitro biophysical properties such as inherent surface activity may also not predict in vivo response to surfactant (Kresch et al 1996). Alternatively, the properties of a surfactant responsible for the improved lung mechanics could be quite different from those contributing to better gas exchange. A study performed in preterm rabbits found that different surfactants, with varying surface properties, were effective in improving the lung pressure-volume recordings and lung histology (Nohara et al 1986).

The 'anti-stick' properties of a particular formulation, instead of its surface tension lowering ability, may be as important in improving gas exchange and lung compliance (Kresch et al 1996). For instance, tension-active agents different from surfactant and with a high surface tension and poor compressibility, such as Tween 20 and perfluorocarbon (3% FC-100, 3M, St. Paul, MN) were as effective as natural surfactant in improving lung expansion and gas exchange in preterm and full-term lambs with respiratory failure (Gladstone et al 1990). Another study compared the ability of Tween 20 or saline to reverse the respiratory failure in the surfactant-deficient preterm lamb. Tween 20 did not alter the alveolar wash minimal surface tension when compared with that of control animals (saline treated) but significantly improved gas exchange and lung mechanics (Jacobs et al 1985). Futhermore, the intratracheal administration of repeated doses of

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FC-100 resulted in significantly better lung function results and survival of surfactant deficient premature lambs compared to lambs treated with Exosurf (Moya et al 1997). Although natural surfactants containing SP-B (<1%), and synthetic surfactants (lipid mixtures) are devoid of peptides, all are effective in restoring various indicators of lung function, lung physiology and histology, researchers have increasingly shifted their focus towards developing surfactant formulations with superior performance both *in vitro* and *in vivo*. The ultimate aim is to develop a totally synthetic formulation that mimics the function of native lung surfactant. The efficacy of combinations of lipid (DPPC) with synthetic peptides (McLean et al 1993), natural bovine surfactant with additional DPPC (Nohara et al 1986), DPPC and low molecular weight apoproteins (Suzuki et al 1986, Robertson et al 1988), bovine surfactant supplemented with SP-B, or SP-B/C (Mizuno et al 1995, Walther et al 1997), or recombinant SP-C (Davis et al 1998), as well as a mixture of lipids with SP-A segment residues (Walther et al 1996) have now been studied. Although SP-A is the most abundant surfactant protein in native surfactant (Walther et al 1996), its effect on biophysical properties have been shown to be less striking than are the effects of SP-B and SP-C (Hall et al 1992, Mizuno et al 1995). A fact borne out by the severe respiratory failure observed in newborn infants dying from congenital SP-B deficiency (Nogee et al 1993). To altogether dismiss the intact SP-A protein as "unimportant" is short sighted. As stated before, the severity of RDS, in preterm infants, correlates with the magnitude of the deficiency of SP-A rather than that of disaturated PC (Floros et al 1989). SP-A is secreted as a large polypeptide (248 residues) and is important for the formation of tubular myelin, which again includes lipids and SP-B and SP-C (Suzuki et al 1989, Wright and Clements 1987). As mentioned

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before, tubular myelin acts as a reservoir facilitating the adsorption of the surfactant lipids to the monolayer at the liquid-air interface. Initial results with various peptides, including a synthetic peptide based on residues 81-102 of SP-A and the hydrophobic peptide gramicidin, were all ineffective as surfactant mixtures (McLean et al 1992). However, an analogue peptide with substitution of leucine residues for aspartat and threonine of SP-A, when mixed with DPPC mixture, restored quasi-static lung compliance to 90% of the pre lavaged state in the excised rat lung model (McLean et al 1993). Walther and co-workers (Walther et al 1996) identified functional domains within the SP-A molecule, modelled a synthetic peptide based on the sequence identified, and tested its in vitro and in vivo activity. The authors found that the synthetic surfactant dispersion containing aminoacids 114-144 improved lung compliance in spontaneously breathing, premature rabbits to a greater degree than surfactant dispersions containing SP-B/C (Survanta) and synthetic lipids alone.

Recently, the recombinant production of the hydrophobic surfactant protein's SP-B and SP-C has been the focus for the synthesis of synthetic surfactants. Recombinant surfactant protein-C (rSP-C) surfactant, containing phenylalanine instead of cysteine, and isoleucine instead of methionine of the SP-C protein, proved to be as effective as natural (sheep) surfactant in two standard preterm animal models of surfactant deficiency (Davis et al 1998). Significantly better oxygenation (higher mean arterial-alveolar oxygen tension ratios), and to a lesser extent, higher lung compliance (in situ pressure-volume curves) were described in the group treated with a combination of synthetic surfactant B and C peptides. In surfactant-deficient lungs in vivo, Survanta, was less effective at improving gas exchange and pressure-volume curves than natural surfactant or organic

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solvent extracts of calf lung lavage (Infasurf) (Mizuno et al 1995). However, the addition of SP-B or SP-B and C (isolated from sheep surfactant by differential solvent extraction and from organic solvent extracts on Sephadex columns, respectively) to Survanta, augmented its short-term in vivo performance to equate that of sheep surfactant. These results therefore demonstrated that Survanta contains insufficient SP-B for so-called optimal in vivo treatment.

Merritt and co-workers analysed the distribution of a peptide of lysine and leucine in combination with phospholipids and palmitic acid (KL4 surfactant; Scripp's Research Institute, La Jolla, CA), combined with positive end-expiratory pressure (PEEP). They also reported on its effect on lowering oxygen requirements and mean airway pressure during mechanical ventilation of preterm monkeys (Merritt et al 1995). KL4 surfactant distribution was better among infants having instillation accompanied by PEEP (KL4 surfactant + PEEP). In both the control animals (KL4 surfactant – PEEP) and the treatment group the arterial to alveolar oxygen ratios (a/A), lung compliance, and mean airway pressures improved above pre-treatment values. A surfactant preparation containing both synthetic B and C peptides performed better than preparations containing only B and KL4 peptides in surfactant deficient rats (Walther et al 1998).

When evaluating the in vivo response to a particular surfactant it should be remembered that the response to replacement therapy (RT) (improvement in physiological parameters and histological findings) is dependent on many interrelating factors. These factors include: the composition of the surfactant agent (Ikegami et al 1998, Cummings et al 1992); the volume of fluid in which surfactant is suspended (Van der Bleek et al 1993); the timing (Ikegami et al 1998, Heldt et al 1992) and rate of administration (Fernandez-

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Ruanova et al 1998, Krause et al 1998, Segerer et al 1996); the model being utilized (preterm animal, preterm human, adult animal) (Heldt et al 1992, Ikegami et al 1998); duration of studies (Walther et al 1987, Heldt et al 1992) as well as the manner in which assisted ventilation is applied during surfactant administration (or thereafter) (Merritt et al 1995, Krause et al 1997, Kobayashi et al 1984, Hafner et al 1993). The aforementioned confounding variables present us with difficulties when data from different studies are compared. For instance, some studies in preterm lambs (Ikegami et al 1998) and preterm rabbits (Heldt et al 1992), show that exogenous surfactant is more uniformly distributed if given at birth before ventilation or if followed by high frequency ventilation (HFV). Ikegami and co-workers (Ikegami et al 1998) found that in preterm lambs ventilated for a 24 h period after ST, the extreme styles of ventilation (high Vt, low Vt, HF oscillation) had minimal effects on lung function, surfactant function or surfactant metabolism. These findings are at odds to those of measurements of the effects of ventilation style (HF oscillation (O) versus conventional ventilation) following surfactant treatment on lung injury (Jefferies et al 1993), and to those of clinical trials showing better pulmonary outcomes for surfactant-treated human preterm infants with RDS that were ventilated with HFO than conventional ventilation (Gerstmann et al 1996, Plavka et al 1999). When newborn infants (Krause and Hoehn 1998) and animals (Vilstrup et al 1992), with established RDS, are treated with surfactant, oxygenation improves in accordance with mechanical recruitment of lung volume i.e., from previously unventilated (atelectatic) terminal air spaces (Vilstrup et al 1992) as well as the stabilization of already ventilated air spaces at higher end-expiratory volumes (Krause et al 1997). It has been shown that more homogeneous distribution of instilled surfactant result when the surfactant is

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administered without interruption of assisted ventilation and maintaining PEEP during instillation (Merrett et al 1995, Kobayashi et al 1984, Rider et al 1993).

In neonatal RDS, surfactant deficiency is the major cause of respiratory failure. In contrast, in acute respiratory distress syndrome (ARDS) a variety of insults culminate in lung capillary damage and increased permeability, often associated with diffuse damage to the alveolar epithelium and flooding of the air spaces with proteinaceous edema (Ashbaugh et al 1967, Van Golde 1994, Lachmann 1989, Pfenninger et al 1991). Independent of the primary cause of capillary damage, and as a direct consequence of the pathological events (high permeability lung edema and activated inflammatory cascade) in the alveolar space, a slow failure of the 'surfactant system' occurs (Hallman et al 1989, Hallman 1991, MacIntyre 1995, Raymondos et al 1999, Lachmann 1989). Compositional surfactant changes consist of alterations in surfactant phospholipids (Harris et al 1989, Hallman et al 1989, Bersten et al 1998), direct damage to SP-A (Baker et al 1999), decreased amounts of surfactant-associated proteins (Raymondos et al 1999, Veldhuizen et al 1995), increased ratio of poorly functioning light aggregate surfactant to superiorly functioning heavy aggregate surfactant (Raymondos et al 1999, Veldhuizen et al 1995, Gunther et al 1999), and increased concentrations of inflammatory cells (Raymondos et al 1999, Harris et al 1989) in bronchoalveolar lavage (BAL) fluid. The surfactant changes promote alveolar instability and atelectasis, alter hydrostatic forces, and increase further alveolar flooding (Petty 1994). Concomitant with the increase in lung fluid and atelectasis there are deterioration in lung compliance and gas exchange (Bersten et al 1998). Histologically, the early ARDS-picture somewhat resembles that of

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severe neonatal RDS and is characterized by diffuse alveolar damage, pulmonary edema and hyaline membrane formation (Ashbaugh et al 1967).

When considering treatment options for patients with ARDS it is perceived that the early identification of patients at risk of ARDS and the ability to detect surfactant abnormalities may assist in tailoring specific therapies in order to improve individual patient outcome (Richman et al 1989, Nicholas et al 1997). In addition to standard treatment, therapies aimed at increasing the amount of aerated lung (volume recruitment by high frequency oscillation or the combination of high PEEP with low tidal volume ventilation, with or without the instillation of surfactant, or other surface-tension active material such as perfluorocarbon) could reduce oxygen and ventilator-induced lung injury (Mcculloch et al 1988, Thome et al 1998, Dreyfuss et al 1999).

The value of surfactant replacement in models of acute lung injury, or humans with this condition, other than quantitative surfactant deficiency states, are currently under investigation (Taeusch 2000).

Secondary surfactant dysfunction and/or deficiency have convincingly been demonstrated in experimental models of acute lung injury as well as in human patients with ARDS (Harris et al 1989; Lewis and Jobe 1993, Veldhuizen et al 1995). The clinical response of animals or patients with this condition to surfactant replacement therapy has however until recently been discouraging and conflicting (Lachmann 1989, Richman et al 1989, Baudouin 1997, Anzueto et al 1996, Warren 1998). Some studies reporting on the tracheal instillation of natural surfactant in patients with ARDS show modest but transient improvement in blood oxygenation (Richman et al 1989, Spragg et al 1994,

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Gregory et al 1997) without changes in FRC or static respiratory compliance (Richman et al 1989). In a pilot study, mortality, in a bovine surfactant treated group of patients with ARDS was 18.8%, as compared with 43.8% in the control group (p=0.075) (Gregory et al 1997). An aerosolized synthetic surfactant had no effect on physiological parameters in sepsis-induced ARDS (Anzueto et al 1996).

Early instillation of a natural bovine surfactant in a study of children, aged between 1 month and 201 months, with acute hypoxemic respiratory failure due to a wide variety of diagnoses (sepsis, ARDS near drowning, respiratory syncytial virus pneumonia, aspiration pneumonia, pulmonary hemorrhage), resulted in a rapid and sustained improvement in oxygenation, translating into earlier extubation and discharge from the intensive care unit, when compared to a control group with similar disease profiles (Willson et al 1999). Natural porcine surfactant instillation in another study of paediatric patients with ARDS resulted in a less dramatic improvement in oxygenation, with some children in the onco-haematological group requiring additional doses after 12-18 hours (Marraro et al 1999).

The reason for this inconsistency is unclear, but as in the case of established neonatal RDS, probably related to several interrelating factors. These factors include some of the issues already discussed in the section on neonatal RDS as well as the models used to mimic ARDS. Animal models of acute lung injury (ALI) do not necessarily demonstrate the entire picture of ARDS (Rosenthal et al 1998). For instance, of 4 animal models of ALI, two were superior in producing hypoxemia (saline lavage and hydrochloric acid infusion), though none of the four models were associated with the production of tumour necrosis factor (TNF), thought to be an important mediator in ARDS (Hanley and Repine

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1994). Other problems include: the composition of a specific surfactant preparation (different natural surfactants versus synthetic surfactant), the dose (low dosages, high dosages or repeated doses), the timing of surfactant treatment over the course of injury (early versus late treatment), the method of delivery (aerosol, bolus, lavage, or diluted), and the susceptibility of the administered surfactant to inhibition by plasma-derived proteins (Berry D et al 1986, Seeger et al 1985), proteases, or phospholipases (Touqui and Arbibe 1999) released into the alveolar space upon injury. In addition, lung inflammation, whether associated with ventilator assistance during the course of early neonatal RDS or ARDS, is another important modulator of surfactant function as well as determinant of the response to exogenous surfactant replacement (Groneck et al 1994, Papoff et al 2001, Sarafidis et al 2001, Jaarsma et al 2001, Zimmerman et al 1983). In the clinical situation, lumping patients together under 'hypoxemic respiratory failure', with the aim of assessing their response to exogenous surfactant replacement, may lead to confusion. It is evident that suboptimal surfactant function may be due to diverse mechanisms that operate during the course of lung diseases due to different etiologies.

The complexity of the relationship between ARDS and SRT was further underscored by a study that showed that when surfactants are delivered in diluted form through a lavage technique to a piglet model with acute lung injury, the resultant improved oxygenation and lung volumes were independent of the type of surfactant used (Balaraman et al 1998). Bronchoalveolar lavage (BAL) with a diluted surfactant suspension prior to a proper surfactant instillation at a late stage (3h) during the ventilation of rats with ARDS, resulted in a similar improvement in oxygenation seen

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after surfactant instillation immediately after inducing acute lung injury (Gommers et al 1998).

Methods to enhance the effect of exogenous surfactant in ALI are also under investigation. For instance, the addition of alpha-1-antitrypsin to Survanta exerted a positive effect on oxygenation and surfactant metabolism in surfactant-deficient rats (Belai et al 1999). Alpha-1-antitrypsin is a protease inhibitor and the addition to surfactant reduces the conversion from LA to SA in the lung (Belai et al 1999). Combined use of surfactant and inhalational nitric oxide (INO) in an animal model of ARDS appeared to be more effective in improving intrapulmonary shunting and oxygenation (Zhu et al 1998). Under these circumstances, INO probably improved ventilation-perfusion matching.

The protective role of surfactant replacement therapy on oxygen toxicity has been studied. In primate animals mechanically ventilated with 100% oxygen the administration of exogenous protein-free artificial surfactant by nebulization improved arterial oxygenation, decreased the intrapulmonary shunt and improved lung edema (Huang et al 1995). The histology of the lungs of adult baboons who were treated with 100% oxygen and ventilation and who received a tracheal instillation of artificial surfactant (Exosurf, Glaxo Wellcome), showed a decrease in the inflammatory response (decreased neutrophil infiltration) in the lung and a protective effect of the surfactant on endothelial and epithelial cell integrity (Piantadosi et al 1995). Thus, during the exposure of primate animals to hyperoxia, both artificial synthetic and natural surfactant replacement appears to correct the functional disturbance of native surfactant caused by exposure to 100% oxygen, but does not completely ameliorate the effects thereof.

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In summary, two decades have passed since the first successful human trial was conducted with natural surfactant. We know that the present natural surfactants offer the advantage of saving more infant lives (need to treat 32 patients in order to save 1 additional life) than some simple synthetic formulations. These statistics are bound to change as better or improved formulations are designed and tested. Concerning ARDS, much more research in terms of clinical trials will have to be performed before SRT can be considered to become an integral part of the standard therapy. The next generation of surfactants is generally described to be composed of defined lipids enriched by hydrophobic proteins or peptides (Griese 1999). The aim of developing newer surfactants is to supply formulations that are biophysically more active, less susceptible to in vivo inactivation, and hopefully less expensive (Taeusch 2000). Novel synthetic surfactants would have the additional benefit that they are not derived from animal tissue and therefore contain no infectious agents.

Surfactant inactivation has been the focus of recent workshops and studies and inactivation-resistant surfactant mixtures have been studied, but are not commercially available. Natural surfactants (Survanta, Abbott Laboratories) in a mixture with nonionic polymers (dextrans, polyethylene glycols or polyvinylpirrolidones) have the ability to reduce surfactant inactivation in the presence of meconium or other inactivating substances (Taeusch et al 1999). There is however, little information on the role of improved or altered synthetic surfactants in conditions where surfactant may be inhibited or inactivated. More research, both basic and clinical, is required to increase the knowledge base in this area of the neonatal and adult respiratory distress syndrome (Robertson et al 1999, Almaas et al 1999, Rauprich et al 1999).

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The aim of this study is the development of an effective synthetic surfactant. In addition, the generated data should significantly contribute to the scant research data on the interaction between carbohydrate and surfactant phospholipids in living animals and humans. We therefore undertook to study the *in vivo* physiological effects of a biocompatible sugar in a mixture with DPPC, in an animal model with respiratory distress syndrome. **The hypothesis of this study is to show that a sugar, which is unrelated to any region within the known lung surfactant chemical composition, in combination with DPPC, improves arterial oxygenation in an in vivo- lavaged lung model of surfactant depletion.**

The *in vivo* study protocol was performed in two parts. The first study examined the hypothesis that a novel synthetic surfactant has a similar effect as a commercially available synthetic product (Exosurf, GlaxoSmithKline) in an animal model of respiratory distress syndrome. The second study assessed the contribution of Trehalose to the surfactant formulation. The *in vitro* study was performed to test the hypothesis that the surface activity of DPPC could be improved by the addition of trehalose and / or hexadecanol, tyloxapol, and $CaCl_2$. Our hypothesis was that the addition of a carbohydrate (trehalose) and / or tyloxapol, hexadecanol, and / or $CaCl_2$ to DPPC, would improve, in a stepwise manner, the *in vitro* surface activity of the DPPC-based formulation. In addition to studying the activity of surfactant at body temperature (37°C), we tested the hypothesis that LPM-1 may act as a surface tension-lowering agent under hypothermic conditions.

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2. Methods

2.1 General

The study protocol was reviewed and accepted by the Research Committee of the Faculty of Medicine of the Stellenbosch University (96/096). All procedures described in this study conformed to the guidelines on the use of animals in biomedical research of the South African Medical Research Council (1993).

2.1.1 Synthesis and purification of a novel surfactant

A synthetic, protein-free surfactant, containing both DPPC and trehalose as major components by weight and composition were prepared by the Department of Medical Biochemistry of the Faculty of Health Sciences of the Stellenbosch University (Lipid mixture-1). The 1,2-dipalmitoyl-*sn*-phosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids (Alabaster, USA). Trehalose, a nonreducing disaccharide of glucose, was obtained from Pfanstiehl Ltd. (Davenham, Cheshire, UK). Hexadecanol (cetyl alcohol) and tyloxapol were purchased from Sigma Chemical Co (St Louis, MO, USA). Merck (Darmstadt, Germany) was the supplier of the chloroform and NaCl used. All of these reagents were of the highest purity (99%) available and certificates of analysis were provided with each purchase. The purity of DPPC was checked by chromatography. All reagents were also inspected to conform to sensory characteristics (i.e. sight, taste, smell) as described in the US Pharmacopoeia.

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2.1.2 Experimental surfactant preparations

2.1.2.1 Lipid mixture-1 (LPM-1):

Dipalmitoylphosphatidylcholine in chloroform (5.4ml) at a concentration of 200mg/ml and cetyl alcohol in chloroform (1.2ml) at a concentration of 100mg/ml were mixed and taken to dryness under a stream of N_2 gas (Afrox, Medical grade) at 37°C. To the dried lipids was added 5.4ml trehalose sugar in sterile distilled water (100mg/ml) and 5.4 ml 1M NaCl. The resulting mixture was resuspended to homogeneity by probe sonication (Virtis VirSonic Probe sonicator), with a 1-minute cooling interval on ice between each sonication event (pulse: 23 cycles / sec; total sonication 4 X 23 = 92 pulses). Thereafter, 0.81 ml 10% tyloxapol and 42 ml sterile water were added to the suspension, followed by mechanical vortexing for approximately 30s at room temperature. The resulting suspension was freeze-dried on a Virtis Freezemobile 6. The dried powder was pulverised with a spatula while scraping out of the freeze-drying flask and weighed out into clear glass vials. Dry surfactant was reconstituted with 6 ml distilled, pyrogen-free water immediately before clinical use. Each reconstituted vial contained 20 mg/ml DPPC, 2.2 mg/ml cetyl alcohol, 1.5 mg/ml tyloxapol, 10 mg / ml trehalose and 100 mM NaCl. The surfactant was then administered at a dose of 70 mg DPPC / kg body weight.

2.1.2.2 Lipid mixture 2

Lipid mixture 2 is a **protein-free synthetic surfactant**, resembling the composition of commercially available Exosurf (Glaxo Wellcome). The formulation was prepared according to the protocol mentioned above (Department of Medical Biochemistry of the University of Stellenbosch). The final preparation consisted of DPPC (20mg / ml) in

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combination with tyloxapol (1.5 mg / ml), hexadecanol (cetyl alcohol) 2.2 mg/ml and NaCl (100mM). The formulation was administered at a dose of 70 mg DPPC / kg body weight

2.1.2.3 Exosurf

Exosurf (GlaxoWellcome), is a **protein-free, synthetic surfactant** in clinical use and consists of dipalmitoylphosphatidylcholine (DPPC) alternatively known as colfosceril palmitate, 13.5 mg/ml, with 1.5 mg/ml tyloxapol (formaldehyde polymer with oxirane and 4-[1,1,1,3,3-tetramethylbutyl]phenol) and 2.2 mg/ml hexadecanol. It is supplied as a sterile lyophilised powder that requires solubilisation with 8 ml of sterile water. The solution is then administered at a dose of 5ml / kg (67.5 mg DPPC / kg) body weight by direct instillation into the trachea.

2.1.2.4. Trehalose

Trehalose ($C_{12}H_{22}O_{11}$) (TRE), a **nonreducing disaccharide of glucose** (1–alpha-D–glucopyranosyl–1–alpha–D–glucopyranoside) was obtained from Sigma Chemical Co (St. Louis, MO, USA). The dry powder was dissolved in 100mM NaCl at a final concentration of 10 mg/ml (1%). The preparation was administered at a dose of 3.5 ml / kg bodyweight (35 mg / kg).

2.2 Animal protocol

Adult New Zealand White rabbits were studied during a 3-hour protocol. At the start of each experiment, rabbits were placed on their backs and anaesthetised with 50 mg/kg ketamine hydrochloride 1% (intramuscular). A second dose of ketamine (25 mg/kg) was

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administered if the animal showed signs of distress or restlessness. A peripheral venous catheter was placed in a vena auricularis and an infusion of 5% dextrose in Ringers lactate commenced at a rate of 5 ml/kg/hour. A tracheostomy was performed under local anesthetic infiltration (1-2 ml lignocaine 1%). After the tracheostomy was performed and the trachea intubated with an uncuffed 3.5 mm (internal diameter) endotracheal tube, intravenous sodium pentobarbitone (6 mg/kg body weight) and pancuronium bromide (0.2 mg/kg body weight) were administered. The endotracheal tube was advanced to the mid-trachea and secured by ligature to avoid leakage around the tube. Anaesthesia was maintained with an infusion of sodium pentobarbitone at a dose of 6 mg/kg/h. For additional muscle paralysis, pancuronium bromide (0.1 mg/kg/h) was used.

In the first study, rabbits were artificially ventilated using the time-cycled pressure-limited mode (Drager Babylog 8000, Dragerwerke, AG, Germany) with 100% oxygen, a peak inspiratory pressure (PIP) sufficient to ensure a tidal volume of 8-12 ml/kg body weight at a rate of 40 breaths per minute (bpm) and an inspiration: expiration ratio of 1:2 and a positive end-expiratory pressure (PEEP) of 2 cmH_2O. In the second study, rabbits were ventilated with a Bear Cub 750 VS, ventilator (Respiratory Care, Africa) at the exact described settings as above. A tidal volume (V_t) between 8-12 ml/kg was selected since this setting results in a partial arterial pressure of carbon dioxide ($PaCO_2$) between 4.5 and 5.3 kPa (Rider et al 1993, Dizon-Co et al 1994, Zhu et al 1998). Arterial oxygenation status was continuously monitored throughout the experiment with a pulse oximeter with the probe affixed to one of the rabbit's hind legs (Ohmeda Biox 3700e, Louisville, USA) (Schnapp and Cohen 1990).

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The left carotid artery was cannulated for continuous monitoring of blood pressure (Datex Cardiocap, CCI 104, Helsinki, Finland) and for intermittent determination of arterial blood gases. Rectal temperature was maintained between 38.5 and 41°C using a heating pad.

After baseline measurements (see below), respiratory failure (surfactant deficiency) was induced by repeated (2-5 min intervals) whole-lung lavage (5-7 times) with 25 ml/kg aliquots of warm isotonic saline (37ºC-38ºC) (Lachmann et al 1982, Lachmann et al 1980, Sandhar et al 1988). The animal was shaken gently as the lavage fluid was instilled by syringe. The instilled saline was then withdrawn with the animal briefly disconnected from the ventilator. A lavage procedure was successful when the arterial oxygen saturation fell below 80% (in 100% oxygen) and remained there for 5 minutes. In a pilot study, this value correlated with a PaO_2 below 12 kPa (unpublished data). Following the first lavage, the PEEP was increased to 5 cmH_2O (maintained at 5 cmH_2O throughout the study) and the PIP adjusted to maintain the tidal volume between 8-12 ml/kg. The FiO_2 was kept at 1 throughout the study. A recovery period of 5 min was allowed after the last lavage was performed. After reaching 'steady state' (5 minutes), surfactant-depleted rabbits were randomised into treatment groups. Randomisation by random number allocation was done by the biochemist, blinded to the procedures. The instillation of bolus dosages of surfactant or saline was completed within a 1-minute period. After receiving the weight-adjusted doses of study-drugs, the PIP was finally adjusted to maintain tidal volumes in the same range as before lavage (8-12 ml/kg). The animal's electrocardiogram and systemic blood pressure were

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continuously monitored. Volume expansion (Ringers lactate) was indicated when the mean arterial blood pressure was below 55 mmHg.

The experimental protocol was continued until the animal died or for a maximum of 3 hours. Animals that were alive at the end of the experiment, received a lethal injection of intra-arterial potassium chloride, while still under general anaesthesia.

2.3 Study 1

In this study rabbits were randomised into 4 groups of 6 rabbits each. Three groups received an intratracheal bolus dose of synthetic surfactant. In the one group, animals received lipid mixture 1, and the second group, lipid mixture 2. Both of these groups received DPPC at a dose of 70 mg/kg. In the third group, Exosurf was administered at a dose of 67.5 mg/kg DPPC. The fourth group of animals served as controls and received 5 ml / kg body weight of isotonic saline.

2.4 Study 2

Fifteen adult New Zealand White rabbits were studied during a 3-hour protocol with the addition that the first broncho-alveolar lavage fluid was used to analyse the cell and phosholipid profiles of the epithelial lining fluid of the rabbits (see below).

Surfactant-depleted rabbits were randomised into 3 groups of 5 rabbits each. One group received an intratracheal bolus dose of LPM-1 (70 mg / kg DPPC), one group received Trehalose (35 mg / kg), and one group of animals served as controls, receiving 5 ml / kg body weight of isotonic saline.

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Immediately prior to natural or induced death, a final broncho-alveolar lavage (10 ml) was performed (see below).

2.5 Physiological measurements

2.5.1 Blood gases

During the 3-hour protocol, arterial blood samples in both *in vivo* studies were collected at the following times: before lavage; 5 min after the last lavage; 15, 30, 60, 90, 120, and 180 min after surfactant treatment. Measurements in the saline control group were done at the same intervals.

Samples were analysed using a pH / blood gas system (IL 1306, Instrumentation Laboratory, Italy). All arterial blood gases were analysed on an inspired O_2 fraction of 1.0. The IL 1306 calculates oxygen saturation of available haemoglobin from the measured PO_2, pH, and pCO_2.

To further quantify pulmonary lung injury and gas exchange, the oxygenation index, arterial alveolar oxygen tension ratio and pulmonary shunt were determined.

The oxygenation index (OI), a measurement of the amount of inspired oxygen and the driving pressure required to oxygenate the arterial blood were calculated for each time point using the equation:

OI = [Mean airway pressure (MAP) x FiO_2/ PaO_2 x 100; where FiO_2 is the fractional inspiratory concentration of oxygen (Robertson and Lynam 1992). Mean airway pressure is expressed in cmH_2O, and the PaO_2 in mmHg.

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The arterial-alveolar tension ratio (a/A ratio) was calculated from the formula:

$PaO_2/ PAO_2 = PaO_2/[(P_B-47) \times FiO_2- PaCO_2/RQ]$, where P_B is barometric pressure, FiO_2 is the fraction of inspired oxygen, and RQ is the respiratory quotient (0.8). The PaO_2 and $PaCO_2$ are expressed as mmHg.

The pulmonary shunt was determined by the following equation:

$Q_s/Q_t = 88.77-2.4 (20.4 \log PaO_2/FiO_2)$ (Coetzee 1987).

2.5.2 Lung function measurements in the first study

Inspiratory tidal volume was continuously displayed by the Dräger ventilator. The compliance of the respiratory system (CRS) was determined by dividing the tidal volume displayed by the infant ventilator by the difference between PIP and PEEP (Ueda et al 1994, Lewis et al 1991, Mizuno et al 1995, Baboolal and Kirpalani 1990). This method of determining CRS was previously validated against the single-breath occlusion technique resulting in a significant correlation ($r^2 = 0.97$) (Kirpalani and Baboolal 1990). Further evaluation of this correlation by proper statistical analysis was not possible because the original data was not published.
All of the measurements were corrected / standardised for body weight.

2.5.3 Lung function measurements in the second study

Tidal volume, airflow, airway pressure and dynamic compliance of the respiratory system (CRSdyn) were measured through a calibrated pneumotachograph (Bicore CP-100, Bicore Monitoring Systems, Irvine, CA) before and after lavage. The model is a microprocessor-controlled respiratory monitor developed for newborn infants and

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paediatric patients (Berman et al 1995). The on-line compliance values, displayed by Bicore CP-100, were checked against a standardized brass test lung model with known compliance prior to studying each rabbit. The displayed values of compliance were within 10% of the known value. All of the measurements were corrected / standardised for body weight.

2.6 Pathological evaluations

2.6.1 Light microscopy / histology

At the end of the study or in cases of death prior to the 3h-study period, the thoracic cage was opened under water to exclude a pneumothorax. The lungs were then excised en-bloc. One main-stem bronchus was tied close to the carina and cut distal to this tie. Sections of this lung were placed in 3% glutaraldehyde for electron microscopy examination. The remaining lung was then fixed transtracheally with 10% buffered formalin, and then immersed in formaldehyde. Fixation blocks of lung tissue representing upper, middle and lower lobes were sectioned and stained with haematoxylin and eosin (HE). Where the right middle lobe was clearly divided into two segments, separate samples were taken and labelled. A semi-quantative morphometric analysis of lung changes / injury was performed by a pathologist (Roger James) who was unaware of the coding of individual specimens to ensure that findings were reported in a blinded manner. Since there are no standard accepted criteria for grading the severity of 'hyaline membrane disease', five features, previously described by researchers, were assessed.

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They included the following:

a) Presence of hyaline membranes,

b) Lymphatic dilatation,

c) Neutrophilic infiltration,

d) Necrotic airway debris, and

e) Vascular congestion.

Sections were surveyed by light microscopy and hyaline membranes, lymphatic dilatation and the presence of neutrophils were graded on a scale from 0 to 3 [0 absent; 1 mild, 2 moderate, 3 severe (present in the majority of fields examined)]. To improve quantification of these grades visual analogue scales similar to those used in the grading of gastritis by the Sydney System (Dixon et al 1996) were devised. Debris and congestion were graded 0 or 1 (absent or present) (Fig 2.6.1.1-2.6.1.4). Scores were given for each criterion on every section by two pathologists.

2.6.2 Electron microscopy

Pieces of lung were fixed by immersion in 3% glutaraldehyde buffered to pH 7.4 with 0.1 M phosphate. Tissue were postfixed in 1% osmium tetroxide and processed for electron microscopy. Semi-quantitive assessment of the ultrastructural features was performed and an attempt made to grade these findings (RW James, MMed Dissertation 2000, Department of Anatomical Pathology, Stellenbosch University). Since the sections could not be regarded as representative of the case as a whole, a general descriptive study of the EM features was decided on.

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2.7 Processing and analysis of bronchoalveolar lavage (BAL) fluid

In study 2, BAL fluid was sampled during the first lavage and at the end of the experiment to isolate and characterize phospholipids and cells (saline-treated, n = 5; TRE-treated, n = 5; LPM-1, n = 5). The phospholipid analysis was performed in order to provide a basis for meaningful comparison with the human surfactant system.

Briefly, BAL fluid was centrifuged at 250 g for 10 min to remove cells. The total cell count was determined in a haemocytocytometer and the differential count was performed on smears made in a cytocentrifuge and stained with H & E and a non-specific esterase.

The cell free supernatant was spun at 48,000 g for 1 h to produce a pellet. This crude surfactant pellet (CSP) was resuspended in 400 microliter of a solution containing 0.15 mmol/L NaCl. Three hundred microliters of the CSP was extracted with chloroform/ methanol (2:1; v/v) according to a modified method of Folch et al (1957). All the extraction solvents contained 0.01% butylated hydroxytoluene (BHT) as an antioxidant. Heptadecanoic acid (C17:0) was used as internal standard to quantify the individual fatty acids. Individual phospholipid classes were separated by TLC on pre-coated silica gel 60 plates (10 X 10 cm) without a fluorescent indicator (Art. 1.05721, Merck, Darmstadt, Germany) using chloroform / ethanol / triethylamine / water (40:50:40:10, by vol) as solvent. BBOT (2,5-bis-(5'-tert.-butylbenzoxazolyl-[2'] thiophene; 10 mg/ml; Sigma Chemical Co.) was added to the solvent to visualise the lipid bands with longwave ultraviolet light after developing the plates in a filter lined TLC chamber. Lipids were identified by co-migration with standards. The bands were scraped off and the lipid phosphorus content of total and individual phospholipids was quantified as described by

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Taya and Ui (1966). An aliquot of the lipid extract was used for total CSP fatty acid composition. After transmethylation, the lipids were cooled and the resulting fatty acid methyl esters (FAME) extracted. Thereafter the top hexane layer was evaporated to dryness, redissolved, and analysed by GLC (Varian Model 3300, equipped with flame ionizing detection). The FAME was identified by comparison of the retention times to those of a standard FAME mixture (Nu-Chek-Prep Inc., Elysian, Minnesota). An aliquot of the CSP was used to measure the total protein content by a modified Lowry procedure using bovine serum albumin as standard (Markwell et al 1978).

2.8 Study 3: *In vitro* surface tension measurements

2.8.1 Materials

The preparation of the different formulations was according to the previously described methodology (2.1). DPPC (20 mg / ml) was prepared and tested with varying chemicals as follows:

2.8.1.1 Only DPPC;

2.8.1.2 2.8.1.2 DPPC plus hexadecanol, tyloxapol and TRE (LPM-1);

2.8.1.3 DPPC plus hexadecanol and tyloxapol (LPM-2);

2.8.1.4 DPPC plus hexadecanol, tyloxapol and 2 mM calcium chloride (LPM-2 + $CaCl_2$);

2.8.1.5 DPPC plus TRE;

2.8.1.6 and DPPC plus trehalose (TRE), tyloxapol and $CaCl_2$.

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2.8.2 Apparatus and procedure

The surface tension of the film was measured with a Du Nüoy tensiometer (Cambridge Instrument Co. England) with a platinum ring of 1cm in diameter. A calibration curve with water at varying temperatures was used to calibrate the tensiometer (Table 2.8.2.1). The curve followed: $y = -0.1597x + 75.955$ ($R^2 = 0.999$).

Surface tensions were measured in triplicate at 20ºC and 37ºC. Temperature was maintained within ± 0.5ºC by performing the studies in a temperature and humidity-controlled room. In this controlled environment no air currents were present. The pool used to simulate the aqueous hypophase was 10 ml of deionised water (Millipore Corporation, Bedford, Massachusetts) covering an area of 33.18 cm^2. The surface area was not altered during the study.

The parameters controlled were: the temperature of the surfactant-deionised water mixture (20ºC and 37ºC), room temperature and humidity, and surfactant concentrations. Because some of the preparations were not readily wetable and therefore slow to spread (DPPC and DPPC + TRE), surfactant formulations were initially solubilized in the deionised water for 24 hours at 25°C and then gently poured into a sterile petri dish. Ten minutes was allowed for temperature equilibration for measurements at 20°C and 37°C. Static maximal surface tensions were then measured by lowering the specified platinum ring onto the surface of the air-water interface, thereafter withdrawing the ring. The ring detachment method of Du Noüy (Sekabunga et al 1969, Barrow and Hills 1979) measures a unique relationship at the critical point of detachment and surface tension as the peak pull per unit perimeter necessary to pull out the ring. In addition, it avoids

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contact-angle artefact, something that is a problem with the Wilhelmy balance studies. The maximum force (FR) needed to pull or 'pluck' the ring out of the surface, is related to (L), the total wetted perimeter (inside + outside) and surface tension (γ) as:

$F_R/L = \gamma$ (Barrow and Hills 1979)

The measurement is recorded at the moment when the ring loses contact with the surface. Each experiment was performed in triplicate. After pouring the mixture into the dish, the pH was monitored (only during the 20°C study) (Table 2.8.2.2).

2.9 Primary outcome measures of the first *in vivo* study

The primary outcome variables considered for by statistical analysis were:

1) PaO_2, a/A ratio, oxygenation index, compliance of the respiratory system, and intrapulmonary shunt.
2) Survival / Mortality.
3) Histological and ultrastructural changes between surfactant treated rabbits and saline treated rabbits.

2.10 Primary outcome measures of the second *in vivo* study

The primary outcome variables considered for by statistical analysis were:

1) PaO_2, a/A ratio, oxygenation index, compliance of the respiratory system, and intrapulmonary shunt
2) Survival / Mortality

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2.11 Primary outcome measures of the *in vitro* surface-tension study

The description of the surface behaviour of:

1) DPPC
2) DPPC plus trehalose (TRE)
3) DPPC plus hexadecanol, tyloxapol and TRE (LPM-1)
4) DPPC plus hexadecanol and tyloxapol (LPM-2)
5) DPPC plus hexadecanol, tyloxapol and 2 mM calcium chloride (LPM-2 + $CaCl_2$)
6) DPPC plus trehalose (TRE), tyloxapol and $CaCl_2$.

2.12 Statistical analysis

In order to establish comparability of the experimental groups before surfactant instillation, pre-lavage and baseline (post-lavage) measurements were compared using a standard nonparametric method (Wilcoxon Rank Sum test). Data are summarized as means ± SD's. Longitudinal data were generated by measurements of individual animals at successive time points. Since missing values occurred over time and not according to any pattern, variables, and its relation over time in this study represents 'irregular' longitudinal data. To examine these data, an exact distribution-free procedure is used in which a summary regression curve is fitted to the observed data, and confidence limits for the curve are found by a permutation argument (Maritz et al 1998). The results of testing groups for homogeneity are represented graphically, and as approximately chi-squared distributed statistics. In most cases the regression curves fitted are second order polynomials so that the number of degrees of freedom associated with the chi-squared

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statistics is 3 (k-1), where k is the number of groups. The graphs are constructed to show a central curve, expected under the null hypothesis of homogeneity, and two enclosing curves between which the observed curve should fall with probability 0.95. It should be noted that the chi-squared test is a global test, whereas the graphical test is pointwise. Thus it may happen that a graph indicates a significant deviation from the expectation when the chi-squared test indicates acceptance of the null hypothesis. In such cases it is appropriate to focus on the pointwise test as it refers to a more specific hypothesis.
The graphs in the first *in vivo* study are presented in the following order: 1) All four groups (LPM-1, LPM-2, Exosurf, saline), 2) the surfactant groups without the saline group, and 3) the LPM-1 and Exosurf groups. The graphs are presented in the described manner since when all four products are analysed and the individual graphs representing the time profiles for the a/A ratio, OI and PaO_2 are displayed in the figure, the graphs of two products, namely, saline and Exosurf, lie totally or partially outside the grey zone (confidence interval). For instance, in figure 3.1.8.5, the graph representing the saline group is below the grey zone. This implies that the saline group is largely responsible for the observed lack of homogeneity. The greatest Kpa values are exhibited by the Exosurf and LPM-1 groups. A test of Exosurf versus LPM-1 therefore has to be performed without the distorting influence of the saline group.

In the second *in vivo* study only the graphs of the time profiles of LPM-1 and trehalose are presented. In that study the data of the saline group of rabbits were omitted because of their early mortality, rendering too little data to include this group in the final analysis.

The data of the *in vitro* surface tension study analysis are the means of the three observations at each point. In the figures, means (mean surface tension values) are plotted

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against $\log_e$ (dilution). These plots indicate that each of the substance sequences follow straight line trends and can, therefore, be summarized in the slope (β) and intercept (α) of a linear regression. In the regression calculations the $\log_e$ (dilution) origin was taken to be dilution 250, thus α is the ordinate at $\log_e$ (dilution = 5.52). Differences between substances were confirmed by three-way analysis of variance.

Lung injury data were analysed using the Kruskal-Wallis nonparametric ANOVA test, followed by Dunn's multiple comparisons test if a difference was found. Statistical significance was accepted at p-values < 0.05.

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3. Results

Study 1: Evaluation of a novel surfactant (1,2-dipalmitoyl-*sn*-phosphatidylcholine and trehalose [$C_{12}H_{22}O_{11}$]) and comparison with other synthetic formulations.

3.1 General condition and description of the animals immediately before and after lavage (point 0)

Twenty-four adult New Zealand White (NZW) rabbits were studied. Their physiological and ventilation characteristics, blood gas results and calculated respiratory indices, before and directly after lavage (point 0), are displayed in Table 3.1.1, 3.1.2, 3.1.3, and 3.1.4, respectively. No differences were found between the different groups in any of the studied variables.

The injury induced by 5-7 lavages in this model produced a significant pulmonary shunt (Qs / Qt: p=0.00001) that resulted in a mean PaO_2 value of 8.5 ± 1.9 kPa, 5 minutes after the final lavage (point 0). At this point the mean pH, a/A ratio, and CRS, had decreased to a significant extent, compared to the pre-lavage values ($p< 0.05$; post-lavage versus pre-lavage). Simultaneously, the mean $PaCO_2$, OI, and intrapulmonary shunt (Q_s/Q_p) increased significantly from pre-lavage values ($p<0.05$, post-lavage versus pre-lavage).

There were no significant differences with regard to the total administered dose of DPPC between the surfactant recipients (p = 0.17; Kruskal-Wallis ANOVA). The LPM-1 group received a mean total dose of DPPC (±SD) of 172 mg (11), the LPM-2, 178 mg (27), and the Exosurf group 149 mg (20.5).

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3.1.1 Analysis over ensuing 3 hours

Tidal volume (Figure 3.1.1) and minute ventilation (Figure 3.1.2) before and after lavage remained stable.

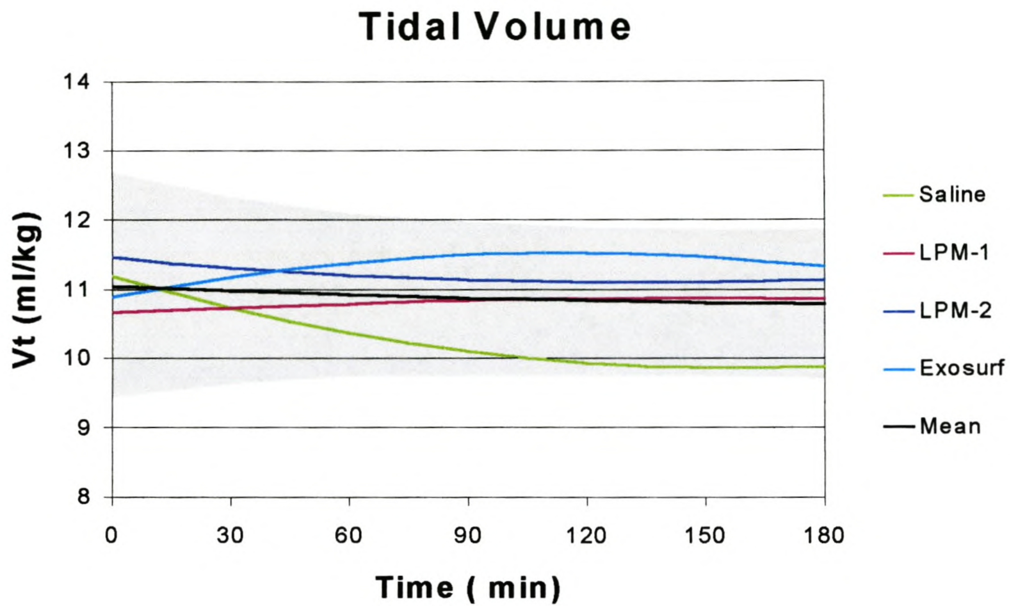

Figure 3.1.1. Tidal volume. The solid line (black) shows the expected regression curve and grey zone the 95% pointwise confidence limits. The time profile of the four four groups did not differ significantly.

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3.1.2 Minute ventilation

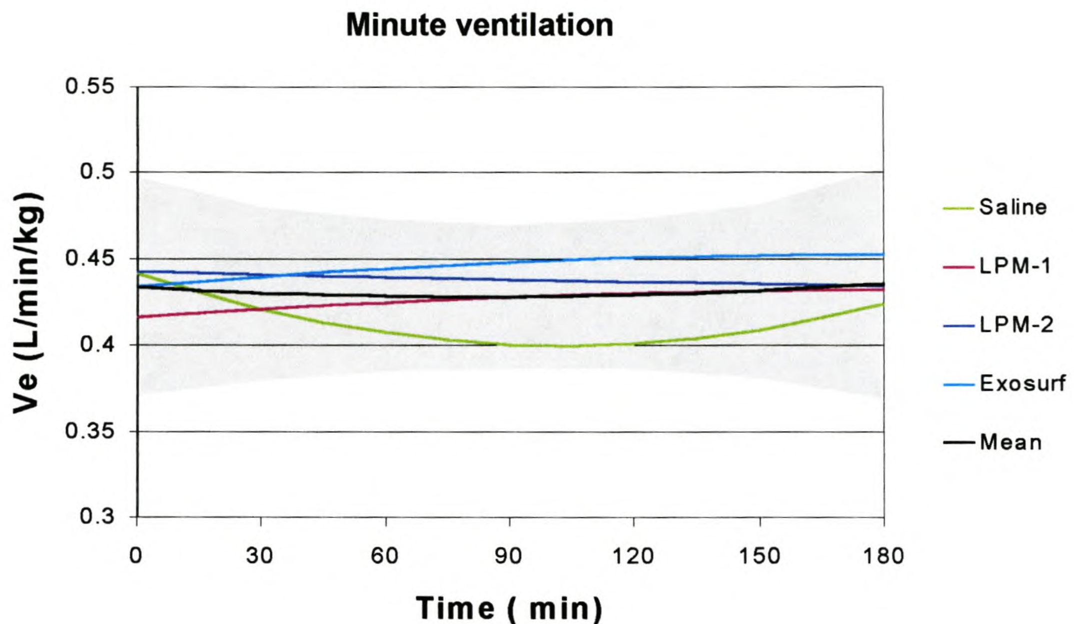

Figure 3.1.2. Minute ventilation. The solid line (black) shows the expected regression curve and grey zone the 95% pointwise limits. The time profiles of the four groups do not differ significantly.

3.1.3 The mean arterial blood pressure

There was no significant change between the mean systemic arterial blood pressure (MABP) at baseline (91.5 ± 20.4 mmHg) and after the final lavage (85.5 ± 21.3 mmHg; p=ns). During the study period the MABP values for the whole group of animals varied between 81.1 ± 15.5 mm Hg and 93.8 ± 15.3 mm Hg. The time profile for the MABP did not differ between the groups (Figure 3.1.3; $\chi^2 = 5.71$, p=0.12).

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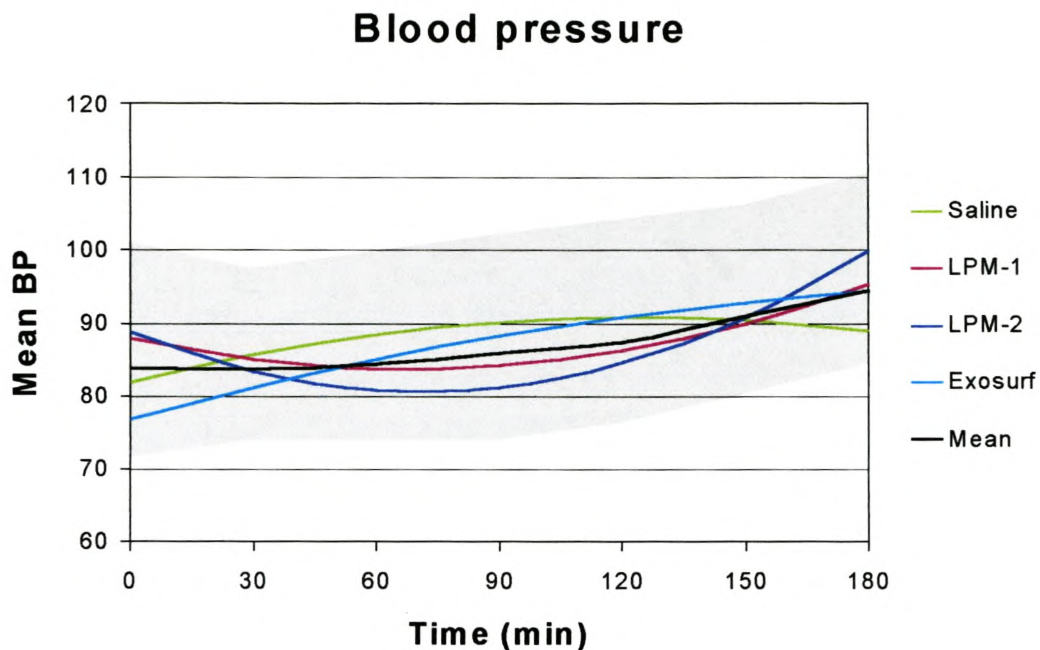

Fig 3.1.3. The time profile of the mean arterial blood pressure. The solid line (black) shows the expected regression curve and grey zone the 95% pointwise confidence limits. The time profiles were not different between groups.

3.1.4 Heart rate

Heart rate remained stable between baseline and point 0 (p=ns). The time profiles for the heart rate were similar in all groups. The saline group experienced a gradual downward trend, deviating significantly from the other groups after 120 min (χ^2 = 10.97, p= 0.27; refer to Statistical methods 2.12).

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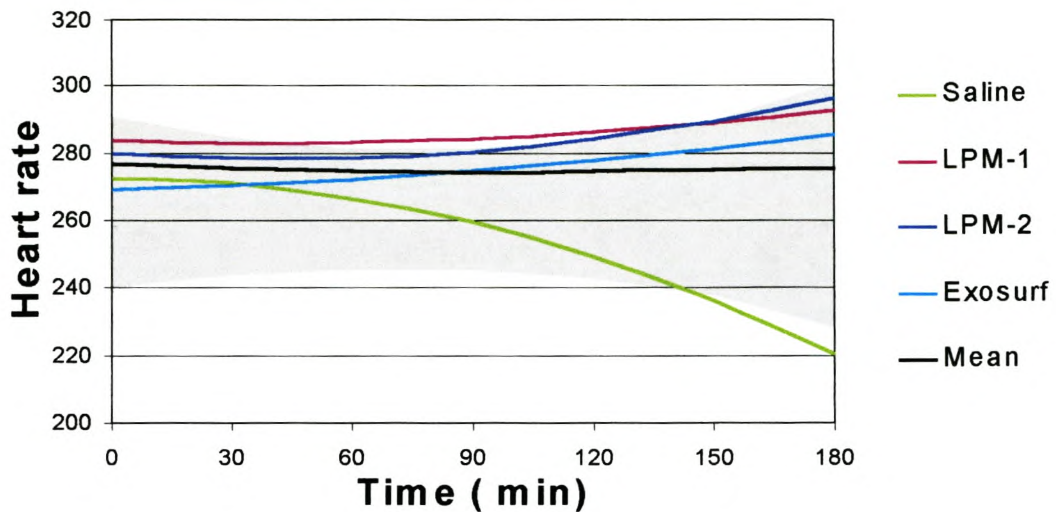

Fig 3.1.4. The mean heart rate. The solid line (black) shows the expected regression curve and grey zone the 95% pointwise confidence limits. The time profile was different between groups. The observed curve for the saline group deviated significantly from the expectation, falling below the 2.5 confidence limit, after 120 min.

3.1.5 The $PaCO_2$

The time profile of the mean $PaCO_2$ was not significantly different between the surfactant groups (Q = 4.76, p=ns). The $PaCO_2$ in the saline group however, deviated significantly from the surfactant groups between 60 and 160 min.

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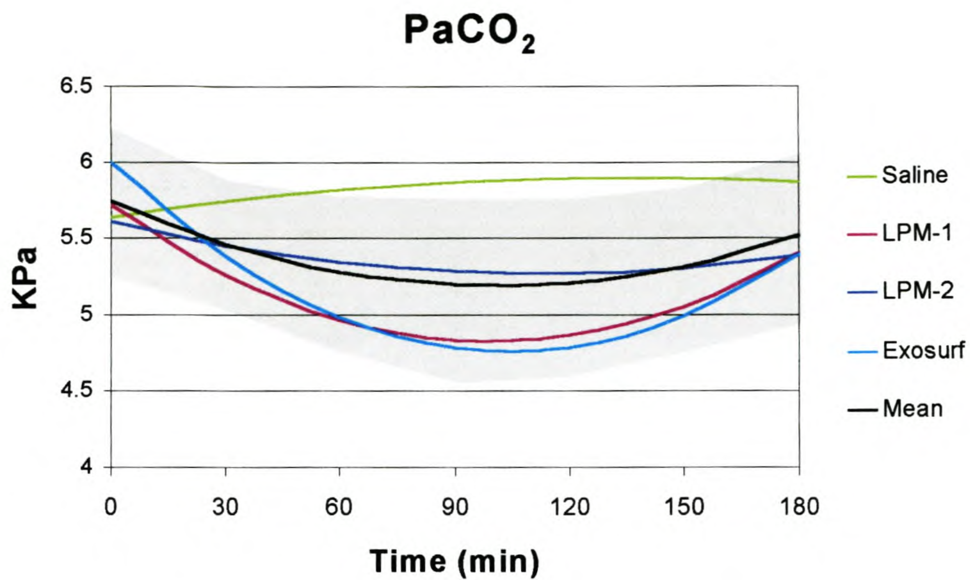

Fig 3.1.5. The mean $PaCO_2$ (kPa). The solid line (black) represents the expected regression curve and the grey zone the 95% pointwise confidence limits. The time profile was not different between the surfactant groups. The saline group had significantly higher values between 60 min and 160 min.

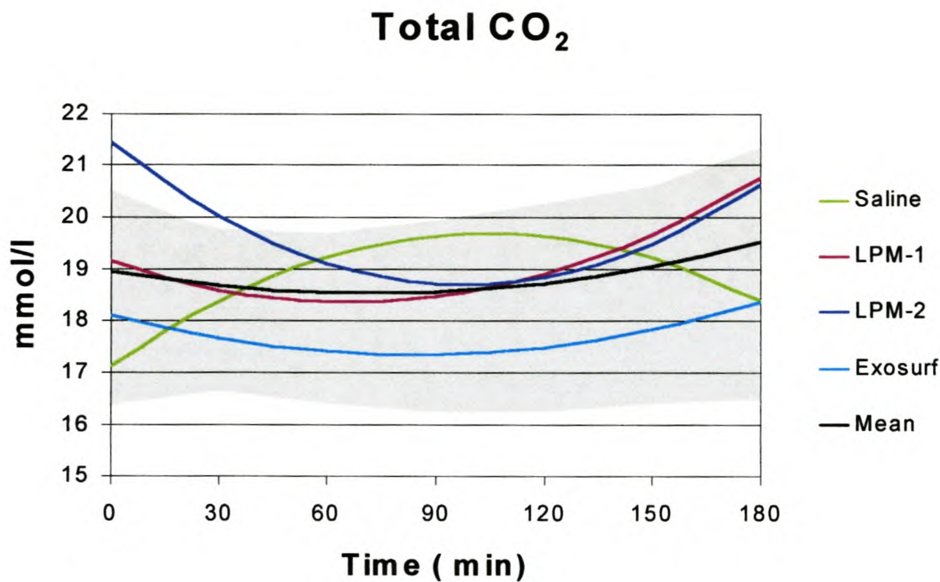

Fig 3.1.6. Total CO_2 content (mmol/L). The solid line (black) represents the expected regression curve and the grey zone the 95% pointwise confidence limits. The time profile was not different between groups.

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3.1.7 pH

The mean pH in the LPM-1 group showed a progressive improvement over time. In the LPM-2 group the mean pH followed a hyperbolic pattern:-decreasing from prelavage levels to its lowest value at 100 min. Thereafter the mean pH progressively increased between 100 min and 180 min, to above the expected value of the group. The pH curve in the Exosurf group followed a parabolic course: initially increasing to reach expectation at 60 min, thereafter decreasing below the expected value between 150 and 180 min.

Because of the wide confidence intervals, the differences were however, non-significant ($\chi^2 = 13.8$, p=0.12).

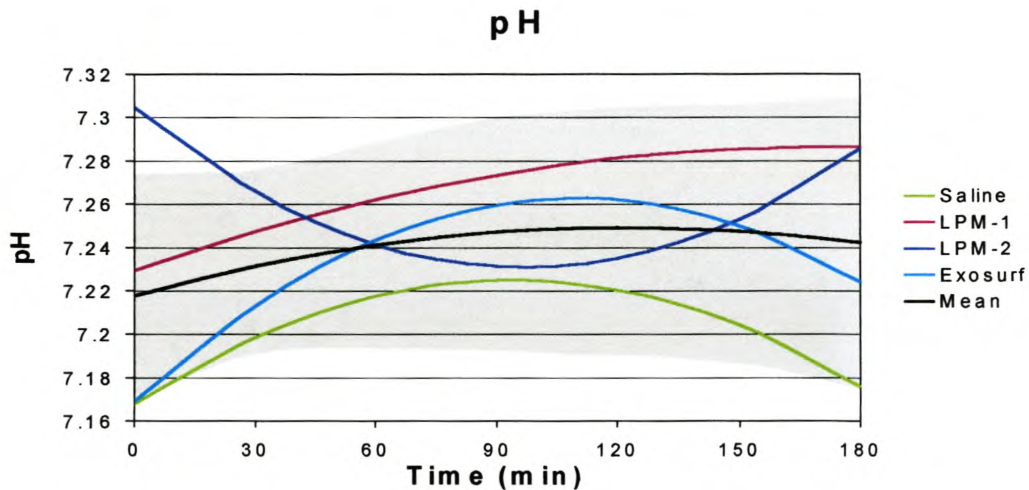

Fig 3.1.7. The mean pH. The solid line (black) represents the expected regression curve and the grey zone the 95% pointwise confidence limits. The time profile was not different between groups.

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3.1.8 Main outcome variables

3.1.8.1 The PaO2 (kPa) (all four groups)

Sequential PaO_2 values for the four treatment groups are shown in Fig 3.1.8.1. These values demonstrate significant differences between groups, already evident at 15 min after treatment. In particular, three surfactant groups, LPM-1, LPM-2 and Exosurf, demonstrated significant increases in PaO_2 within 15 min of administration. The observed curve for the Exosurf group diverts significantly upwards and crosses the 97.5% pointwise limit within minutes after Exosurf instillation. The curve for the saline group diverges downwards from the expected curve and crosses the 2.5% pointwise limit within 15 minutes after saline instillation.

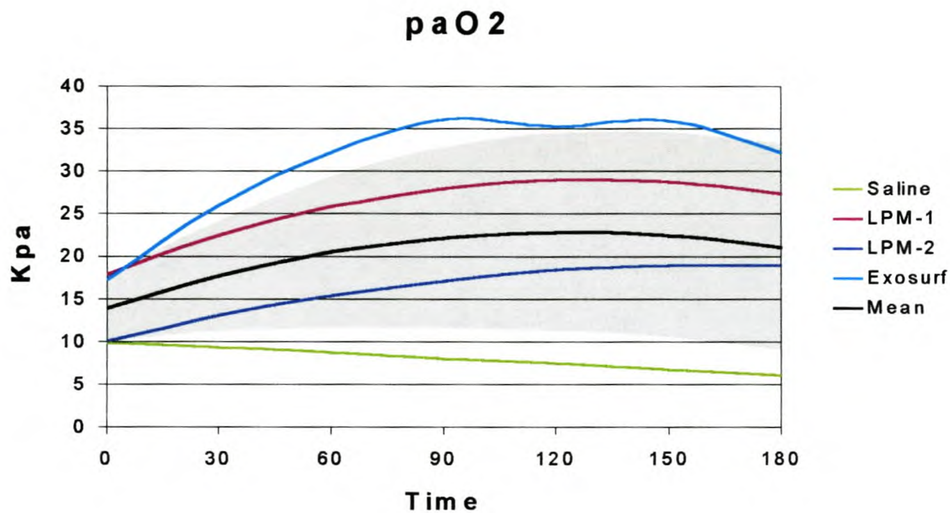

Fig 3.1.8.1 The time profile of the mean PaO_2 (kPa). The solid line (black) shows the expected regression curve and grey zone the 95% pointwise confidence limits. The time profile was different between the groups (see text).

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3.1.8.2 Oxygenation index (all four groups)

The time profile of the oxygenation index (OI) was significantly more favourable in both the LPM-1 and Exosurf groups (χ^2=34.12, p<0.000). In these two surfactant groups, between the time-points 15 min and 180 min, the regression curves are below the expected (black line). The regression curve for the Exosurf group deviates significantly from expectation within 30 minutes after treatment (refer to Statistical methods section 2.12). The regression curve for the saline group diverges from expectation and crosses the 95% pointwise limits within 15 to 30 minutes after the instillation of the saline.

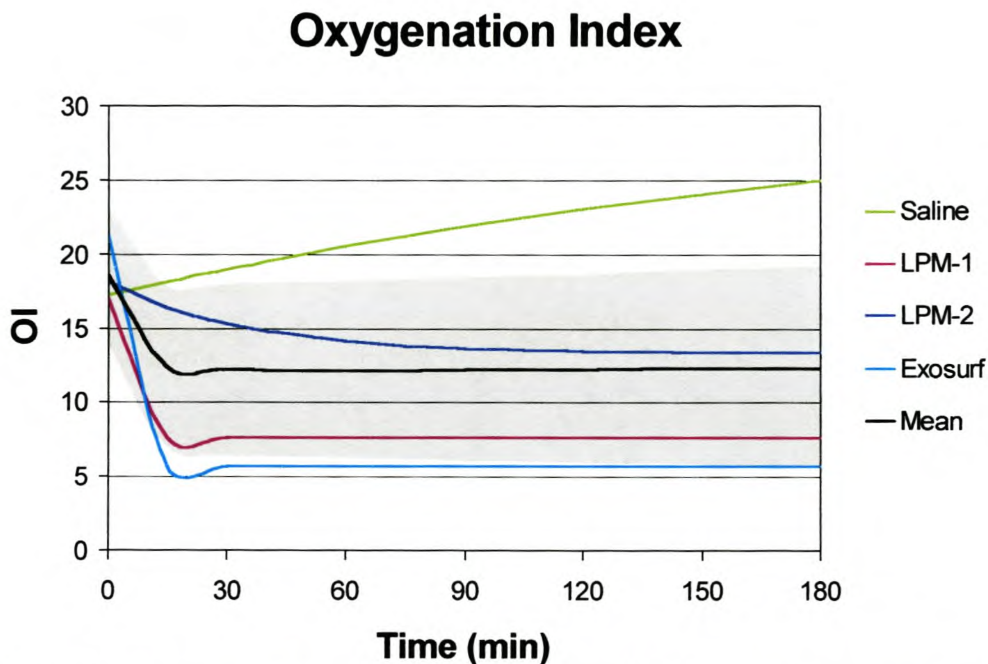

Fig 3.1.8.2. The oxygenation index (OI). The solid line (black) represents the expected regression curve and the grey zone the 95% pointwise confidence limits. Significant differences were noted between groups (p = 0.000).

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3.1.8.3 The OI of the synthetic surfactant groups without the saline group

Since the saline group is largely responsible for the observed lack of homogeneity in Fig 3.1.8.2, this group is omitted from further analysis in order to study true differences or similarities between surfactant groups. The manner in which the results are presented, from now onwards, follow this trend (see 2.12).

Except for a timepoint close to zero, the observed regression curve for the LPM-2 (DPPC) group was either above, or on the 97.5% pointwise limit. One-way analysis of variance of the mean values between 15 min and 180 min confirms the mean value of LPM-2 (14.8) to be significantly different to that of LPM-1 (Biosurf) (7.68) and Exosurf (5.57) ($p < 0.031$).

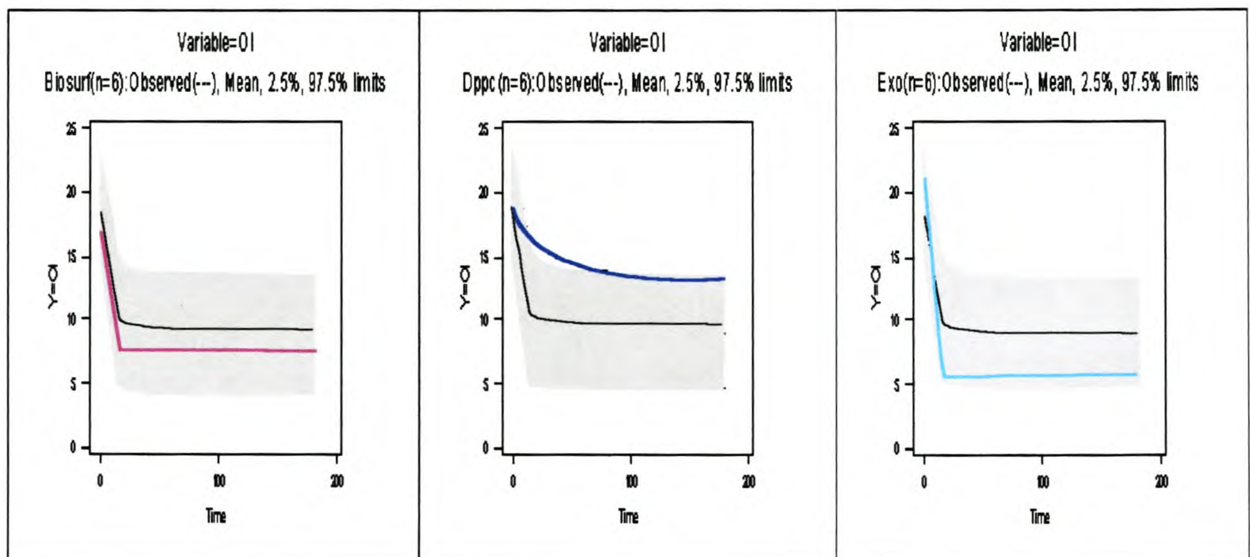

Fig 3.1.8.3 The mean OI of LPM-1 (Biosurf), LPM-2 (DPPC) and Exosurf (Exo). The solid lines represent the expected regression curve and the 95% pointwise confidence limits. The time profile was significantly different between the LPM-2 (DPPC) group and the LPM-1 (Biosurf) and Exosurf groups.

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3.1.8.4 The oxygenation index (OI): time profile of Exosurf and LPM-1

In order to assess true differences or similarities between the two surfactant groups, Exosurf and LPM-1, a separate analysis of only these two groups was performed (see 2.12). There were no differences between the LPM-1 and Exosurf groups with regard to the time profile of the OI. This finding supports the null hypothesis of the present study.

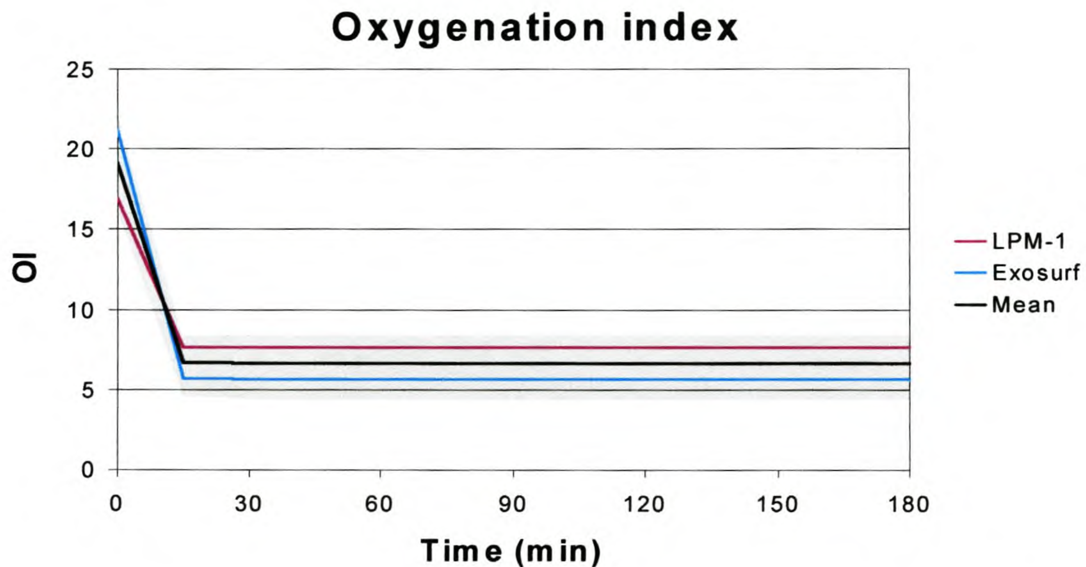

Figure 3.1.8.4 The time profile of the OI for the LPM-1 and Exosurf groups. The solid line (black) represents the expected regression curve and the grey zone the pointwise limits. There are no differences in the time profile between the two groups.

3.1.8.5 The time profile for the a/A ratio: comparison of all four groups

The time profile for the mean alveolar-arterial oxygen tension ratio was more favourable in the Exosurf group (following the 97.5% pointwise limits), followed by the LPM-1 group (between the expected mean and the 97.5% limit) and the LPM-2 group (between the expected mean and the 2.5% limit), and worse in the

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saline group (χ^2=13.54, p<0.13). The observed regression curve for the saline group diverges from the expected regression curve (black line) within minutes after the instillation of the saline.

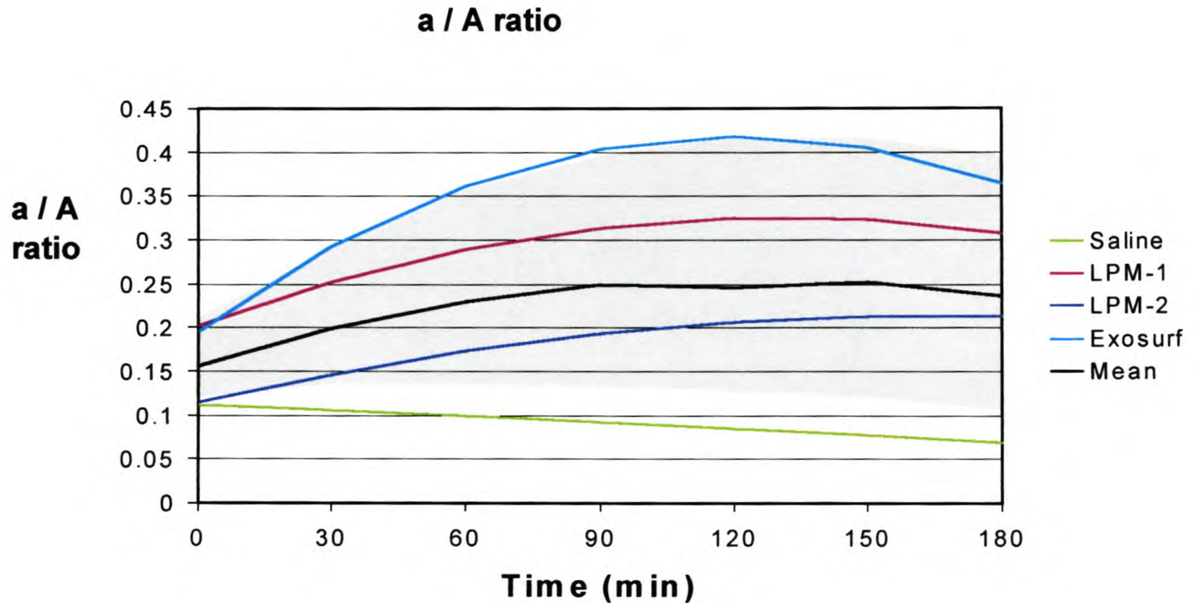

Fig. 3.1.8.5 The time profile for the arterial-alveolar oxygen tension ratio. The solid line (black) shows the expected regression curve and the grey zone the 95% pointwise confidence interval. There were no significant differences between the surfactant groups. The time profile for the a/A ratio in the saline group however, differed significantly from the three surfactant groups.

3.1.8.6 The time profile for the a/A ratio of the three lipid mixtures without the saline group

The graphs of the observed regression curves in the figure below show the a/APO_2 ratio of the Exosurf group to be significantly higher than the expected regression curve (above and following the 97.5% pointwise confidence limit), that

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of the LPM-1 (Biosurf) group above the expected value, and that of the LPM-2 (DPPC) group below the expected regression curve ($\chi^2 = 7.82$, $p<0.05$).

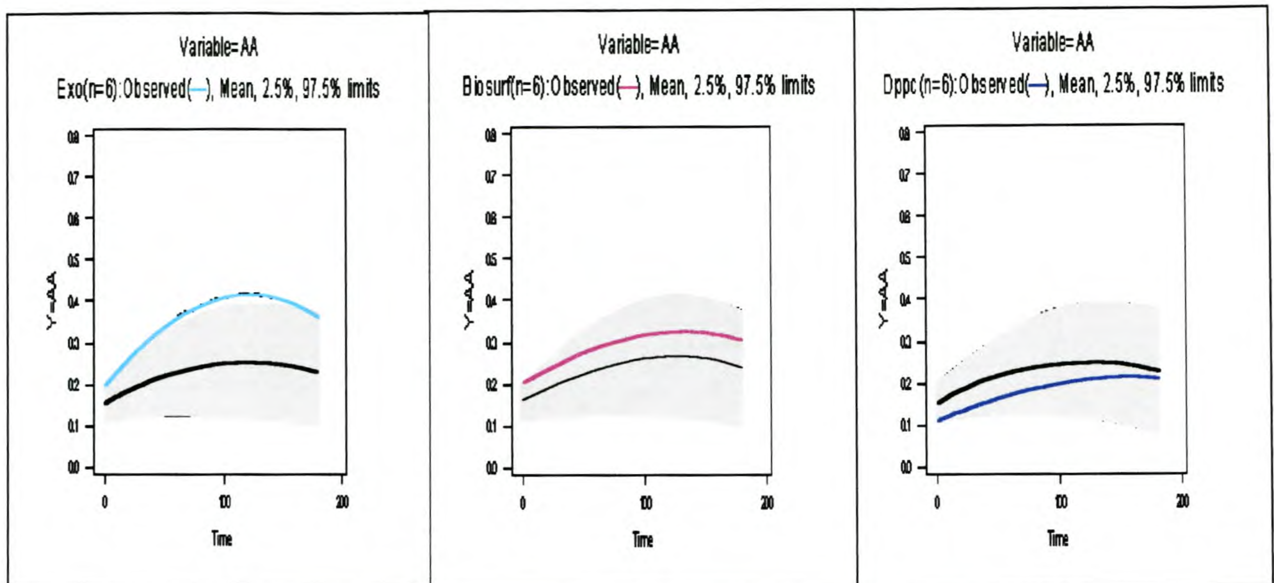

Fig 3.1.8.6. The a/A ratio of Exosurf (Exo), LPM-1 (Biosurf) and LPM-2 (DPPC) without the saline group. Solid lines show the regression curve and 97.5% pointwise confidence limits. The time profile was significantly different between groups ($p < 0.05$, $Q = 7.82$). The observed regression curve of the Exosurf group was above the 95% pointwise limit, that of LPM-1 above the expected, and that of LPM-2, below the expected regression curve.

3.1.8.7 The time profile for the a/A ratio: comparison between LPM-1 and Exosurf

Again, when the saline group and the LPM-2 group are omitted from the analysis, no differences with regard to the time profile of the a/A ratio existed between the LPM-1 and Exosurf groups. This finding supports the null hypothesis of the present study.

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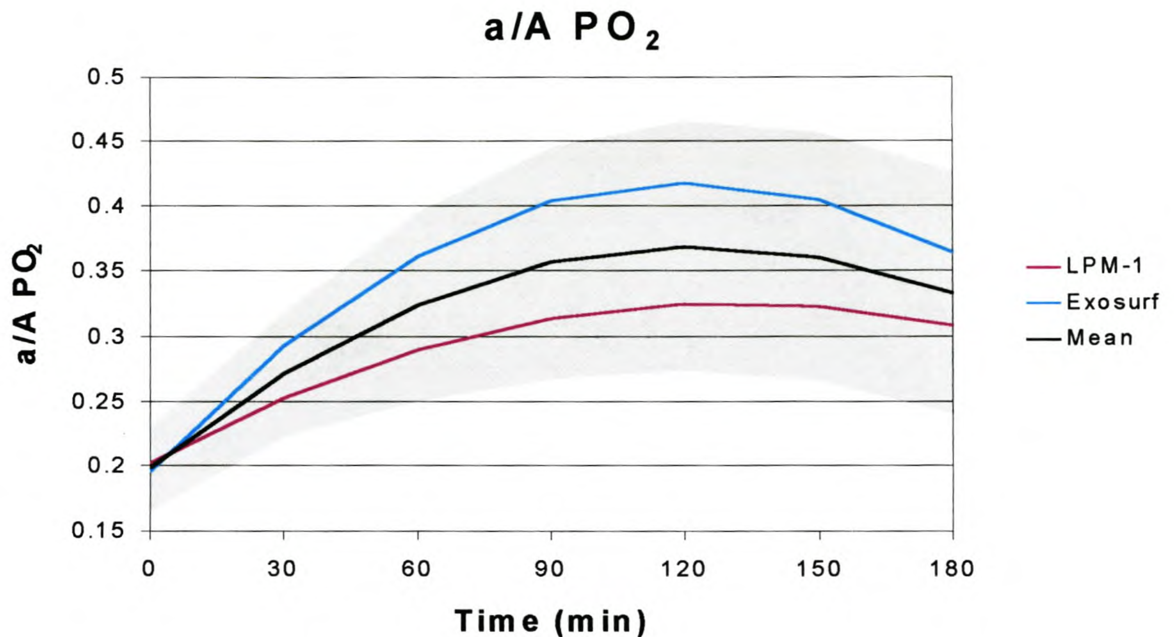

Figure 3.1.8.7 The time profile for the a/APO_2 ratio of the LPM-1 and Exosurf groups. The solid line (black) represents the expected regression curve and the grey zone the pointwise limits. There are no differences in the time profile between the two groups.

3.1.8.8 The time profile for the percentage pulmonary shunt (Qs / Qt): comparison of all four groups

The time profile for the pulmonary shunt differed significantly between the groups. The observed regression curve for the saline group diverged from the expected regression curve (black line) and crossed the 97.5% pointwise limit approximately 30 minutes after the instillation of the saline. The observed curve for the Exosurf group was below the 2.5% pointwise limit within 30 minutes after the instillation of the Exosurf.

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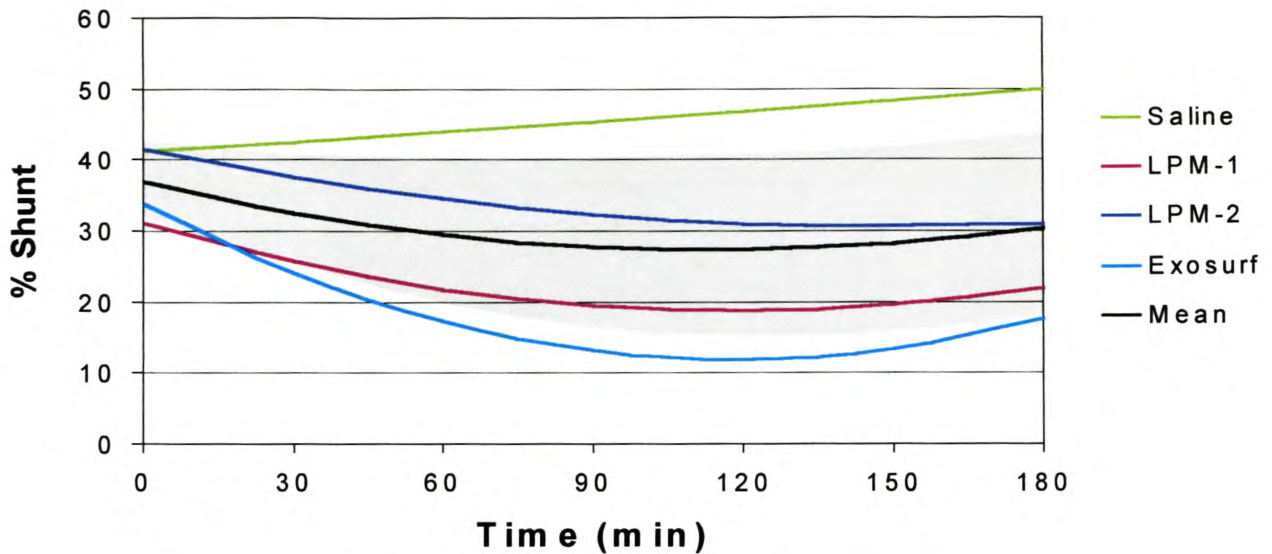

Figure 3.1.8.8 Time profile for the percentage pulmonary shunt in 100% oxygen. The solid line (black) shows the expected regression curve and the grey zone the 95% limits. The time profile was significantly different between groups.

The observed regression curve for the saline group was above the 97.5% pointwise limit, whereas the observed regression curve for the Exosurf group was below the 2.5% pointwise limit.

3.1.8.9 The time profile of the pulmonary shunt in the lipid mixture groups without the saline group

Significant differences in the percentage pulmonary shunt were evident between the three study groups. The time profile of the pulmonary shunt was significantly more favourable in the LPM-1 (Biosurf) group and Exosurf (Exo) group ($p < 0.05$) by individual regression and by ANOVA ($p < 0.0$).

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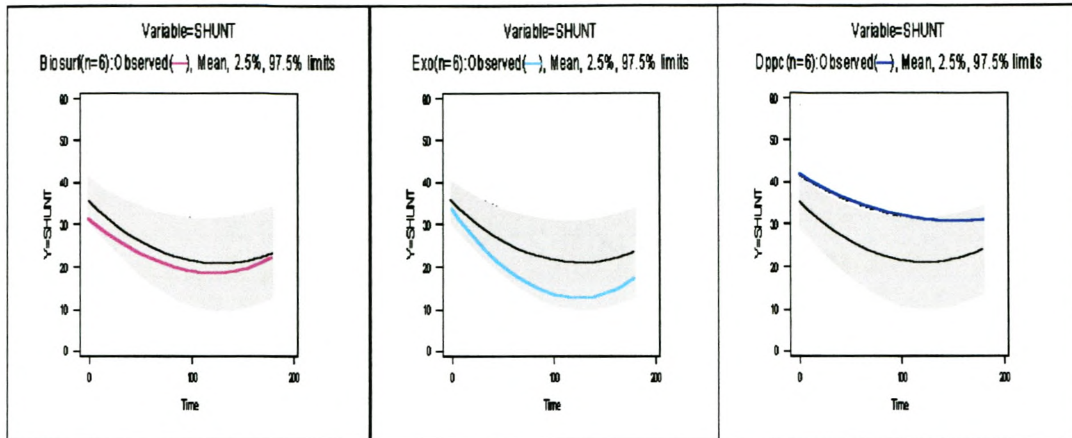

Figure 3.1.8.9 Comparison of the percentage pulmonary shunt between LPM-1 (Biosurf), LPM-2 (DPPC) and Exosurf (Exo). Solid lines represent the expected regression curve and 95% pointwise confidence intervals. The time profile was significantly different between groups ($Q = 8.2$, $p < 0.05$).

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3.1.8.10 Pulmonary shunt in the LPM-1 and Exosurf group

There were no differences in the time profile of the pulmonary shunt between the LPM-1 and Exosurf groups, thus supporting the null hypothesis.

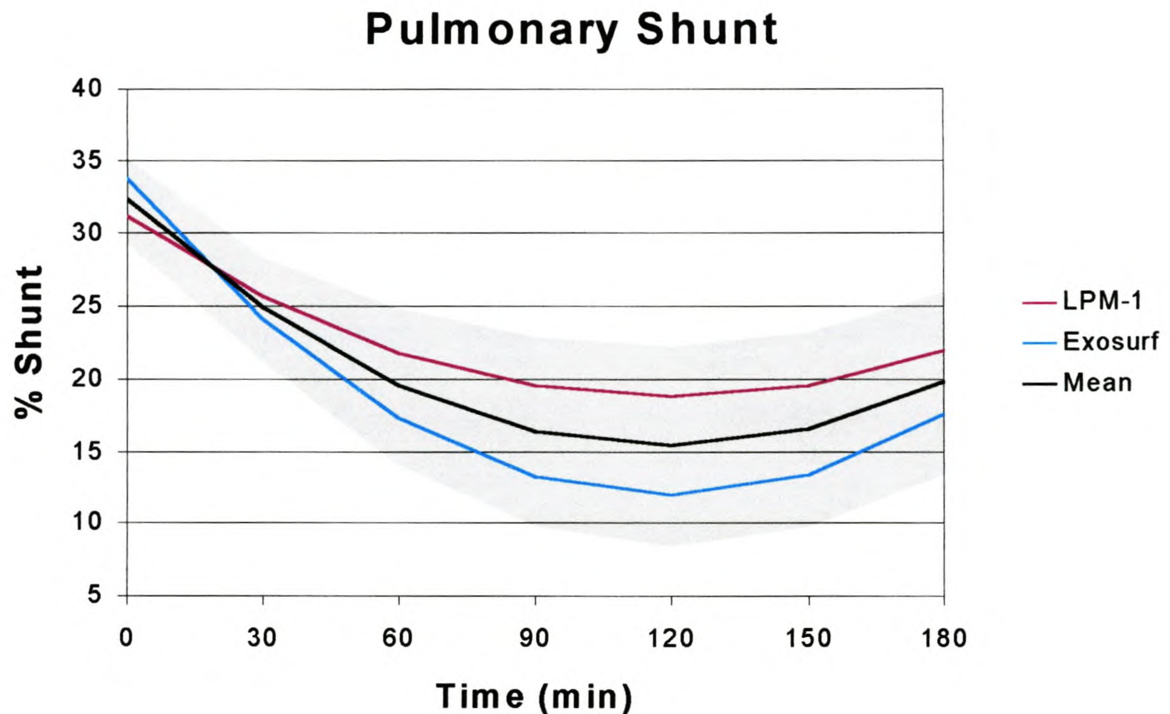

Figure 3.1.8.10 The time profile of the percentage shunt in 100% oxygen. The solid line (black) represents the expected regression curve and the grey zone the pointwise 95% limits. There were no differences between groups.

3.1.8.11 The time profile of the Respiratory compliance (CRS) of all four groups

The overall mean respiratory compliance (CRS) of the rabbits decreased by 52% after lung lavage (0.86 ± 0.04 vs 0.45 ± 0.019 ml/cmH_2O/kg, $p < 0.05$).

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Following surfactant administration, Exosurf increased the compliance to above the 97.5 confidence interval, after 150 min. Compliance in the LPM-1 group remained above the expected, whereas the compliance of the LPM-2 and saline group showed a decline.

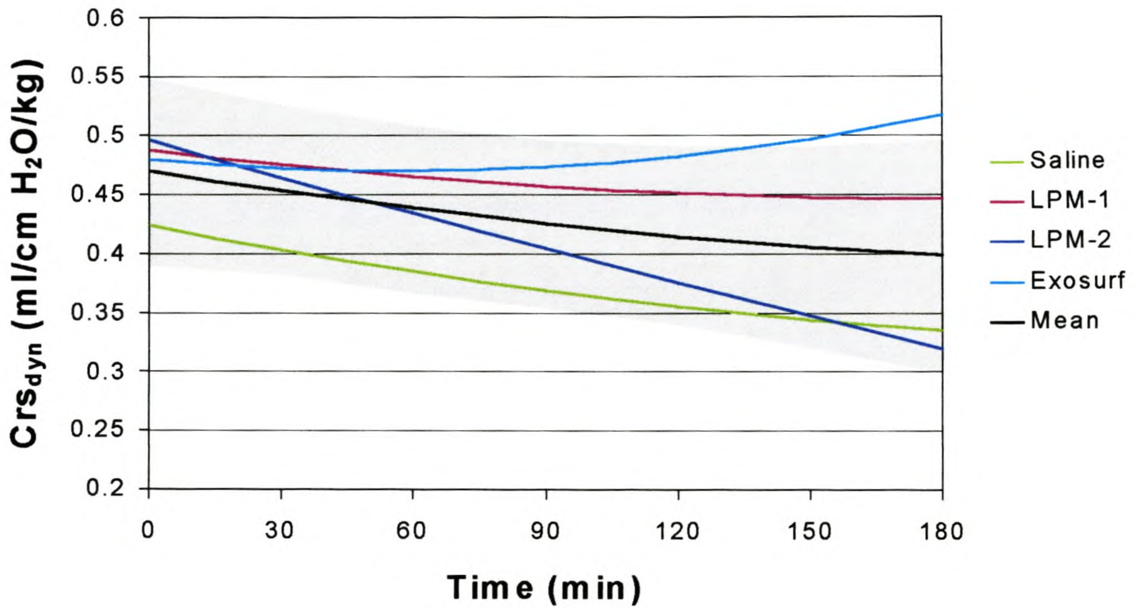

Fig 3.1.8.11 The compliance of the respiratory system (CRS). The black solid line shows the regression line and the grey zone the 95% pointwise confidence limits. The time profile for the CRS of the Exosurf group diverted from the expected curve and crossed the 97.5% limit after ~ 150 minutes.

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3.1.8.12 The time profile of the respiratory compliance of the lipid mixture groups without the saline group

Omitting the saline group from the analysis, reveals significant worsening of respiratory compliance in the LPM-2 group over time when compared to the LPM-1 group and Exosurf group ($\chi 2 = 8.16$, $p<0.05$).

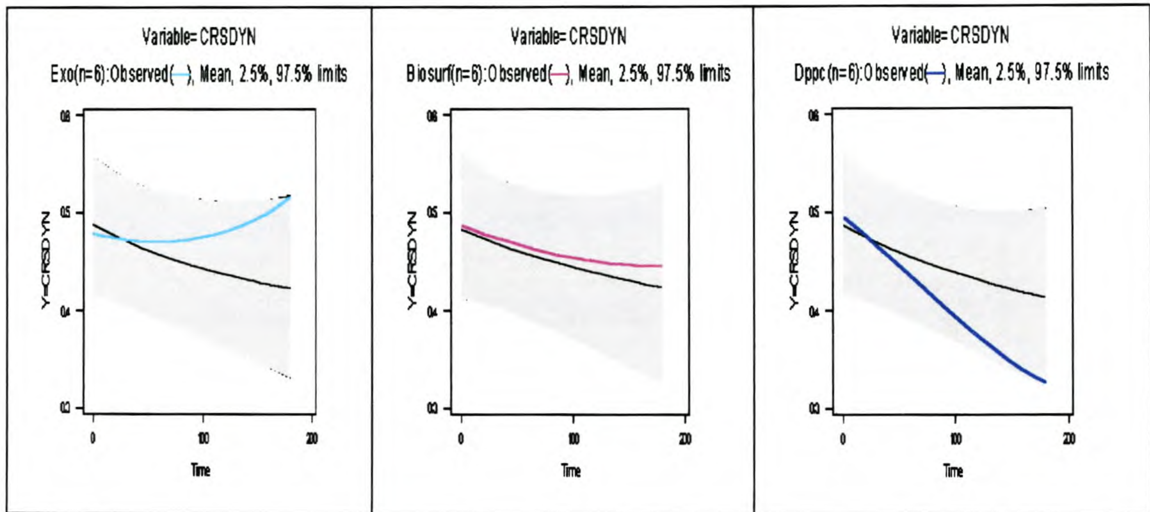

Figure 3.1.8.12 The respiratory compliance (CRS) of LPM-1 (Biosurf), LPM-2 (DPPC), and Exosurf (Exo) in the absence of the saline group. Solid lines represent the observed regression curve and 95% pointwise confidence limits for the regression lines. The time profile was significantly different between groups ($Q = 8.16$, $p < 0.05$). The observed regression curve for the LPM-1 group and Exosurf group is above expectation, while that of the LPM-2 group diverges from, and decrease below expectation before the 100 min time point.

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3.1.8.13 Dynamic respiratory compliance: comparison between LPM-1 and Exosurf

There were no differences between the LPM-1 and Exosurf groups in the time profile of the CRS, thus supporting the null hypothesis.

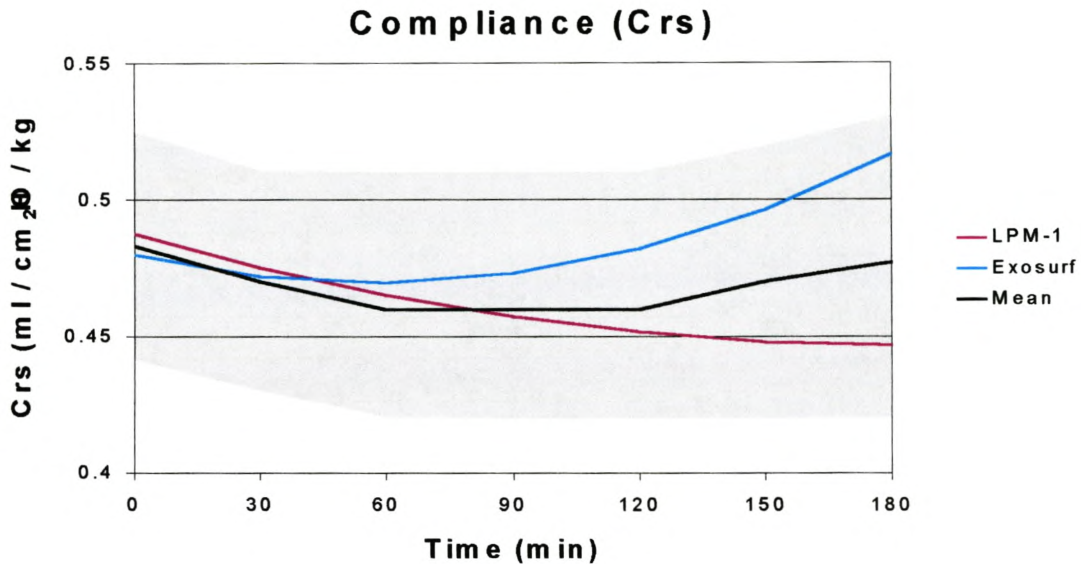

Figure 3.1.8.13 Time profile of the CRS of the LPM-1 and Exosurf group. The solid line (black) represents the expected regression curve, whereas the grey area denotes the pointwise 95% limits. There were no differences between the two groups.

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3.1.8.14 The correlation between the OI and percentage shunt

A good correlation, at time points: pre-lavage, post-lavage, 15 min, 30 min, 60 min and 90 min after lavage, was obtained between the OI and percentage pulmonary shunt ($r^2 = 0.95$).

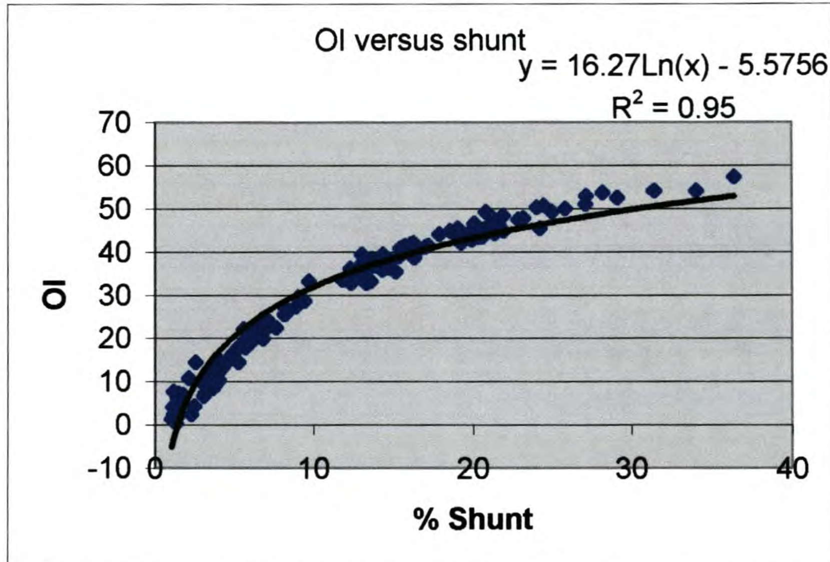

Fig 3.1.8.14 Relationship between oxygenation index (OI) and percentage pulmonary shunt on 100% oxygen at time points: pre-lavage, post-lavage, and at 15 min, 30 min, 60 min and 90 min after lavage. There is a log-linear correlation between the OI and the % shunt ($r^2 = 0.95$).

3.1.9 Survival / mortality

Five of 6 rabbits (83%) survived in each group until 3 hours. One rabbit each in the LPM-1 group and the Exosurf group died of a pneumothorax at time points 144 min and 100 min, respectively. The rabbits that died in the saline group and LPM-2 group presented with pulmonary haemorrhage, systemic hypotension and severe metabolic acidosis. In addition, the rabbit that died in the saline group

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developed preterminal hypercarbia ($PaCO_2$ 8 kPa). It is speculated that these findings may indicate inadequate lung perfusion due to acute pulmonary hypertension and right heart failure.

3.1.10 Histological observations

Hyaline membrane formation was significantly less in surfactant treated animals compared to the control group of animals (saline) who did not receive surfactant treatment. However, *the surfactant-treated groups* did not differ significantly *from each other.* The difference in the degree of hyaline membrane formation in the control group (saline) in comparison to the pooled data of the surfactant groups: LPM-1, LPM-2, and Exosurf, is displayed in Table 3.1.10.1. There was a significantly greater presence of membranes in the control group compared to the pooled data of the surfactant-recipients. Although present, atelectasis was not a prominent finding (Fig 3.1.10.1).

All animals demonstrated mild to moderate infiltration of polymorphonuclear neutrophils (Table 3.1.10.2), mild congestion (Table 3.1.10.3) lymphatic dilatation (Table 3.1.10.4), and airway debris (Table 3.1.10.5) (p=ns between groups by ANOVA). In addition to the difference in the degree of hyaline membrane formation between the surfactant treated groups and the untreated group (saline / control) when "membranes" were used as only feature, a significant difference between the treated and untreated groups could also be shown using a "total score" obtained from the 5 variables (hyaline membranes, congestion, lymphatic dilatation, debris, and neutrophil infilration) (Table 3.1.10.6).

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3.1.11 Electron microscopy

With lavage-induced surfactant deficiency, inhomogeneous lung disease may result. Focal changes are brought about which may be missed when ultrastructural examination of selected tissue is peformed. With this in mind, a descriptive analysis of the ultrastructural features is presented.

The most striking finding was the variability in the ultrastructural morphology in the hyaline membranes, with different membrane types seen within the same case. Two basic membrane types were common to all groups, those being granular (Fig 3.1.11.1) and fibrillar (Fig 3.1.11.6). However, membranes in the lungs of animals in the control group (no surfactant) were denser and more granular when compared to the other groups (Fig 3.1.11.2). The LPM-2 group had thick membranes of various types. A finely granular substance was seen in airspaces, interpreted as oedema fluid. When the groups were compared, this luminal oedema appeared more prominent in the control animals and the LPM-2 group (Fig 3.1.11.3).

Organelles were consistently seen entrapped within the membranes in all of the groups. The organellar debris could have originated from lysis of epithelial cells and inflammatory cells (Fig 3.1.11.4). Tubular myelin was not seen in any of the cases. In the present study epithelial damage was a consistent finding in all groups. This damage was more evident by electron microscopy examination and included hydropic changes, most readily observed in the mitochondria (Fig 3.1.11.5), and organellar debris, probably originating from lysis of epithelial cells.

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All of the cases showed mitochondrial swelling with no difference between groups. These changes were ascribed to the tissue preparation, fixation, as well as secondary to the disease process.

In summary, with respect to the oedema and membrane formation, the overall impression was that the control group (saline) and LPM-2 group appeared to have more severe ultrastructural disease.

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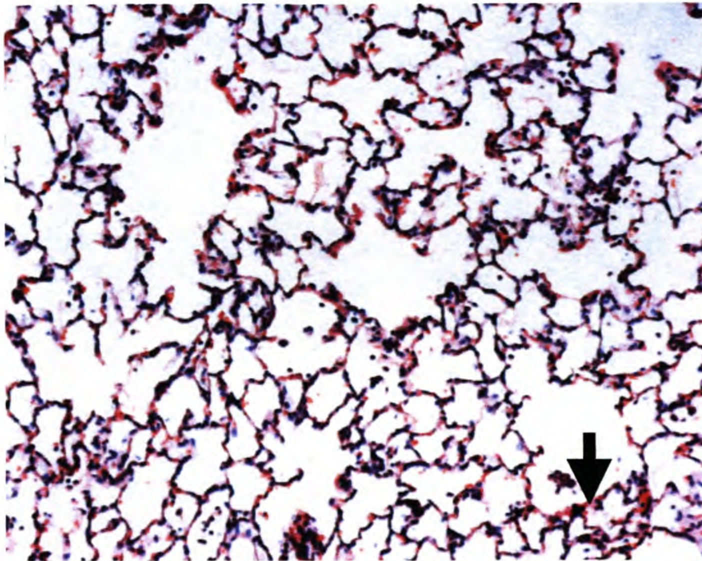
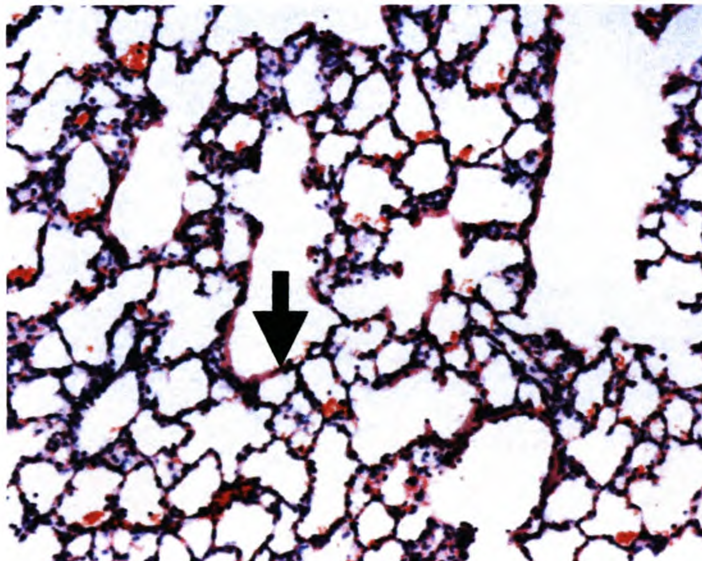
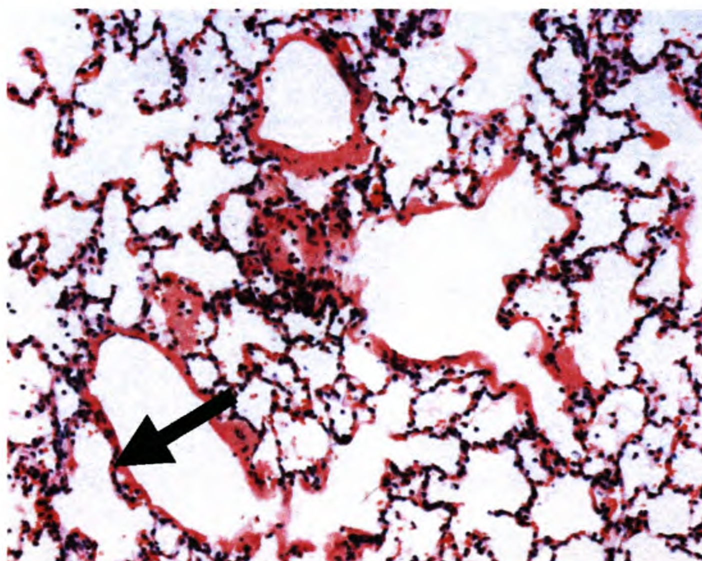


Grade 1: Scattered thin adherent membranes are present



Grade 2: Moderate presence of thicker membranes



Grade 3: Extensive presence of membranes in airspaces

Figure 2.6.1.1 Hyaline membrane classification.

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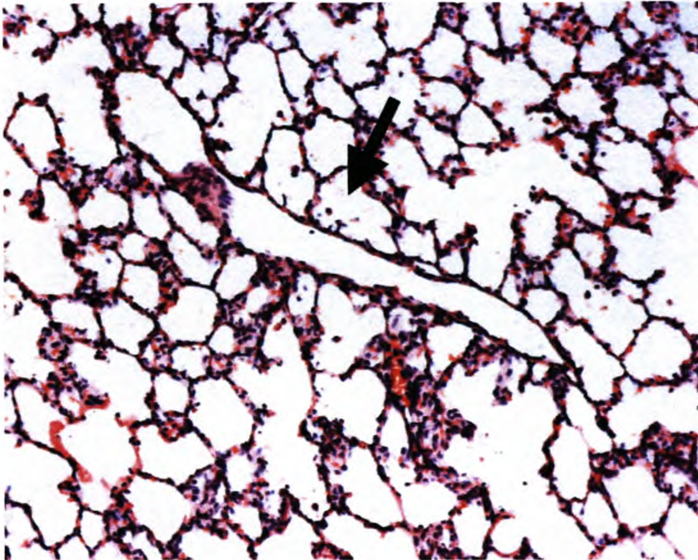
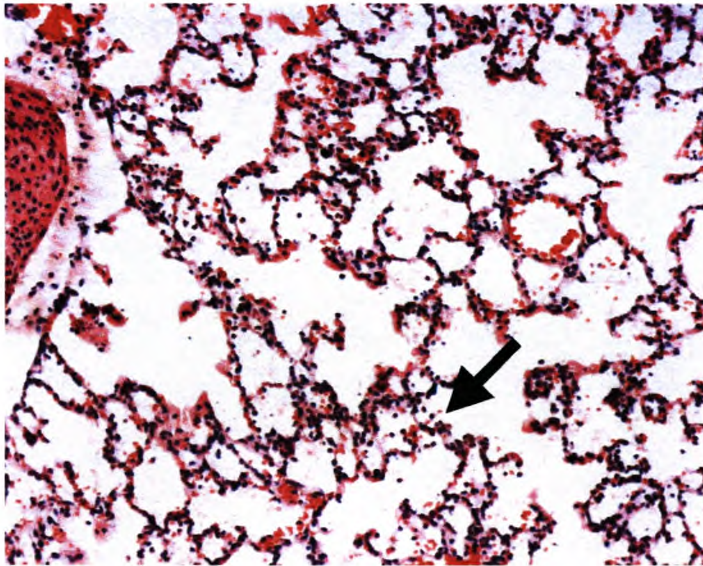
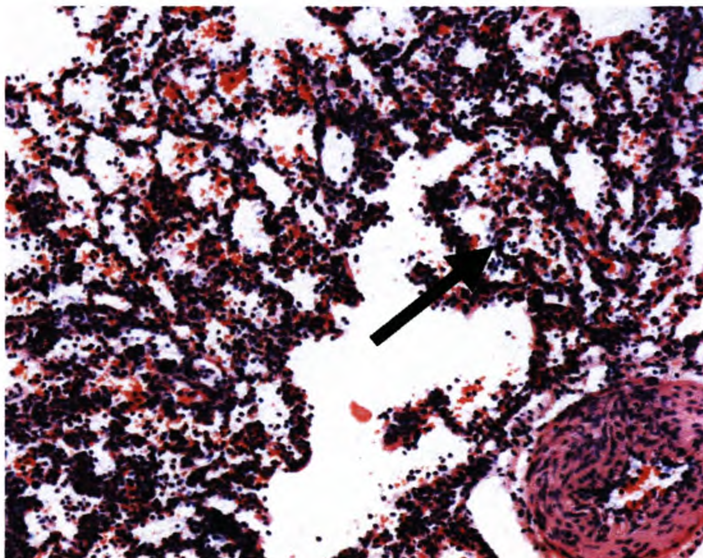


Grade 1: Scattered neutrophils



Grade 2: Moderate numbers of neutrophils



Grade 3: Numerous neutrophils forming 'aggregates'

Figure 2.6.1.2 Neutrophil classification

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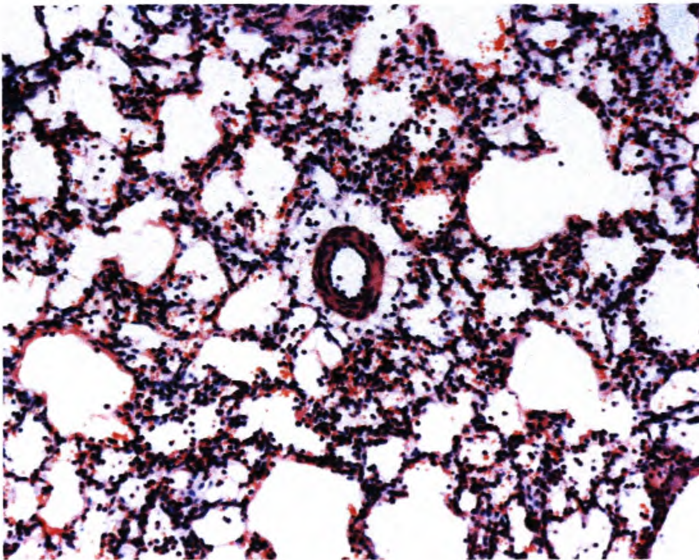
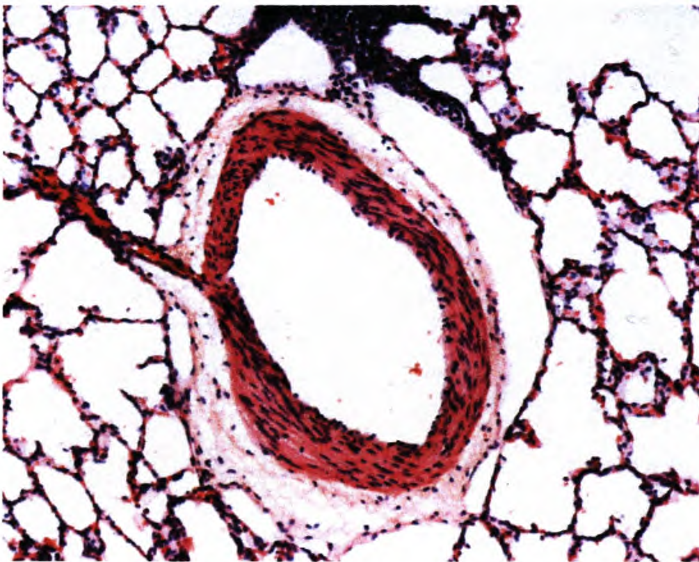
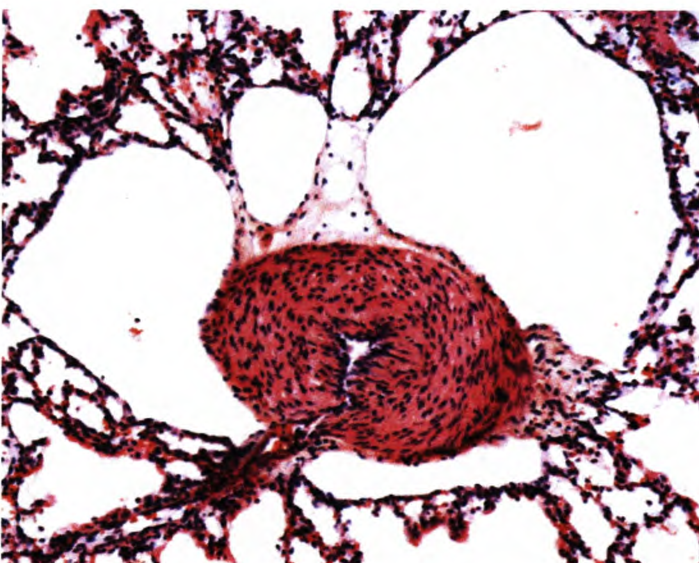


Grade 1: Mildly dilated perivascular lymphatics



Grade 2: Moderately dilated lymphatics



Grade 3: Marked lymphatic dilation

Figure 2.6.1.3 Lymphatic vessels

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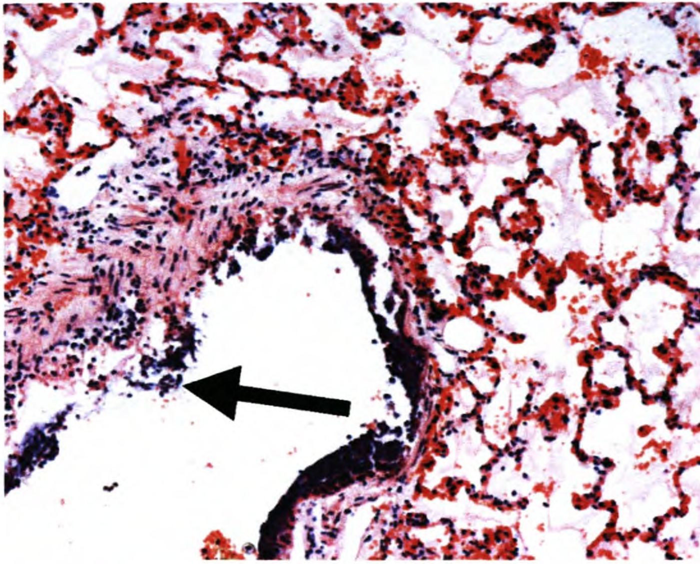
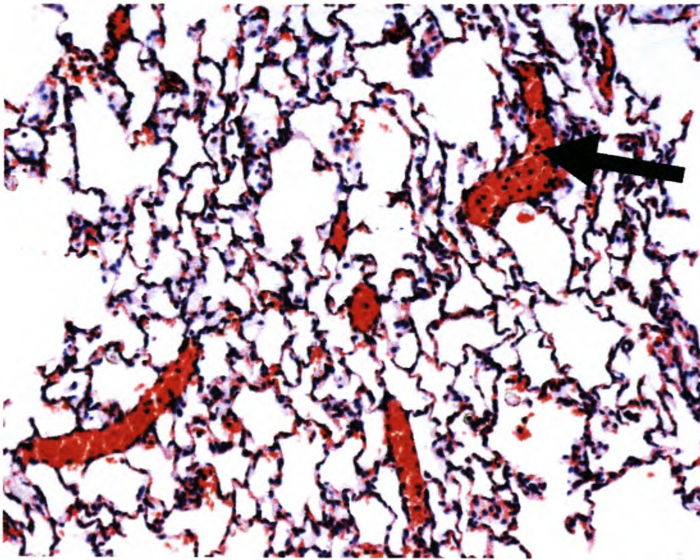


Necrotic cellular debris



Dilated congested small blood vessels

Figure 2.6.1.4 Necrotic airway debris

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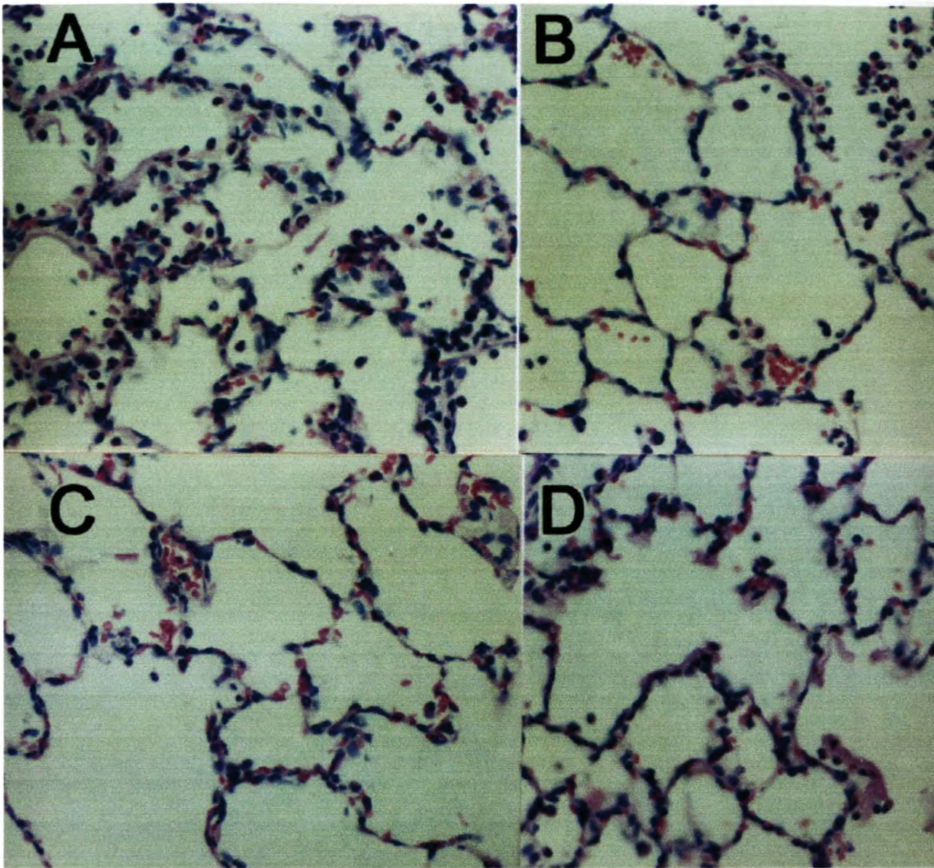

Figure 3.1.10.1: Light micrograph of lung parenchym. Hematoxylin and eosin stained sections (magnification 400 X). A: LPM-2; B: control; C: Exosurf; D: LPM-1.

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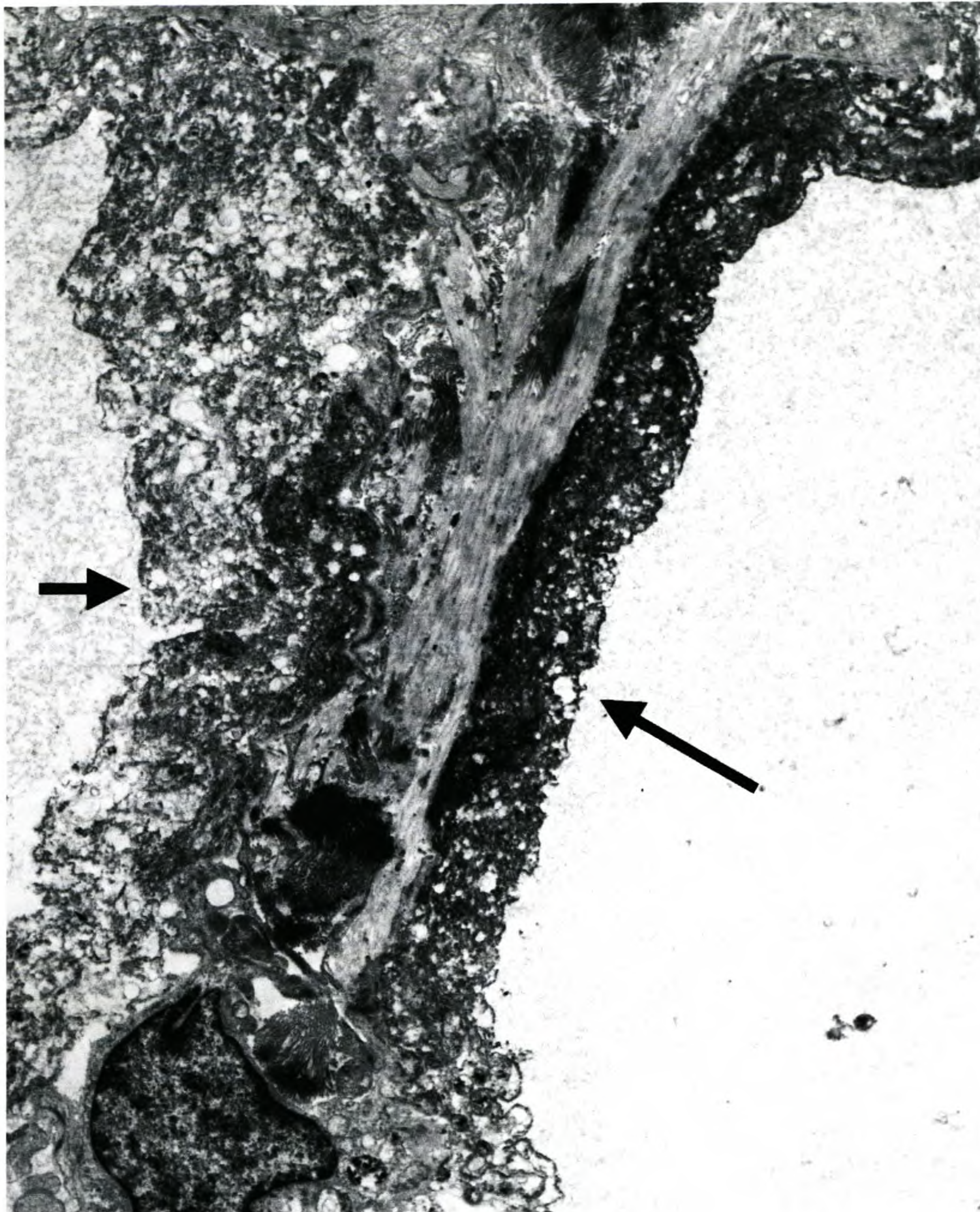

Figure 3.1.11.1: Example of two different membrane types in adjacent alveoli-note vesicular structures contained within membrane: LPM-2 recipient (magnification 9000 X).

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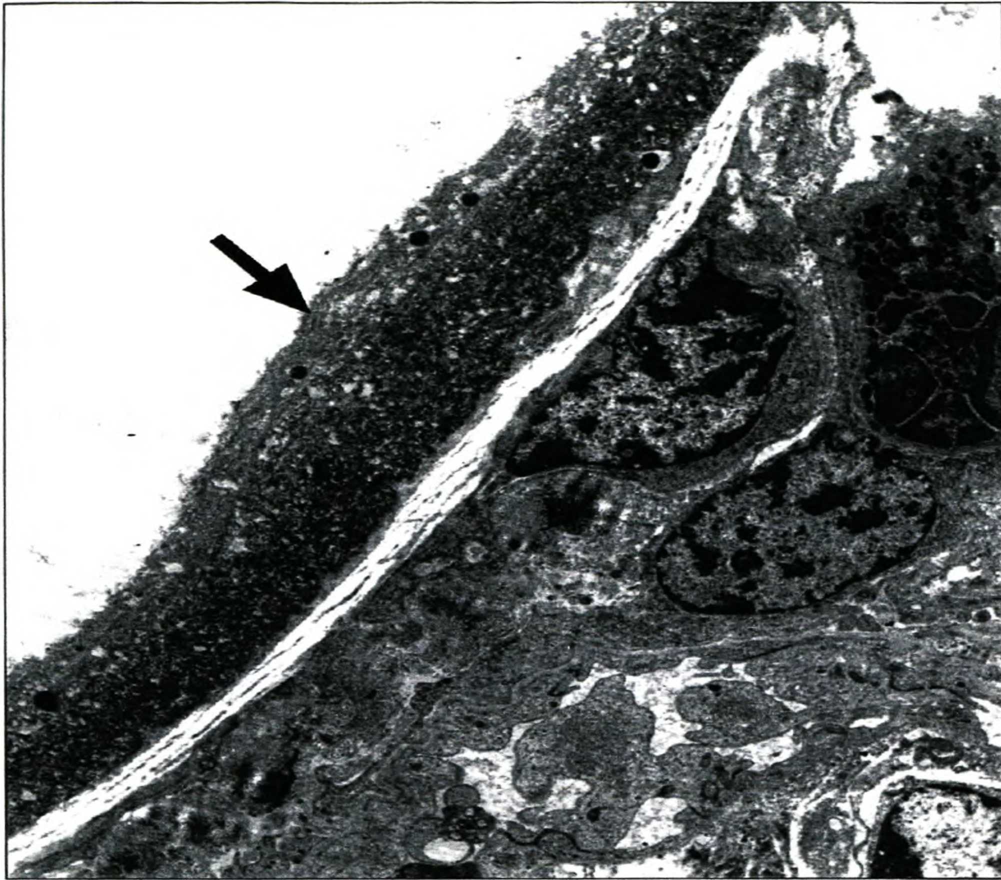

Figure 3.1.11.2: Adherent granular membrane seen lining alveolar space: control rabbit (magnification 9000 X)

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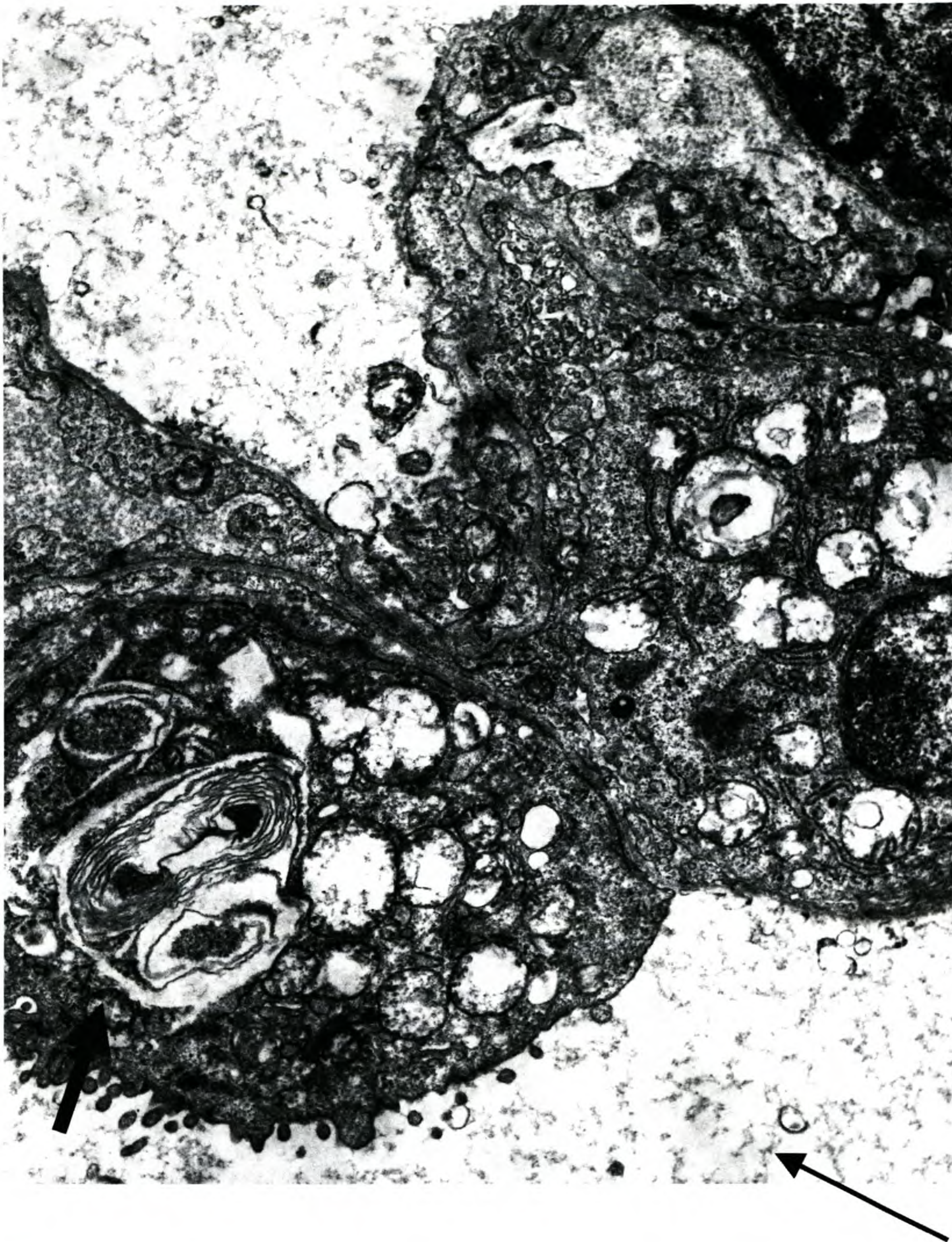

Figure 3.1.11.3: Lumenal oedema (thin dark arrow) and lamellar body in type 2 pneumocyte (thick dark arrow) (magnification 18000 X).

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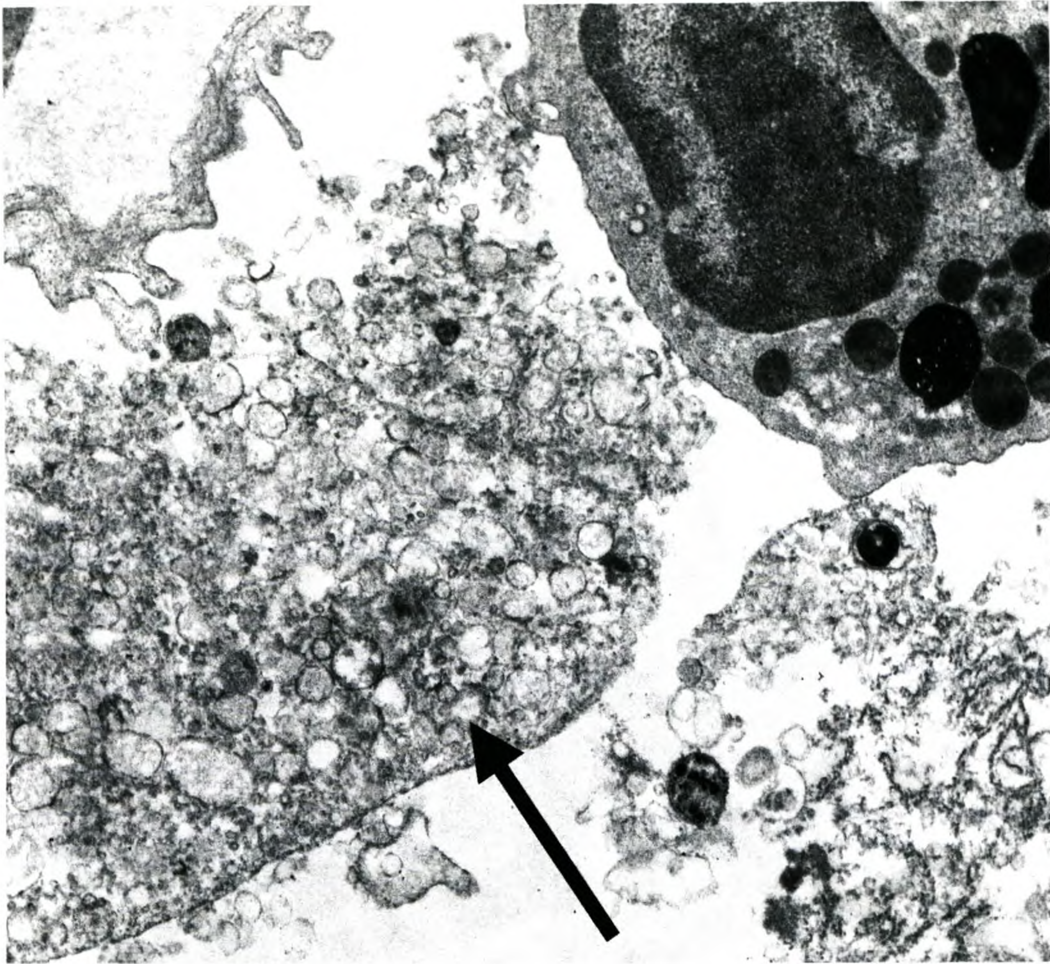

Figure 3.1.11.4: Cell lysis with cytoplasmic contents extruded into airspaces (dark arrow): LPM-2 recipient (magnification 24000 X).

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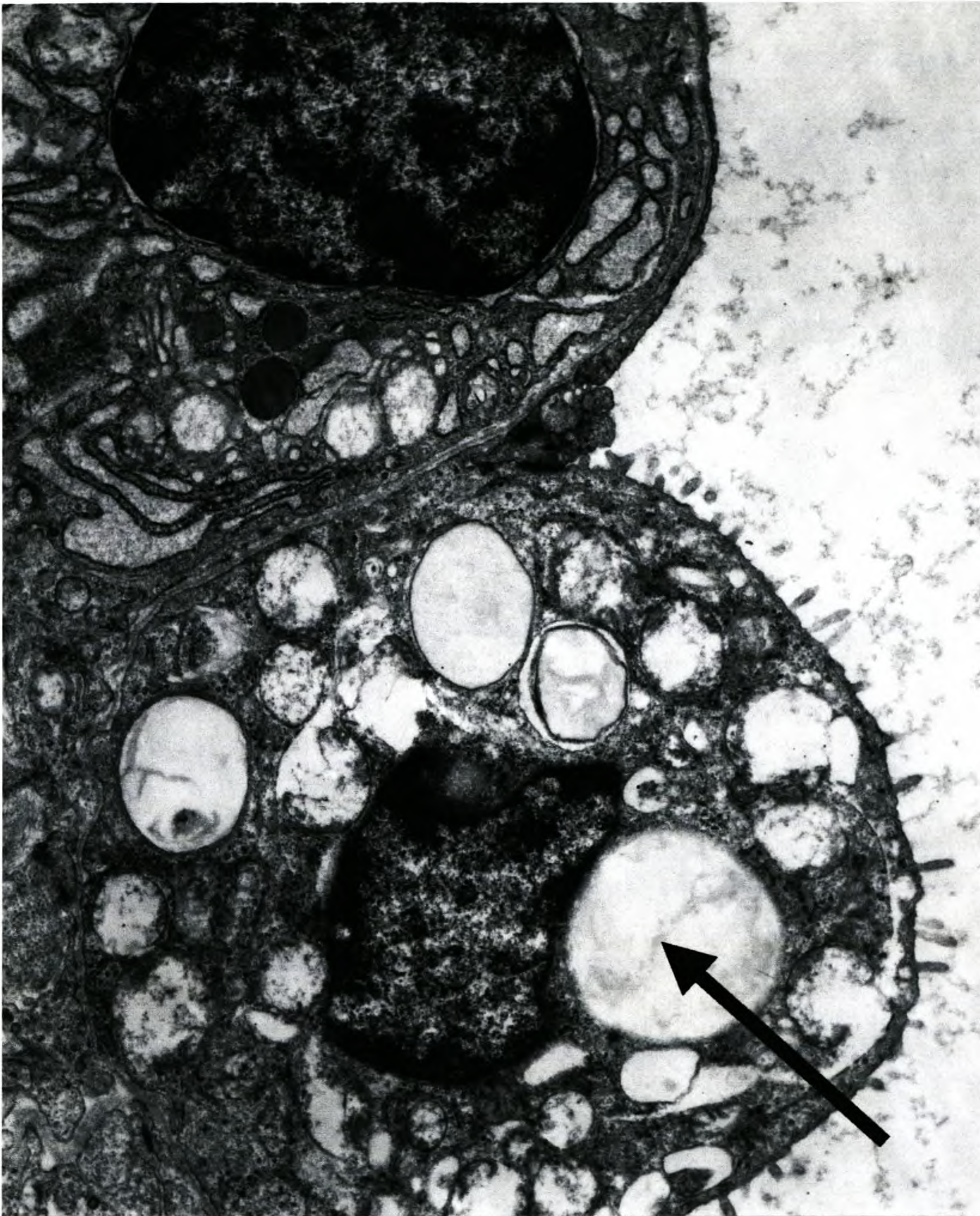

Fig 3.1.11.5 Type 2 pneumocyte with organelles showing marked hydropic swelling (dark arrow): LPM-2 recipient (magnification 18000 X).

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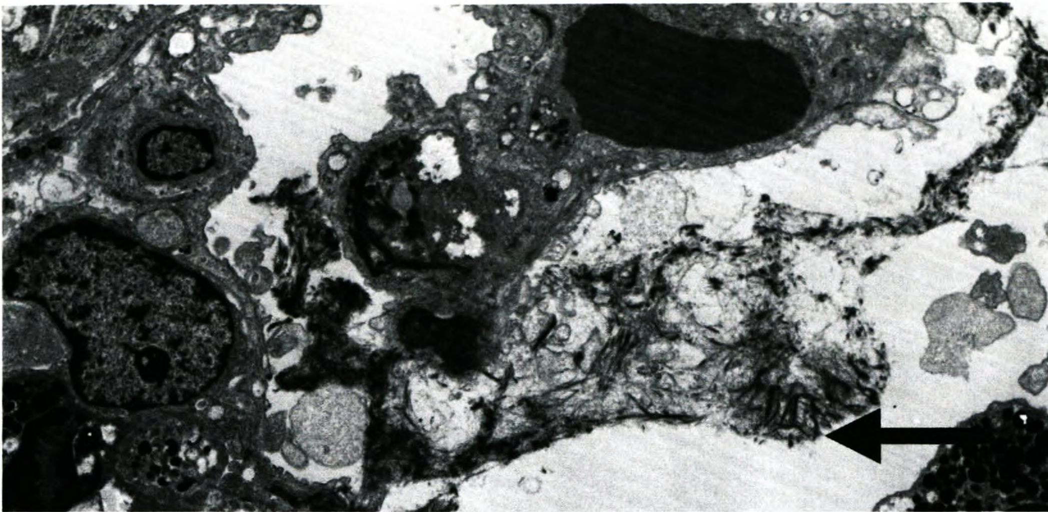

Fig 3.1.11.6 Fine fibrillar membrane (arrow): Exosurf recipient (magnification 6000 X).

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TABLE 3.1.1

Pre-lavage physiological and ventilation variables (mean ± SD).

Variable	ALL (n=24)	SALINE (n=6)	LPM-1 (n=6)	LPM-2 (n=6)	EXOSURF (n=6)
Weight (kg)	2.4 ± 0.37	2.4 ± 0.54	2.48 ± 0.16	2.55 ± 0.38	2.21 ± 0.30
MABP (mmHg)	91.5 ± 20.4	89.5 ± 12.7	95 ± 29.9	89.8 ± 28.5	91.6 ± 13.7
Heart rate (per min)	265 ± 41.1	240 ± 50.1	270 ± 26.8	265 ± 48	285 ± 31.41
Paw (cmH_2O)	5.51 ± 0.6	5.56 ± 0.69	5.51 ± 0.89	5.46 ± 0.61	5.51 ± 0.22
Vt (ml/kg)	10.5 ± 2.48	10.4 ± 2.7	10.6 ± 2.49	10.1 ±2.62	11.2 ± 2.59
V_E (L/kg/min)	0.41 ± 0.09	0.42 ± 0.13	0.39 ± 0.07	0.40 ± 0.08	0.44 ± 0.10
CRS ($ml/cmH_2O/kg$)	0.82 ± 0.32	0.67 ± 0.15	0.86 ± 0.20	0.95 ± 0.64	0.80 ± 0.11

MABP: mean arterial blood pressure; Paw: mean pulmonary airway pressure; Vt: tidal volume; V_E: minute ventilation; CRS: compliance of the respiratory system.

P=ns, for any variable between groups.

TABEL 3.1.2

Pre-lavage blood gases and indices (mean ± SD).

Variable	ALL (n=24)	SALINE (n=6)	LPM-1 (n=6)	LPM-2 (n=6)	EXOSURF (n=6)
PaO_2 (kPa)	52.95 ± 7.6	51.2 ± 8	51.1 ± 10.4	53.9 ± 5.9	55.4 ± 6.6
$PaCO_2$ (kPa)	4.46 ± 0.73	4.48 ± 1.0	4.51 ± 0.89	4.53 ± 0.77	4.33 ± 0.19
CO2 total (mmol/L)	20.3 ± 2.9	20.0 ± 1.8	19.8 ± 3.5	22 ± 1.5	19.5 ± 4.0
pH	7.35 ± 0.08	7.36 ± 0.09	7.34 ± 0.05	7.36 ± 0.09	7.34 ± 0.11
OI	1.42 ± 0.32	1.48 ± 0.33	1.5 ± 0.52	1.37 ± 0.26	1.33 ± 0.12
a / A ratio	0.59 ± 0.08	0.57 ± 0.08	0.57 ± 0.11	0.60 ± 0.06	0.61 ± 0.07
% Qs / Qt	4.6 ± 3.32	5.3 ± 3.3	5.5 ± 4.8	4.0 ± 2.3	3.5 ± 2.5

All p–values were non-significant.

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TABLE 3.1.3

Post lavage (baseline: point 0) physiological and ventilation variables

(mean ± SD).

Variable	ALL (n=24)	SALINE (n=6)	LPM-1 (n=6)	LPM-2 (n=6)	EXOSURF (n=6)
MABP (mmHg)	85.5 ± 21.3	78.5 ± 15.2	91.5 ± 23.8	90. 6 ± 18.5	81.6 ± 28.3
Heart rate (beats per min)	276 ± 38.5	265 ± 55	280 ± 30.9	285 ± 31.4	275 ± 39.8
Paw (cmH_2O)	11.7 ± 0.99	12 ± 0.89	11.8 ± 1.4	11.5 ± 0.5	11.5 ± 1.0
Vt (ml/kg)	10.9 ± 2.4	11.2 ± 3.3	10.6 ± 2.3	11.2 ± 2.2	10.6 ± 2.4
V_E (L/kg/min)	0.425 ± 0.09	0.443 ± 0.128	0.410 ± 0.085	0.426 ± 0.083	0.420 ± 0.09
CRS ($ml/cmH_2O/kg$)	0.46 ± 0.12	0.41 ± 0.14	0.51 ± 0.09	0.47 ± 0.19	0.46 ± 0.08

MABP: mean arterial blood pressure; Paw: mean pulmonary airway pressure; Vt: tidal volume; V_E: minute ventilation; CRS: compliance of the respiratory system. P=ns, for all variables between groups.

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TABLE 3.1.4

Post lavage (baseline: point 0) blood gases and indices (mean ± SD).

Variable	ALL (n=24)	SALINE (n=6)	LPM-1 (n=6)	LPM-2 (n=6)	EXOSURF (n=6)
PaO_2 (kPa)	8.5 ± 1.9	8.5 ± 2.1	9.8 ± 2.2	8.4 ± 1.7	7.4 ± 1.4
$PaCO_2$ (kPa)	5.9 ± 0.77	5.6 ± 0.98	6.0 ± 0.83	5.8 ± 0.60	6.3 ±0.62
pH	7.19 ± 0.95	7.14 ± 0.06	7.19 ± 0.02	7.27 ± 0.08	7.15 ± 0.12
CO_2 total (mmol/L)	18.6 ± 3.0	16.2 ± 2.4	18.9 ± 3.3	20.6 ± 1.53	18.8 ± 3.3
OI	19.1 ± 4.4	19.6 ± 4.5	16.9 ± 5.3	18.8 ± 3.6	21.2 ± 4.4
a / A ratio	0.09 ± 0.02	0.09 ± 0.02	0.11 ± 0.02	0.09 ± 0.01	0.08 ± 0.01
Qs / Qt	43.6 ± 4.8	43.7 ± 5.4	40.5 ± 4.5	43.8 ± 4.5	46.4 ± 4.2

P=ns, for all variables between groups.

TABLE 3.1.10.1

Analysis of hyaline membranes (light microscopy) by ANOVA.

GROUP (n)	Mean score	SD
LPM-1 (6)	1.53	0.34
Exosurf (n=6)	1.70	0.60
Saline (n=6)	2.58	0.46
LPM-2 (n=6)	1.69	0.53

P=0.008, Saline versus groups LPM-1, LPM-2, and Exosurf.

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TABLE 3.1.10.2

Analysis of neutrophils (light microscopy) by ANOVA.

GROUP (n)	Mean score	SD
LPM-2 (n=6)	1.4	0.49
Exosurf (n=6)	1.79	0.87
Saline (n=6)	2.0	0.44
LPM-1 (n=6)	1.62	0.62

p=ns, between groups.

TABLE 3.1.10.3

Analysis of congestion (light microscopy) by ANOVA.

GROUP (n)	Mean score	SD
LPM-1	0.19	0.30
Exosurf	0.0	0.0
Saline	0.04	0.10
LPM-2	0.12	0.20

P=ns, between groups.

TABLE 3.1.10.4

Analysis of lymphatics (light microscopy) by ANOVA.

GROUP (n)	Mean score	SD
LPM-1 (n=6)	0.98	0.50
Exosurf (n=6)	1.04	0.33
Saline (n=6)	1.58	0.60
LPM-2 (n=6)	1.61	0.90

P=ns, between groups.

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TABLE 3.1.10.5

Analysis of debris (light microscopy) by ANOVA.

GROUP (n)	Mean score	SD
LPM-1 (n=6)	0.55	0.28
Exosurf (n=6)	0.40	0.32
Saline (n=6)	0.50	0.47
LPM-2 (n=6)	0.52	0.28

P=ns, between groups.

TABLE 3.1.10.6

Analysis of the difference between the pooled total histological score of LPM-1, Exosurf and LPM-2 (Pooled) and the Saline group, by ANOVA.

GROUP (n)	Mean score	SD
Pooled (n=18)	5.09	1.4
Saline (n=6)	6.7	1.3

P=0.025, between groups.

3.2 Results: Study 2

3.2.1 General

Since the time profile for all the tested variables was significantly more unfavourable in the saline group (animals died early) compared to that of the trehalose and LPM-1 groups after randomisation, we omitted them from further analysis (Fig 3.2.1).

Baseline and immediate post-injury ventilator settings, gas exchange, and lung and systemic parameters were not different between treatment groups (Table 3.2.1, 3.2.2, 3.2.3, 3.2.4). During the treatment period, mean airway

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pressure remained similar between the two analysed groups, i.e. TRE and LPM-1 (Fig 3.2.2). The time profiles for tidal volume (Fig 3.2.3), minute ventilation (Fig 3.2.4), arterial pH (3.2.5), and PaCO2 (Fig 3.2.7) differed significantly between the groups after 90-120 min. Moreover, oxygenation improved significantly (3.2.3.1), whilst percentage shunt decreased significantly (Fig 3.2.3.2), immediately following surfactant instillation in comparison to TRE treatment.

3.2.2 Survival

All of the animals in the saline and trehalose (TRE) groups died before 180 min, whereas 4 of the five animals in the LPM-1 replacement group survived to 180 min. In general, the animals in the TRE group died at a significantly later stage during the study, compared to animals in the saline group (124 ± 23 min vs 73.6 ± 21.2 min, $p<0.05$, see Kaplan-Meier graph in Fig 3.2.1). Causes of demise in the TRE-group and saline group were progressive hypoxemia, systemic hypotension and metabolic acidosis, and pneumothorax (2/5). Because of the poor survival rates in the saline group, we omitted them from the final statistical analysis.

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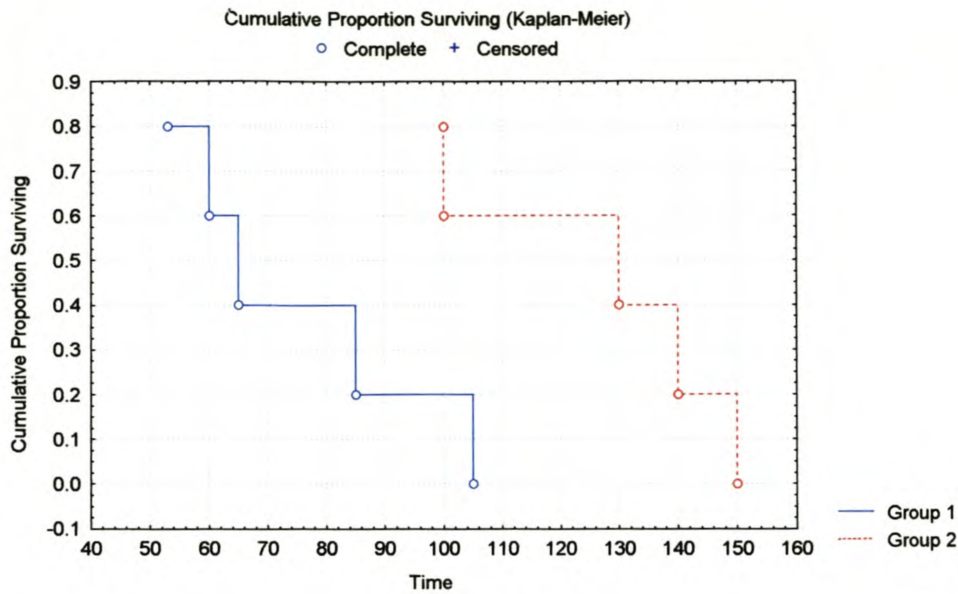

Fig 3.2.1 The rates of survival according to the Kaplan-Meier graph. Group 1: Saline, Group 2: Trehalose ($p < 0.05$, TRE vs Saline).

TABLE 3.2.1

Pre-lavage physiological and ventilation variables (mean ± SD).

Variable	ALL (n=15)	SALINE (n=5)	LPM-1 (n=5)	TREHALOSE (n=5)
Weight (kg)	2.43 ± 0.11	2.4 ± 0.18	2.48 ± 0.17	2.42 ± 0.19
MABP (mmHg)	87.9 ± 13.1	89 ± 16	92.4 ± 13.6	82.4 ± 9.6
Heart rate (per min)	248.8 ± 10	254 ± 2.2	246 ± 13.9	246 ± 10.4
Paw (cmH_2O)	5 ± 0.79	5.6 ± 0.83	4.7 ± 0.5	4.6 ± 0.75
Vt (ml/kg)	10 ± 2.3	11.4 ± 3.7	9 ± 0.95	9.8 ± 0.55
V_E (L/kg/min)	0.314 ± 0.07	0.35 ± 0.1	0.296± 0.07	0.288 ± 0.01
CRS (ml/cmH_2O/kg)	0.94 ± 0.24	0.84 ± 0.24	0.95 ± 0.30	1.03 ± 0.16

MABP: mean arterial blood pressure; Paw: mean pulmonary airway pressure; Vt: tidal volume; V_E: minute ventilation; CRS: compliance of the respiratory system. P=ns, for any variable between groups.

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TABEL 3.2.2

Pre-lavage blood gases and indices (mean ± SD).

Variable	ALL (n=15)	SALINE (n=5)	LPM-1 (n=5)	TREHALOSE (n=5)
PaO_2 (kPa)	57.9 ± 7.5	59.4 ± 8.6	55.4 ± 5.8	59 ± 8.7
$PaCO_2$ (kPa)	5.1 ± 1.3	4.7 ± 1.7	5.36 ± 0.97	5.3 ± 1.4
CO2 total (mmol/L)	24.7 ± 4.4	26.7 ± 6.3	22.8 ± 3.6	25 ± 3.5
pH	7.40 ± 0.09	7.47 ± 0.08	7.34 ± 0.09	7.39 ± 0.08
OI	1.16 ± 0.24	1.27 ± 0.27	1.1 ± 0.1	1.08 ± 0.31
a / A ratio	0.65 ± 0.07	0.66 ± 0.08	0.62 ± 0.06	0.66 ± 0.09
% Qs / Qt	2.6 ± 2.7	2.0 ± 3.0	3.4 ± 2.3	2.2 ± 3.2

All p–values were non-significant.

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TABLE 3.2.3

Post lavage (baseline: point 0) physiological and ventilation variables (mean ± SD).

Variable	ALL (n=15)	SALINE (n=5)	LPM-1 (n=5)	TREHALOSE (n=5)
MABP (mmHg)	99.7 ± 13.8	97.6 ± 16.1	97.8 ± 12.4	103.8 ± 15
Heart rate (beats per min)	236.8 ± 24.5	239.4 ± 13.5	235.6 ± 39	235.4 ± 19.9
Paw (cmH_2O)	10.9 ± 1.1	10.6 ± 0.83	11.3 ± 1.9	11 ± 0.46
Vt (ml/kg)	9.8 ± 0.4	9.6 ± 0.52	10.2 ± 0.28	9.8 ± 0.29
V_E (L/kg/min)	0.284 ± 0.04	0.27 ± 0.04	0.284 ± 0.05	0.298 ± 0.01
CRS ($ml/cmH_2O/kg$)	0.40 ± 0.10	0.41 ± 0.08	0.38 ± 0.07	0.41 ± 0.15

MABP: mean arterial blood pressure; Paw: mean pulmonary airway pressure; Vt: tidal volume; V_E: minute ventilation; CRS: compliance of the respiratory system. P=ns, for all variables between groups.

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TABLE 3.2.4

Post lavage (baseline: point 0) blood gases and indices (mean ± SD).

Variable	ALL (n=15)	SALINE (n=5)	LPM-1 (n=5)	TREHALOSE (n=5)
PaO_2 (kPa)	6.5 ± 0.86	6.8 ± 1.1	6.2 ± 0.91	6.6 ± 0.29
$PaCO_2$ (kPa)	7.6 ± 1.2	7.2 ± 0.9	8.4 ± 1.3	7.3 ± 1.1
pH	7.23 ± 0.07	7.25 ± 0.06	7.18 ± 0.05	7.25 ± 0.08
CO_2 total (mmol/L)	26.1 ± 3.6	27.1 ± 5.2	25.6 ± 4	25.9 ± 2.2
OI	22.7 ± 4.6	21.1 ± 3.8	24.9 ± 7.1	22 ± 0.52
a / A ratio	0.07 ± 0.00	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.0
Qs / Qt	48.8 ± 2.8	48 ± 3.6	49.9 ± 3.3	48.5 ± 0.94

P=ns, for all variables between groups.

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3.2.3 Ventilation parameters

The time profiles for the mean airway pressure, tidal volume and minute ventilation are presented in Figure 3.2.2, 3.2.3, and 3.2.4 No differences were noted between the time profiles for mean airway pressure between the groups.

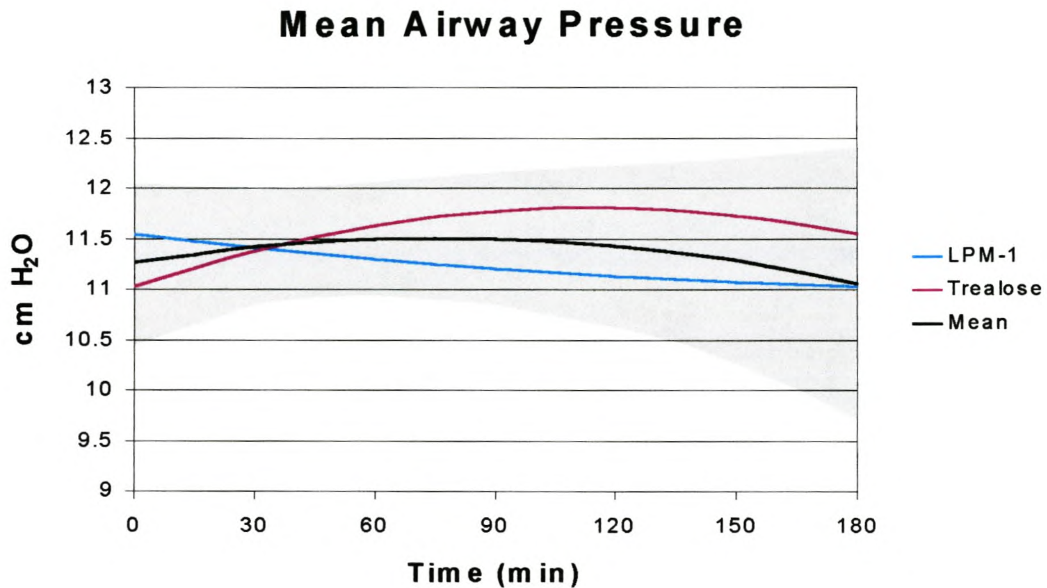

Figure 3.2.2 Time profile of the mean airway pressure. The solid line (black) represents the expected regression curve and the grey zone the 95% pointwise confidence limits. The time profile was not different between groups.

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3.2.3 Tidal volume

In the TRE group, the tidal volume deviated significantly from expectation after 90-120 minutes.

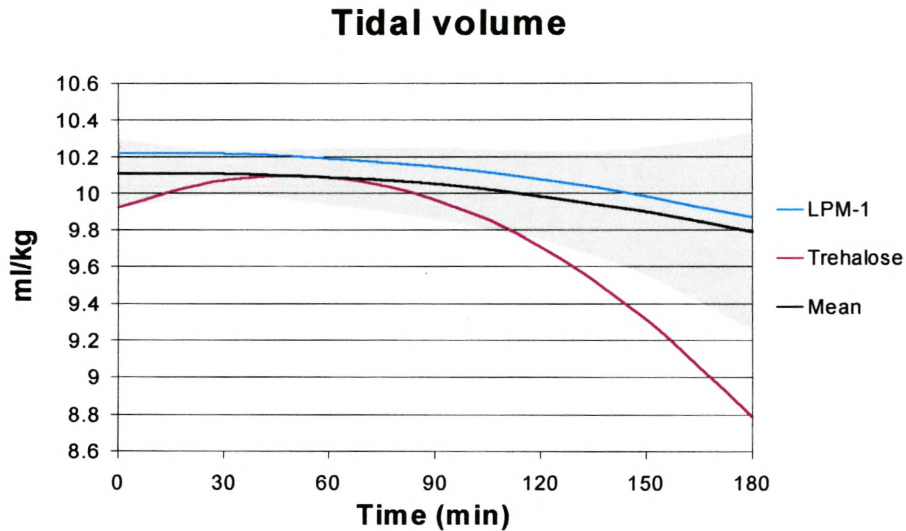

Figure 3.2.3 Time profile of tidal volume. The solid line (black) shows the expected regression curve and grey zone the 95% pointwise limits. Significant differences were noted between the time profiles of the two groups.

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3.2.4 Minute ventilation

Again, significant differences were noted in the minute ventilation-time profiles between the two groups. Significant deviation of the regression curve, to below the 2.5% pointwise limit, occurred after 60-90 minutes in the Trehalose group.

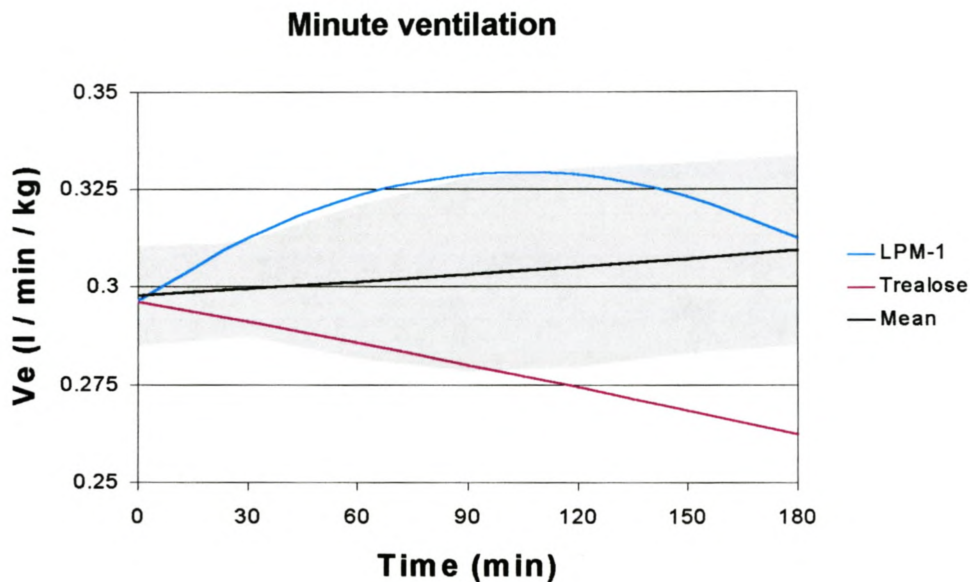

Figure 3.2.4 Minute ventilation. The solid line (black) shows the expected regression curve and grey zone the 95% pointwise limits. The time profiles of the groups differ significantly.

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3.2.5 Blood gases

The arterial pH and $PaCO_2$ are shown in Figure 3.2.5 and 3.2.7. Significant deviation of the pH from the expected occurred in the Trehalose group after 90-120 minutes. Around 120 minutes the regression curve for the ph (TRE group) dropped below the 2.5% pointwise limit.

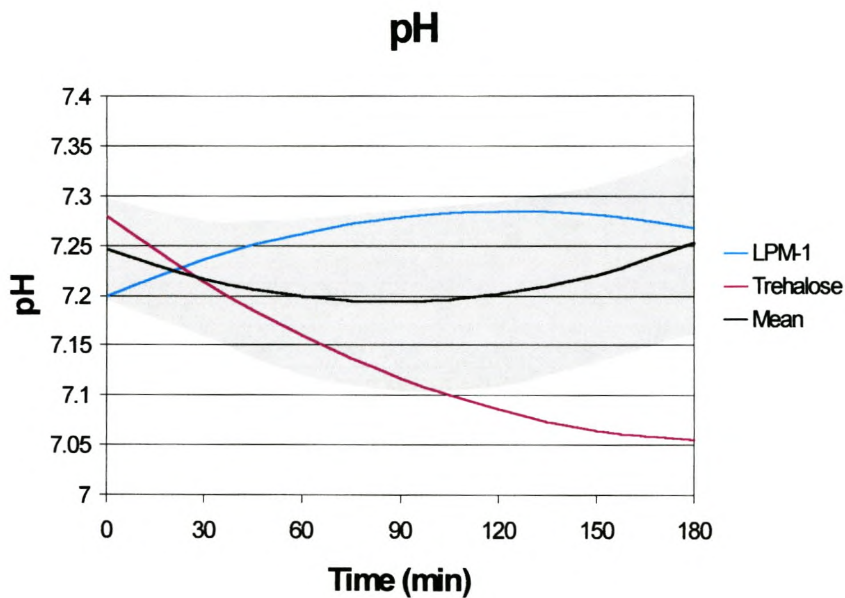

Figure 3.2.5 Arterial pH. The solid line (black) shows the expected regression curve and grey zone the 95% pointwise limits. The time profiles of the groups differ significantly.

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3.2.6 Total CO_2 content

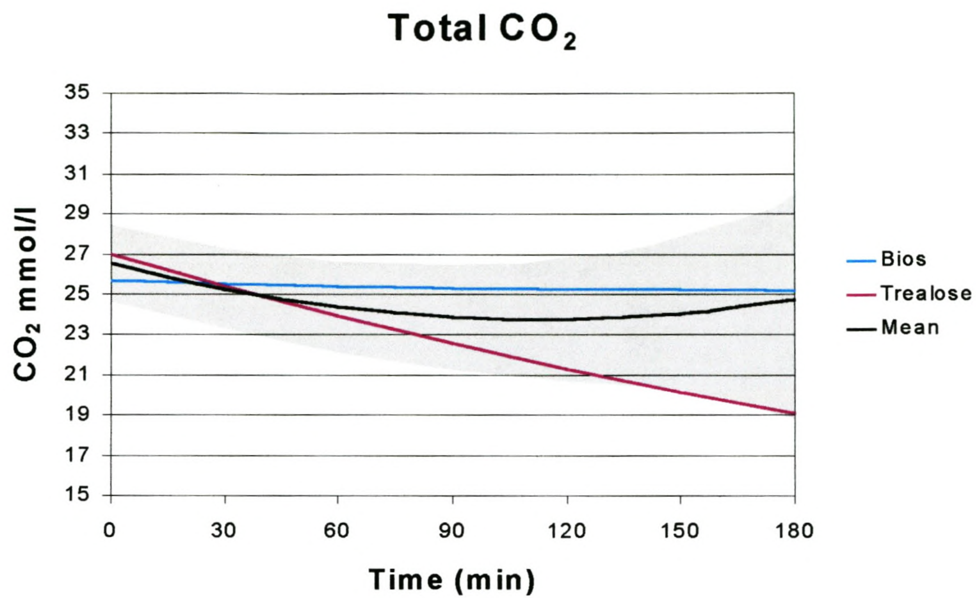

Fig 3.2.6 Total CO_2 content (mmol/L). The solid line (black) represents the expected regression curve and the grey zone the 95% pointwise confidence limits. The time profile of the groups differs significantly after 150 min.

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3.2.7 The arterial $PaCO_2$ (kPa)

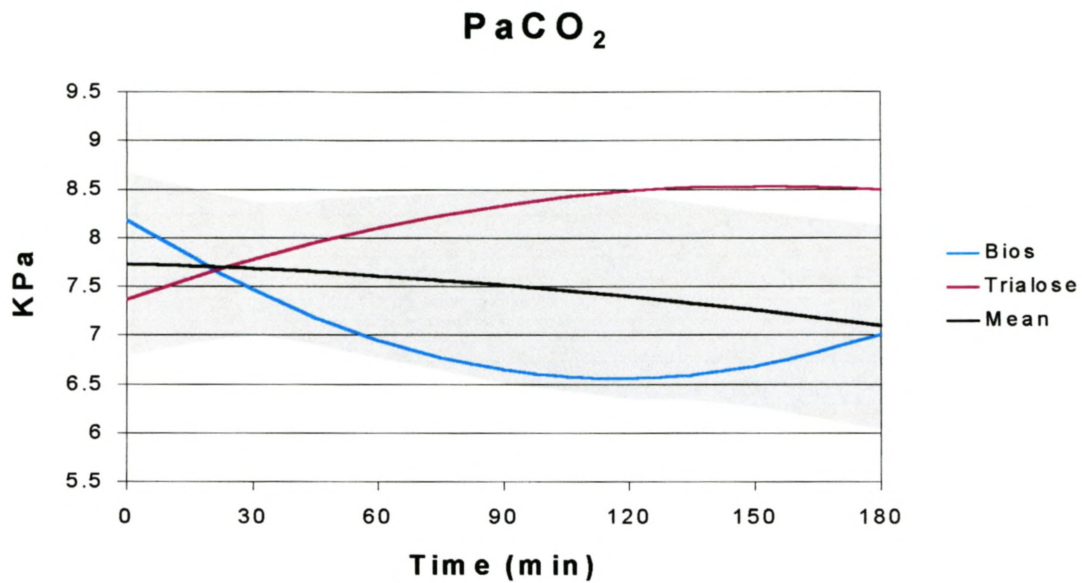

Fig 3.2.7 The arterial $PaCO_2$ (kPa). The solid line (black) represents the expected regression curve and the grey zone the 95% pointwise confidence limits. Between 60 minutes and 120 min, as well as after 120 minutes, the time profile was different between groups.

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3.2.3 Outcome measures

3.2.3.1 Time profile of the a/A ratio

Significant differences in the regression curves were noted between groups. Immediately after instillation of the surfactant, LPM-1, the regression curve crossed the 97.5% pointwise limit, whilst that of the TRE group followed a course below expectation.

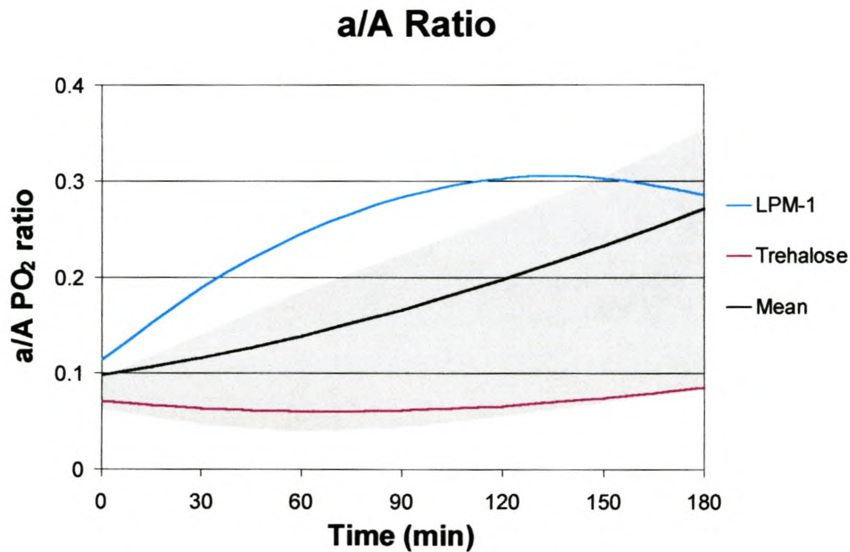

Figure 3.2.3.1 a/A ratio. The solid line (black) represents the expected regression curve and the grey zone the pointwise limits. The time profile of the a/A ratio was significantly more favourable in the LPM-1 group.

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3.2.3.2 Percentage pulmonary shunt

Significant differences for the % pulmonary shunt were noted between the groups. The observed curve for the LPM-1 group diverts significantly upwards and crosses the 97.5% pointwise limit within minutes after instillation. The curve for the TRE group diverges downwards from the expected curve and crosses the 2.5% pointwise limit within 15 minutes after instillation.

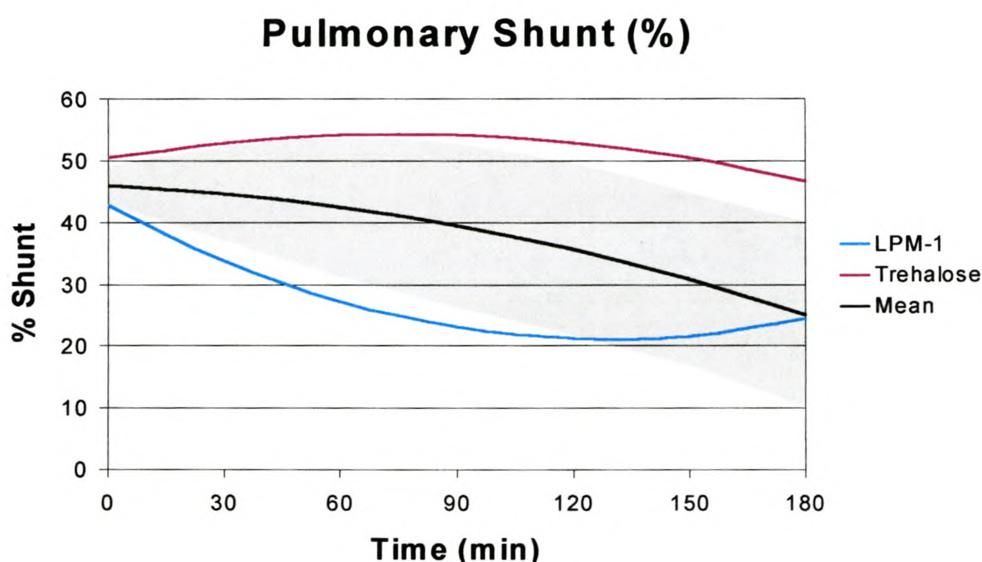

Figure 3.2.3.2 Percentage pulmonary shunt. The time profile of the % shunt on 100% oxygen. The solid line (black) represents the expected regression curve and the grey zone the pointwise 95% limits. There were significant differences between the groups.

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3.2.3.3 Total dynamic respiratory compliance

No differences were noted in the time profiles for the dynamic respiratory compliance between the two groups.

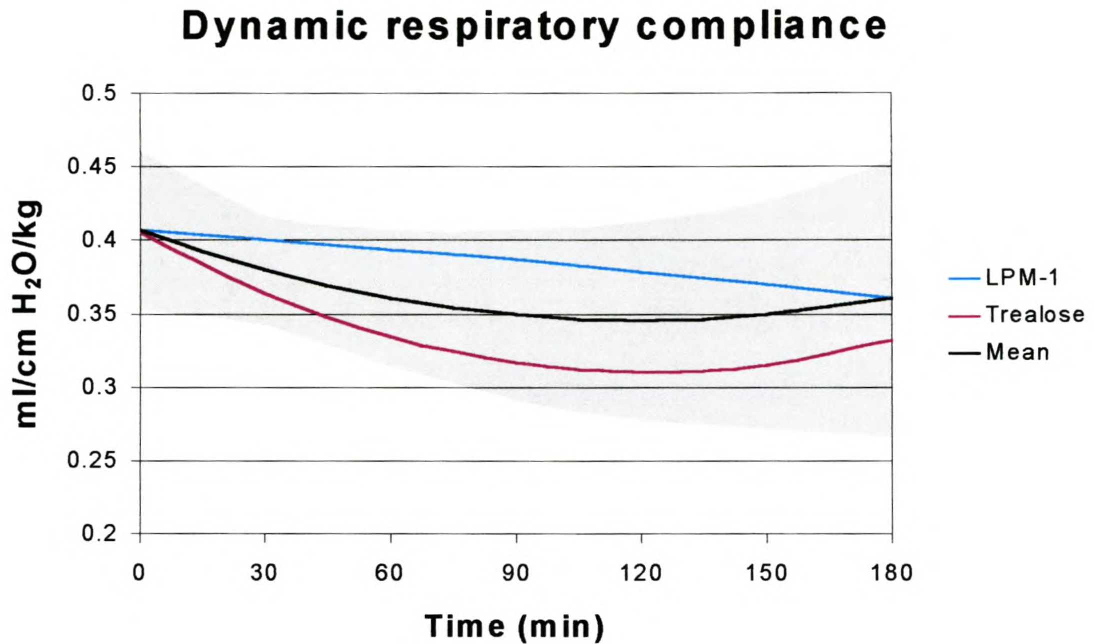

Figure 3.2.3.3 Time profile of the Respiratory compliance of the LPM-1 and TRE group. The solid line (black) represents the expected regression curve, whereas the grey area denotes the pointwise 95% limits. There were no differences between the two groups.

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3.2.4 Bronchoalveolar lavage findings

Phosphatidylcholine (PC) was the most abundant phosholipid, comprising 70.7% of the total phospholipid (Table 3.2.4.1), and contained 70% palmitic acid (C 16:0; PA) (Table 3.2.4.2). The majority of the fatty acids were saturated (73-77%, data not shown). The ratio of saturated fatty acids (FA's) to unsaturated FA's was similar between groups before treatment (~ 3.4%; data not shown). The range of PA (58 – 82%) was similar to that of other mammalian species, including human lung surfactant (~ 73%), whereas the saturated / unsaturated FA ratio (~ 3.4), were slightly lower than that reported for human surfactant extracted from minced lung tissue (~ 4.6%) (Shelley et al 1984). The phoshatidyldimethy-lethanolamine (PEA) level reported in the present study (7.2%) (Table 3.2.4.1) is somewhat higher than what was published by others (Table 3.2.4.3) (Rooney et al 1975, Harwood et al 1975). It may be that the lipid identified as PEA, was in fact partly phosphatidylglycerol, - a lipid whose accurate identification was precluded because of technical reasons.

The ratio of PC / SM in BAL fluid, a rough Lecithin / sphingomyelin ('L/S') ratio, decreased significantly in the TRE- group between the first lavage and final lavage (23.5 vs 6.04; $p = 0.009$). This change in ratio was not significant in the LPM-1 group (23.8 vs 11.3; p=ns) and saline group (15.7 vs 6.4; $p = 0.07$).

Fatty acid composition of surfactant phosphatidylcholine at baseline and at the end (final) of the study is shown in Table 3.2.4.4. In accordance with the findings of other studies, there was a very low amount (< 1%) of fatty acids with chain lenghts greater than 18 carbons.

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Table 3.2.4.1

Bronchoalveolar lavage: Major surfactant phospholipid distribution at the beginning (baseline) and the end of the study (final) (mean ± SD).

Phospholipids	% PC		% PEA		% SM		% LPC	
	Baseline	Final	Baseline	Final	Baseline	Final	Baseline	Final
TREHALOSE	71.3 (7.2)	56 (7.8)	7.1 (2.4)	15.2 (3.6)	3.9 (1.5)	10.4 (4.1)	9.1 (3.1)	4.8 (0.7)
LPM-1	75 (7.2)	69 (11.5)	6.4 (2.4)	8.8 (4.2)	4.9 (3.5)	7.6 (3.7)	7 (3.5)	3.3 (1.8)
SALINE	65.8 (9.1)	56.6 (2.8)	8 (1)	14.6 (1.3)	4.9 (2.3)	9.2 (2.2)	12.2 (6.4)	5.4 (1.1)

PC: phosphatidylcholine; PEA: phosphatidylethanolamine; SM: sphingomyelin; LPC: lysophosphatidylcholine.

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Table 3.2.4.2

Fatty acid composition (%) of phosphatidylcholine (PC) in surfactant isolated by bronchoalveolar lavage compared to published data for rabbits.

Fatty acid	Present study Baseline N = 15	Rooney et al (1975)	Harwood et al (1975)
14:0	3 (0.92)	2.2	2.5
16:0	70 (7.26)	62.8	56.3
16:1	3.57 (1.95)	5.9	5.5
18:0	2.51 (0.63)	2.5	5.4
18:1	14 (3.6)	14.8	19.6
18:2	6 (2.2)	10.6	3.0
Total saturated	75.5	67	64
16:0 / 16:1	19.6	10.6	10.2

Table 3.2.4.3

Composition of surfactant phospholipid isolated by BAL (n = 15), compared to published data for rabbits.

Phospholipid (%)	Present study Baseline mean (SD)	Rooney et al (1975) mean	Harwood et al (1975) mean
PC	70.7 (8.3)	86.2	91.6
PEA	7.21 (2)	3.0	1.2
SM	4.6 (2.4)		2.2
PI	7.9 (2.3)		1.8

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Table 3.2.4.4

Fatty acid composition of surfactant phosphatidylcholine in surfactant isolated by BAL at baseline and at the end (final) of the study- % of total fatty acids (mean ± SD).

FATTY ACIDS	TREHALOSE	LPM-1	SALINE
14:0 baseline	3.2 (1)	3.1 (1.3)#	2.8 (0.2)
final	2.8 (0.6)	1.2 (0.6)	2.1 (1.4)
16:0* baseline	70 (7.9)	67.8 (9.6)#	72.2 (4)
final	66 (2.6)	87.4 (7.3)	53 (29.8)
16:1 baseline	3.3 (1.7)	4.3 (2.8)#	2.9 (1)
final	2.8 (1.1)	0.65 (0.3)	2.1 (1.3)
18:0 baseline	2.2 (0.4)	3 (0.7)	2.2 (0.4)
final	5 (1)	2.8 (2.3)	4.4 (2.7)
18:1 baseline	14.7 (4.7)	13.7 (3.7)#	13.7 (3)
final	12.8 (0.8)	4.1 (2.2)	11 (6.6)
18:2 baseline	5.3 (2.5)	7.3 (2.6)#	5.5 (0.9)
final	9.2 (1.7)	3 (1.6)	6.3 (3.7)

* C 16:0 = palmitic acid (see text)

# $p < 0.05$ intra-group (pre-lavage vs post-lavage).

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3.2.5 Lung lavage fluid cells

The neutrophil count increased from the initial to the final lavage in all three groups ($p = 0.04$ for all groups) (Table 3.2.5.1). There were no differences between the percentage cell counts between groups at the time of the final lavage.

A drop in total cell count between the first and the final lavage was observed in all three groups. This decrease was however, only significant in the Saline group. In the group as a whole (n=15), the total cell count decreased from 337 X 10^3 / ml at the time of the first lavage to 104 X 10^3 / ml at the final lavage ($p = 0.01$). The drop in cell count was mainly due to a decrease in macrophage numbers.

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Table 3.2.5.1

Bronchoalveolar lavage. Mean cells (range).

Cell-type	LPM-1 N=5	TREHALOSE N=5	SALINE N=5
1 st lavage			
Total cells ($X\ 10^3$ / ml)	344 (100-742)	288 (102-498)	380 (103-726)
Neutrophils %	5.2 (2-15)	8.6 (2-22)	4.2 (2-6)
Macrophages %	94.2 (91-97)	87.4 (76-97)	89.6 (83-89)
Epithelial %	7.2 (0-22)	4.4 (0-17)	10.2 (0-33)
2 nd lavage			
Total cells ($X\ 10^3$ / ml)	153 (9-533)	102 (22-186)	58 (22-128)
Neutrophils %	75.6 (32-94)	75.4 (61-94)	61.6 (53-76)
Macrophages %	19.2 (5-56)	22.2 (5-36)	34.2 (21-45)
Epithelial %	4.4 (0-17)	9.8 (0-21)	8 (0-17)

LPM-1, Trehalose, Saline: % Neutrophils and % macrophages, first lavage vs 2 nd lavage: $p = 0.04$.

LPM-1, Trehalose: Total cells, first lavage vs 2nd lavage, p = ns; Saline, first lavage vs 2nd lavage, $p < 0.05$.

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3.2.6 Peripheral blood count (Table 3.2.6.1)

In the study group as a whole (n=13), the total peripheral white blood cell count (WCC) decreased from 5.1 (2.2) at the time of the first lavage, to 1.7 (1.37) at the final lavage (p = 0.0033). This decrease in both the peripheral WCC and neutrophil count, between the baseline values and and final values, were found within all groups. These differences did however, not reach statistical significance.

Tabel 3.2.6.1

The peripheral white blood cell count at baseline and at the end of the study. Mean (±SD)

	LPM-1 N=5		**TREHALOSE N=5**		**SALINE N=4**	
Baseline count						
*WBC X 10^9 / l	4.5	(1.2)	5	(2.5)	5.1	(3.2)
Neutrophils %	42	(30.2)	44.5	(8)	37.5	(38.9)
Final count						
*WBC X 10^9 / l	1.8	(1.2)	1.66	(1.43)	1.92	(2)
Neutrophils %	42	(22)	27.3	(20.3)	26.7	(23.4)

*WCC = white blood cell count.

Between groups, baseline values vs final values; p = ns (p = 0.06, for Saline group variables: baseline-WCC and final WCC).

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Table 3.2.7

Correlation (r^2) between the BAL-derived ratio of phosphatidylcholine (PC) to sphingomyelin (SM) and four variables at the time of the last lavage.

	CRS_{dyn}	OI	a/A ratio	% shunt
PC/SM ratio	0.0006	0.38	0.50	0.45

CRS_{dyn}: dynamic respiratory compliance, OI: oxygenation index, a/A ratio: arterial-alveolar oxygenation ratio.

3.3 In vitro study results

Difficulty was experienced in solubilizing both DPPC and the mixture of DPPC and TRE. After solubilization, it was noted that LPM-1 and LPM-2 + $CaCl_2$ contained fine particulate material.

The results shown in the graphs (Fig 3.3.1, 3.3.2) are the mean maximal surface tension values plotted against dilution (log). At a concentration of 20 mg/ml DPPC (at 20°C and 37°C), surface films consisting only of synthetic DPPC or DPPC in combination with trehalose (TRE), show markedly higher mean maximal surface tension values than those surface films consisting of mixtures of DPPC with other chemicals-the mean ordinate surface tension values of the DPPC-only and DPPC + TRE group are higher, and the slopes of their respective graphs smaller in magnitude than those of the other formulations, implying that the firstmentioned formulations have less surface tension-lowering capability than the other surfactants. The apparent differences in mean ordinate values of the various surfactants were confirmed by a three-way analysis of variance.

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At 20°C (20 mg/ml DPPC-surfactants) the mean ordinate values of DPPC and DPPC + TRE, 70.13 and 69.47, respectively, were not significantly different from each other (Fig 3.3.1). Also, the means of LPM-1 and the formulations containing DPPC + TRE + tyloxapol + $CaCl_2$ were not significantly different from each other. The same applies to LPM-2 (on-site Exosurf) and LPM-2 + $CaCl_2$. From these results it appears that there are three internally homogeneous subgroups, namely DPPC and DPPC + TRE, LPM-2 and LPM-2 + $CaCl_2$, and LPM-1 and DPPC + TRE + tyloxapol + $CaCl_2$. These subgroups differ significantly ($p< 0.001$).

Similar conclusions apply to the ordinate values of the surfactants at 37°C ($p<0.001$) (Fig 3.3.2), and to the mean slope values at 20°C ($p<0.001$) (Fig 3.3.1), with the exception that the subgroups, LPM-2 and LPM-2 + $CaCl_2$, and LPM-1 and DPPC + TRE + tyloxapol + $CaCl_2$, are not so clearly separated. In the ordinate values at 37°C the trends are less clear, with the differences in mean ordinate values not significantly different. A similar analysis of mean slope values was performed. Here too, a significant difference between substances was found, DPPC-only or in combination with TRE, again being significantly different from the other substances.

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Table 2.8.2.1

Calibration values (with deionised water at varying temperatures) for the tensiometer.

Degrees in Celsius	dyn / cm
40	69.56
30	71.97
25	71.18
20	72.75

Table 2.8.2.2

pH measurements of the surfactant-deinonised water at 20 °C (means ± SEM).

DPPC concentration	Dilution: 1000µg	Dilution: 500µg	Dilution: 250µg
10mg/ml	7.47 ± 0.11	7.59 ± 0.10	7.64 ± 0.10
20mg/ml	7.44 ± 0.20	7.48 ± 0.17	7.58 ± 0.13

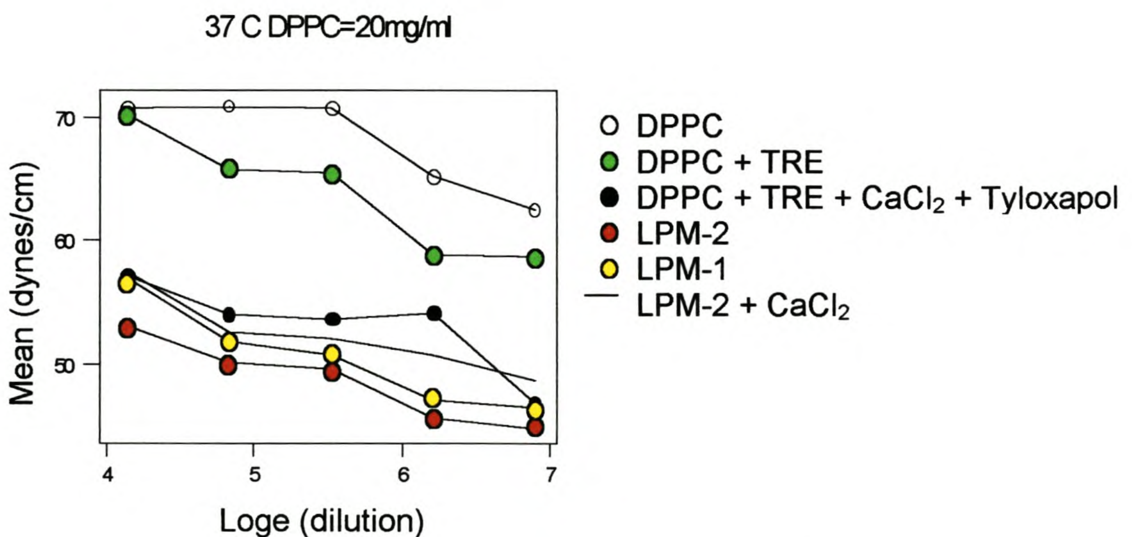

Figure 3.3.1 Adsorption of different synthetic surfactants (DPPC 20mg/ml) after 24 hours at 37°C. The adsorption of LPM-1, LPM-2, LPM-2 plus $CaCl_2$, and

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DPPC plus TRE and tyloxapol and $CaCl_2$, is compared to DPPC and DPPC plus TRE. Data are surface tension determined after 24 hours as a function of surfactant at various dilutions.

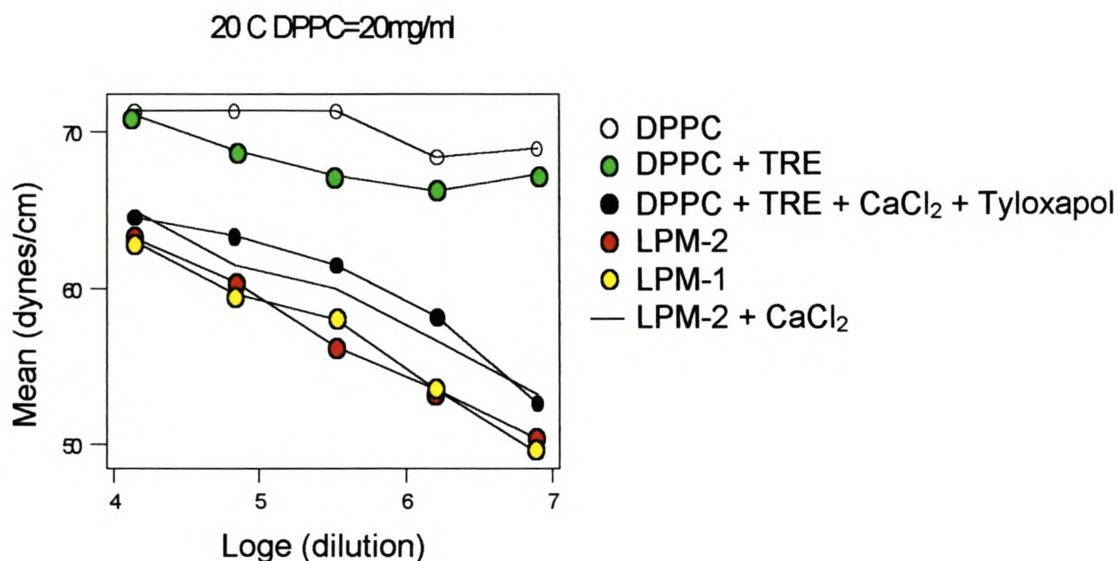

Figure 3.3.2 Adsorption of different synthetic surfactants (DPPC 20mg/ml) at 20°C. Adsorption of LPM-1, LPM-2, LPM-2 plus $CaCl_2$, and DPPC plus TRE and tyloxapol and $CaCl_2$, is compared to DPPC and DPPC plus TRE. Data are surface tension determined after 24 hours as a function of surfactant at various dilutions.

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4. Discussion

The aim of the present study was to develop a clinical useful protein-free synthetic lung surfactant. Towards this end, **a study** was designed to firstly test the hypothesis that the true difference in acute physiological effects between a mixture of DPPC, tyloxapol, hexadecanol and trehalose (LPM-1), and a commercial synthetic surfactant, Exosurf (GlaxoSmithKline), is zero, in a surfactant-deficient animal model. A **second study** addressed the physiological effects of DPPC, hexadecanol, tyloxapol and trehalose (LPM-1) compared to treatment with trehalose (TRE) or saline, in order to determine (1) the contribution of TRE to the mixture of DPPC, hexadecanol and tyloxapol, and (2) to assess the effect of the LPM-1 surfactant replacement on the epithelial lining fluid composition by means of analysing broncho-alveolar lavage fluid. **Thirdly**, the effects of TRE and / or calcium were studied on the surface properties of DPPC suspensions, by *in vitro* analysis using the ring detachment method of Du Noüy (Sekabunga et al 1969, Barrow and Hills 1979). The saline-lavage model used in this study is widely used for studying ventilator settings, experimental surfactant-deficiency syndrome, and for assessing the effects of surfactant replacement (Lachmann et al 1980, 1982, Gommers et al 1993, Makhoul et al 1995, Wenzel et al 1999, Rimensberger et al 1999).

In the first *in vivo* study, three synthetic surfactants, LPM-1, Exosurf and LPM-2, and a saline group were tested. LPM-1 is a new formulation that consists of a mixture of DPPC, TRE, hexadecanol and tyloxapol. Neither the *in vivo* effects, nor the surface behaviour of a mixture of TRE / DPPC, have until now been studied. LPM-2 is a formulation with a composition equivalent to that of

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commercially available Exosurf, prepared on site. The results showed that LPM-1 and Exosurf performed similarly after administration, surpassing that of LPM-2 or saline. The first hypothesis (1.2) was therefore accepted. Overall, intratracheal instillation of both Exosurf and LPM-1, rapidly improved the gas exchange and reduced the intrapulmonary shunt, but did not restore the lung to its prelavage condition. From the 2nd *in vivo* study it was evident that trehalose-only, was inefficient as a lung surfactant, failing to improve oxygenation indices, alleviating the calculated percentage shunt, or influencing respiratory compliance. The addition of the sugar, trehalose (TRE), to the on-site 'Exosurf' mixture (LPM-2) brought the activity of the resultant LPM-1 to the same level as that of commercial Exosurf, but failed to raise the activity above that of Exosurf. These physiological improvements were sustained for up to 3 h. Saline-treated animals had no improvement in gas exchange despite management with variable PIP (to maintain a tidal volume of ~10 ml / kg) and constant PEEP of 5 cm H_2O.

In-vitro results, obtained by the Du Noüy tensiometer, showed higher mean ordinate surface tension values for the DPPC-only and DPPC + TRE mixtures, and the slopes of their respective graphs smaller in magnitude than those of the other formulations. From these findings we gather that these formulations have less surface tension-lowering capability than the other surfactants. At 20°C (20 mg / ml DPPC-surfactants) the mean ordinate values of DPPC and DPPC + TRE, 70.13 and 69.47 respectively, were not significantly different from each other. The mean ordinate values of LPM-1 and the formulation containing DPPC + TRE + tyloxapol + $CaCl_2$ were lower, but similar, as were the values of LPM-2 (~ Exosurf) and LPM-2 + $CaCl_2$. Thus, three internally homogeneous subgroups could be identified which differed significantly, namely: DPPC and DPPC + TRE,

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LPM-2 and LPM-2 + $CaCl_2$, and DPPC + TRE + tyloxapol + $CaCl_2$ and LPM-1. Similar conclusions apply to the ordinate values of the surfactants at 37°C, and to the mean slope values at 20°C, with the exception that the subgroups, LPM-2 and LPM-2 + $CaCl_2$, and LPM-1 and DPPC + TRE + tyloxapol + $CaCl_2$ are not so clearly separated. A similar analysis of mean slope values was performed. Here too a significant difference between substances was found, DPPC alone or in combination with TRE, again being significantly different from the other surfactants. The poor *in vitro* performance of TRE corroborates the *in-vivo* finding that TRE-alone is an inefficient 'surfactant'. LPM-1 and DPPC + TRE + tyloxapol + $CaCl_2$ lowered *in vitro* surface tension, but not to the same extent as LPM-2 (on-site 'Exosurf') and LPM-2 + $CaCl_2$. These findings were, however at odds with that of the *in vivo* studies, in which LPM-1 performed better than LPM-2.

The development of an effective artificial surfactant mixture, devoid of foreign protein, remains a challenge. Many researchers focus on the development of a protein-free synthetic surfactant with the idea that such a product may pose less of a problem than natural, animal-derived, surfactants in terms of antigenicity. The *in vivo* and *in vitro* immunogenicity of natural surfactants have been investigated, but in very limited fashion. Survanta (bovine-derived) and Exosurf (synthetic) surpresses alveolar macrophage production of interleukin-1 and tumor necrosis factor-alpha in a dose-dependent manner (Moore et al 1995). Bovine surfactant has been shown to impair the mitogenic response of peripheral blood lymphocytes of neonates to stimulation, and surfactant-antisurfactant immune complexes have been detected in plasma of infants and adults with RDS (Merritt at al 1988, Bartmann et al 1992). However in another study involving premature infants, no

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trace of a specific immunological response to bovine surfactant proteins after single or multiple doses of exogenous surfactant replacement was detected for up to 6 months after treatment (Whitsett et al 1991). It is not known whether immune complexes from heterologous surfactant replacement would translate into disease in later life.

One of the problems with developing a synthetic surfactant is that DPPC, in isolation, does not possess all the physical characteristics required of a lung surfactant. It adsorbs slowly onto a surface, only undergoes a transition from solid to liquid-crystalline state well above body temperature, i.e. at 41°C, and resists compression (Albon and Sturtevant 1978, van Golde et al 1994). On its own, DPPC forms liposomes that have to be destabilised or 'liquefied' in order to spread and effectively form a monolayer at body temperature. It is known that surface film status (gas, liquid, or solid) is determined by its composition, degree of compression, and temperature. Since DPPC has a melting point above body temperature, it should be combined with other lipids and / or surfactant proteins (SP-B / C) to decrease the melting temperature and to create a stable surface film at the alveolar air-water interface (Palmblad et al 1999). Effective spreading of surfactant is achieved by mixing DPPC with unsaturated phospholipids (such as phosphatidylglycerol and / or cholesterol), or other lipids, or through the addition of SP-B (Morley et al 1980, Longo et al 1993). The addition of unsaturated phospholipids reduces the melting point of DPPC, therefore allowing better 'spreading' at the air-water interface (Bangham et al 1979, Munden and Swarbrick 1973).

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Extrapolating from the aforementioned knowledge, we assessed whether another 'substance', TRE, which had until now not been tested *in vivo,* might affect surfactant function in beneficial ways. Trehalose interacts with DPPC by hydrogen bonding between the OH groups in the sugar and the polar head groups of DPPC and is speculated to inhibit phase transition of phosphatidylcholine to its gel phase (Crowe et al 1984). Trehalose (TRE) was chosen for its potential biocompatibility with mammalian tissue. Trehalose-containing solutions (Euro-Collins solution) have been used for both the preservation of cultured murine endothelial cells (Isowa et al 1996) and as a storage solution to preserve organs of animal models for transplantation purposes (Bando et al 1994, Hirata et al 1994). Trehalose (TRE) is a non-reducing disaccharide, existing in prokaryotes, fungi, yeasts, some desert plants, and insect body fluid (Crowe et al 1984, Bando et al 1994). It is biocompatible, and human studies have shown that it yields glucose on hydrolysis by trehalase (Eze 1989). The enzyme, trehalase is found in many tissues in the human body including; small bowel (Lojda 1981), kidney (Briere et al 1989, Ishihara et al 1997), and human plasma (Eze 1989). The cell membrane-, and endothelial stabilizing effect under stressful conditions (complete dehydration, freezing, high- or low temperature, and ischemia-reperfusion injury) is a unique property of trehalose not shared by other carbohydrates (Crowe et al 1984). Trehalose is a FDA-approved protein stabilizer for lung inhalational particles (Bosquillon et al 2001). Neither trehalase, nor trehalose has as far as the author could determine, been described to be present in the mammalian lung. Trehalose alters the physical properties of membrane- and other phospholipids when present in a dry state by interacting with the polar head groups of DPPC by means of hydrogen bonding. Also, when TRE is added to the subphase of

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phosphatidylcholine *in vitro*, the monolayer is spread (expanded) by the carbohydrate (Crowe et al 1984). The lastmentioned investigators studied the surface pressure changes of the phospholipid mono-molecular films of DPPC at the air / water interface in the presence of various carbohydrates and found that, at a given temperature, the area per molecule of DPPC increased with increasing concentration of TRE. In comparison to glycerol, TRE produces a greater increase in area per molecule, both at a high-and a low temperature. Trehalose appears to alter the phase transition behaviour of the phospholipid by means of a reduction in the gel to liquid-crystalline (fluid) phase transition temperature. (Crowe and Crowe 1988).

By using adult New Zealand White rabbits in which the lung surfactant had been reduced / removed by repeated whole-lung lavage, the physiological effects of synthetic protein-free surfactant replacement were examined. The adult rabbit was selected for both practical reasons, and the fact that they have comparable weight and respiratory physiology to full term newborn infants (Crosfill and Widdicombe 1961). Repeated saline lung lavage, by the method of Lachmann et al (1980), (Lachmann 1980, Lachmann et al 1982), consistently induced respiratory failure characterised by deterioration in gas exchange, increased intrapulmonary right-to-left shunting and venous admixture and impairment of lung mechanics. Both the physiological and histological data indicate that the animals had developed acute lung injury and respiratory failure due to surfactant deficiency, similar to early clinical ARDS and neonatal RDS. In agreement with previous literature, this model was reproducible in terms of hyaline membrane formation and decrease in total respiratory compliance (Lachmann et al 1982, Kobayashi et al 1984,

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Mcculloch et al 1988, Nielsen et al 1991, Makhoul et al 1995, Krause et al 1997, Rimensberger et al 1999, Puligandla et al 2000).

During repeated whole-lung lavage, alveolar surfactant is removed with warm saline and short-term survival of animals ensured by assisted conventional ventilation or high-frequency oscillatory ventilation (Lachmann et al 1982, Kobayashi et al 1984, Mcculloch et al 1988, Boynton et al 1991, Wenzel et al 1999). Broncho-alveolar lavage (BAL) with 30 ml / kg physiological saline removes ~ 80% of the vital capacity of the adult rabbit (Wenzel et al 1999). The present study utilized 5-7 lavages per animal (25 ml / kg), and although accurate effluent-volume recordings were not performed, severe surfactant deficiency was evident by the absence of foam at the end of the washings, concurrent with severe impairment in pulmonary gas exchange and deterioration in respiratory compliance. In addition, the study groups did not differ as to the endpoints of lung damage. Although it is acknowledged that this deterioration in respiratory physiology may partly have been caused by fluid retention in the terminal airspaces or increased lung-tissue water, a number of studies have shown that the reduction in lung surfactant is the major reason for the deterioration (Lachmann et al 1980, 1982, Kobayashi et al 1984). Moreover, Kobayashi and coworkers (Kobayashi et al 1984) have shown that the lung water content was similar, regardless of whether adult rabbits were treated with surfactant or placebo (saline), with or without PEEP.

The respiratory related changes that occur with surfactant-deficiency conditions have been explained by a 3-compartment and a 2-compartment model (Hansen et al 1979, Hand et al 1990). The 3-compartment model depicts the

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surfactant-deficient lung as consisting of well ventilated, or overventilated, and perfused units (V/Q > 1), open, but severely underventilated, perfused units, i.e. fluid filled (V/Q < 1), and atelectatic, underventilated areas (V/Q = 0). In the face of ongoing perfusion of atelectatic lung units, or units that are 'open' but hypoventilated (alveolar hypoventilation), a shunt occurs (Coetzee 1987). These shunt-alveoli are representative of low V/Q units, a common finding in surfactant-deficiency conditions. The 2-compartment model of the neonatal lung suggests that mismatches involving high V/Q units and low V/Q regions coexist, with high V/Q units contributing to increased dead space. The low V/Q unit is further subdivided into a compartment that represents true veno-arterial shunting, with venous admixture from an underventilated compartment (Hand et al 1990). In effect this model appears to be more or less similar to the proposed 3-compartment model. Confirmation that high V/Q regions exist in RDS is inferred from the observed increase in the arterial-alveolar difference in CO_2 tension existing in RDS, a situation that is reversed over hours, following synthetic surfactant (Exosurf) treatment (Billman et al 1994).

Following lavage, alveoli become unstable and tend to collapse abruptly during expiration when the trans-alveolar pressure decreases below the critical closing pressure (Cotton 1994). In the face of continuing capillary perfusion of these unstable gas exchange units, venous admixture (intrapulmonary shunt) increases. Surfactant deficiency therefore, will result in arterial hypoxemia and hypercapnia as a result of right-to-left shunting (Qs/Qt) and alveolar hypoventilation, respectively (Cotton 1994, Krause et al 1998). *Bronchoalveolar lavage* elevates alveolar and physiological deadspace (Wenzel et al 1999), lowers the mean lung volume above residual volume (Boynton et al 1991), decreases arterial PaO_2 and

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increases arterial $PaCO_2$ (Wenzel et al 1999, Puligandla et al 2000), decreases arterial-alveolar PO_2, ratio (a/A ratio), raises systolic right ventricular pressure (Krause et al 1998), increases right-to-left shunting / venous admixture (Boynton et al 1991, Krause et al 1998, Wenzel et al 1999, Schermuly et al 2000), and increases perfusion of low V/Q areas (Schermuly et al 2000). A significant negative correlation ($r^2 = 0.62$) has been established between the arterial-alveolar ratio and the deadspace / tidal volume ratio, and a significant agreement recorded between the alveolar deadspace / tidal volume ratio and the arterial end-tidal PCO_2 difference ($r = 0.98$) (Wenzel et al 1999). In the adult rabbit, whole-lung lavage increases alveolar deadspace by almost 5-fold and alveolar deadspace/tidal volume ratio from zero to one third of the tidal volume (Wenzel et al 1999). If not treated with surfactant, the majority of saline-lavaged surfactant-deficient rabbits, supported by conventional mechanical ventilation after lavage, deteriorate significantly over the next 1-2 hours after the lavage in regard to static lung compliance and arterial oxygenation status (Makhoul et al 1995).

Exogenous surfactant replacement therapy with or without effective volume recruitment strategies is an effective treatment for surfactant deficiency disorders. In general, in prematurely delivered human infants and animals (piglets, lambs, rats, rabbits, baboons), and small or adult animals (rats, pigs, rabbits), rendered surfactant-deficient either by their gestation or by lavage, respectively, synthetic and natural exogenous surfactant results in improved oxygenation, reduced shunt-flow, improved respiratory mechanics and improved aeration of the lungs (Ikegami et al 1979, Robertson and Lachmann 1988, Cotton et al 1993, Gommers et al 1993, Häfner et al 1993, Billman et al 1994, Häfner et al 1995, Wenzel et al 1999, Puligandla et al 2000, Kelly et al 2000, Schermuly et al 2000).

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Surfactant replacement (bovine surfactant) reduces right-to-left shunting, alveolar deadspace/tidal volume ratio and the arterial end-tidal PCO_2 difference (Wenzel et al 1999). However, and in agreement with our findings, surfactant treatment improves gas exchange but does not restore the lung to its prelavage condition (Wenzel et al 1999). In some of these models, pure phospholipid mixtures consistently performed worse than the natural surfactants containing surface-active proteins or when the DPPC was combined with phosphatidylglycerol (Ikegami et al 1979, Morley et al 1980, Häfner et al 1993, Kelly et al 2000). In preterm human infants, the a/A ratio and FRC improved earlier and to a greater extent after bovine surfactant instillation than after synthetic surfactant (Cotten et al 1993). Unfortunately, very few researchers have attempted to study the physiological effects of synthetic protein-free surfactant replacement on the lung of the saline-lavaged adult rabbit model (Kelly et al 2000). It therefore remains problematic to draw comparisons between the physiological findings of the present study and that available in the literature.

Overall, the increase in arterial oxygenation tension following the instillation of exogenous surfactant is largely due to an increase in lung volume, more specifically, the functional residual capacity (FRC) (Gommers et al 1993, Cotton et al 1993, Cotton 1994, Alexander and Milner 1995, Dimitriou et al 1997). It is reasoned that the increase in FRC is due to stabilization of already open, but underventilated airspaces as well as to recruitment of new gas exchange units (Cotton 1994, Krause et al 1997, Wenzel et al 1999). In the presence of surfactant replacement, the relative contribution of these two mechanisms may depend on ventilator settings, i.e. employment of PEEP, mean airway pressure, and other volume recruitment maneuvers (Krause et al 1997). Of interest to note,

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is the finding that acute changes in mean airway pressure during *conventional ventilation,* in *the absence of surfactant* treatment, have little, if any effect on the recruitment of atelectatic alveoli, but causes redistribution of ventilation within already ventilated alveoli (Hand et al 1990). This finding is in contrast to the effects of a high mean airway pressure on oxygenation and lung volume status, achieved by high-frequency oscillatory ventilation in the lavaged model (Boynton et al 1991).

Whether pulmonary vascular reactivity remains unaltered when large lavage volumes, as in the present study, are used, is unlikely. It could be expected that surfactant deficiency would result in alveolar collapse, reduced lung volume, and as a consequence, raised pulmonary vascular resistance. Saline lavage of the lung of the adult rabbit has been shown to have variable effects on pulmonary vascular reactivity. Recently, Rosenthal et al (1998) tested different methods of inducing acute lung injury. In their study, repeated lavage with small volumes of warm saline (6 ml / kg) resulted in surfactant deficiency and acute hypoxemia, without causing hemodynamic instability or acute pulmonary hypertension. In contrast to that study, Krause et al (1998) have shown that repeated airway lavage with larger volumes of normal saline (20 ml / kg) increased the intrapulmonary right-to-left shunt significantly from a baseline of ~3% to ~64% (calculated by Fick equation). Simultaneously, there was a significant increase in systolic right ventricular pressure (SRVP). We speculate that a raised SRVP reflects a raised pulmonary vascular resistance and therefore a situation that may adversely affect cardiac output, with resulting lowered oxygen saturation of the mixed venous blood. In the majority of *in vivo* animal studies in which the effects of lavage and subsequent surfactant replacement have been recorded, no systematic

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measurements of mixed venous oxygenation status, in combination with assessment of the true cardiac output, are available. Mixed venous desaturation becomes an important determinant of arterial oxygen saturation in the presence of an increased shunt (as opposed to normal lungs).

In the absence of a pulmonary arterial catheter, we used an indirect method as 'best' substitute to calculate the percentage shunt and where also able to show that the intrapulmonary right-to-left shunt significantly increased from ~ 5%, at baseline, to ~ 44% after lavage (Coetzee 1987). This estimated shunt fraction values are very similar to the measured values obtained by Boyton et al (1991). These authors determined venous admixture, after saline lavage to the same extent as that of the present study, and found that the percentage venous admixture varied between 35% and 55%, at a mean airway pressure between 10 and 15 cmH_2O, respectively. In order to find the best substitute for true venous admixture, various oxygenation indices have been used by researchers to determine venous admixture when a pulmonary arterial catheter was not in use (Zetterström 1988, Coetzee et al 1995). These indices included the following measures of oxygenation: the PaO_2, $P(A\text{-}a)O_2$, a/A ratio, and PaO_2 / FiO_2, and a calculated estimate of venous admixture (Zetterström 1988). Both of the referred studies included adult patients with ARDS. In the one study (Zetterström 1988), the a/A ratio and calculated PaO_2 / FiO_2 index performed similarly, correlating significantly with the actual venous admixture (shunt), but were influenced by the FiO_2. In the other study (Coetzee et al 1995), both the a/A ratio and the PaO_2 / FiO_2 failed to correlate with the calculated shunt. Again the effect of varying the FiO_2, on the respective indices, was recorded. Both studies found better correlations between shunt and index when patients received 100% oxygen.

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In the absence of a pulmonary arterial catheter we substituted the true calculation of shunt (which require measuring of mixed venous blood) with the calculated pulmonary shunt (Coetzee 1987), supported by the a/A ratio, determined while animals were receiving 100% oxygen. As discussed above, the a/A ratio has been reported to be reasonably accurate in reflecting venous admixture in adult patients with ARDS on 100% oxygen ($r = -0.81$) (Zetterström 1988). It is acknowledged that both the calculated shunt and a/A ratio incorporates the variables, FiO_2 and PaO_2, and that the calculated shunt assumes a normal mixed venous hemoglobin saturation, an unlikely situation in a critically ill animal or patient (Coetzee 1987). In addition, it ignores alveolar ventilation. During the present study the effect of FiO_2 on the indices were taken out of the equation, since the FiO_2 remained at 1.0 during the study protocol. The issue of hypoventilation, as reflected by serial arterial $PaCO_2$ determinations, was addressed by attempts to deliver a standardised tidal volume, at least initially, to all animals. Another index of oxygenation, the oxygenation index (OI), incorporates the FiO_2 and PaO_2, the mean airway pressure (MAP), and a constant, 100. Whether the OI changes, depends on whether the PaO_2 is influenced by the MAP. In the present study, a significant agreement was found between the OI and percentage shunt. This correlation may be accounted for by the fact that both indices incorporate PaO_2 as well as FiO_2, and therefore illustrate some mathematical interdependence. The effect of changing the MAP on oxygenation *during high-frequency oscillatory ventilation* has been studied (Boynton et al 1991). In that study with experimental surfactant deficiency, the venous admixture fractions were more or less inversely related to the applied MAP between 10 cmH_2O and 25 cmH_2O, upon inflation (alveolar recruitment), and while animals received 100% oxygen

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(Boynton et al 1991). During deflation, the inverse correlation was only found at very low mean airway pressures (< 10 cmH_2O). That is, deflating the saline-lavaged lungs of the animals to below the opening pressure for atelectatic alveoli. These findings sugessted that shunt rather than ventilation-perfusion mismatch (venous admixture) was the main cause of the rabbits' hypoxemia. The present study utilized conventional mechanical ventilation with postlavage MAP's varying between ~ 9cmH2O and 13 cmH2O. In the presence of surfactant replacement (surfactant treated groups) these pressures may have been sufficient to open atelectatic alveoli (volume recruitment) in addition to overexpanding already open units, but insufficient to open atelectatic units in the absence of surfactant (saline lavaged animals).

Following synthetic surfactant instillation, we were able to show that LPM-1, LPM-2, and Exosurf influenced PaO_2, OI, a/A ratio and calculated % shunt. Since we did not directly measure pulmonary vascular resistance, mixed venous oxygen content, or assessed lung volume, we speculate that the decrease in shunt after surfactant instillation could be related to either one or more of the following: shunting of blood flow from poorly to better ventilated lung regions (improved ventilation perfusion matching), a decrease in pulmonary vascular resistance (relief of hypoxic vasoconstriction) in the open, but hypoventilated compartment, or lung volume recruitment. The fact that we were able to show a significant correlation between the OI and percentage shunt, may indicate that mean airway pressure changes, and therefore possible alterations in lung volume, were translated into shunt-fraction changes. It has previously been shown that FRC, arterial-alveolar difference in oxygen in 100% oxygen, and true shunt in hyaline membrane disease (diffuse atelectasis) are significantly related (Tori et al 1973)

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and that a low a/A oxygen ratio (in 100% oxygen) suggests intrapulmonary shunting as the primary mechanism (Gilbert et al 1979). In order to decrease the shunt, a clinician usually employs measures such as higher than normal PEEP (> 10 cmH_2O on the inflation limb of the pressure-volume curve) to recruit and maintain lung volume or, as an additional measure, increase the inspired oxygen concentration (Boynton et al 1991). In the absence of accurate shunt fraction measurements and the aceptance of the assumption that cardiac output and oxygen consumption remained reasonably stable, we speculate that the exogenous surfactant treatment was responsible for the decrease in the shunt fraction during the first 60 through 90 minutes of the first *in vivo* study. During this period, variables that influence shunt fractions, such as changing tidal volumes, minute ventilation, PEEP, FiO_2, blood pressure, heart rate (~ cardiac output) and rectal temperature (~ VO_2) of the animals were kept at a constant level. Thereafter, physiological changes occurred and temperatures somewhat changed in the dying animals (influencing VO_2), rendering our speculation inaccurate.

In the first *in vivo* study, three synthetic surfactants, LPM-1, Exosurf and LPM-2, and a saline group were tested. Overall, intratracheal instillation of both Exosurf and LPM-1, rapidly improved the gas exchange and reduced the intrapulmonary shunt. Saline-treated animals had no improvement in gas exchange despite management with variable PIP (to maintain a tidal volume of ~10 ml / kg) and constant PEEP of 5 cm H_2O. In addition, combining the mortality data of the two *in vivo* studies show that 54% of the saline-treated group died in comparison to 9% of the LPM-1 group ($p = 0.03$). These findings lend support to the concept that the effects of low levels of PEEP (~3-5 cmH_2O), or high levels of PEEP (~7-11 cmH_2O), in the absence of surfactant, are limited in acute lung injury

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induced by saline lavage (Kobayashi et al 1984, Sugiura et al 1994). The progression of lung injury after lavage is not prevented by increasing PIP and/or PEEP during *conventional mechanical ventilation* (Sugiura et al 1994). These authors have shown that saline-lavaged animals develop progressive metabolic acidosis during conventional ventilation. In the present study, causes of early demise in the animals were in agreement with the findings of others, and included: pneumothorax (23%), the combination of systemic hypotension, metabolic acidosis and hypoxemia (56%), and hypoxemia with pulmonary hemorrhage (20%). Under similar study protocols as that followed by the present study, the incidence of early pneumothoraces, in control and surfactant treated animals, have been reported to vary between 20% and 75% (Quan et al 1984, Kerr et al 1998, Kelly et al 2000). In saline-lavaged animals, the documentation of a metabolic acidosis as a cause of mortality can be as high as 50% (Quan et al 1984). Although not well documented, pneumothoraces have been mentioned (no data provided) to occur with increased frequency at PEEP-levels of 5 cmH_2O, before or after surfactant treatment (Krause et al 1998). We speculate that the most likely cause for the metabolic acidosis is impaired tissue utilization of oxygen following cardiac output failure in association with acute pulmonary hypertension and right ventricular failure.

Although the addition of TRE to LPM-2 (on-site 'Exosurf) improved the physiological response of the resultant formulation (LPM-1), an improved level over and above that of Exosurf was not obtained. The first *in vivo* study does however, highlight some significant differences between the *in vivo* performance of LPM-2 (on-site 'Exosurf') and true Exosurf in terms of the time profiles of their respective changes in PaO_2, OI, percentage shunt, and dynamic respiratory

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compliance. These differences in performance may partly be explained by the way in which local / on site 'Exosurf' (LPM-2) was prepared. Our preparation process may not be equivalent to that of commercial Exosurf. For instance, suspension techniques involved in preparing artificial surfactants may significantly impact on surfactant activity. Mechanical dispersion methods, including sonication and/or mechanical vortexing, as well as the temperatures at which sonication is performed, influence the microstructure of surfactant dispersions and thus the performance of that particular surfactant, both *in vitro* and *in vivo* (Ikegami et al 1979, Robertson 1981, Notter et al 1985, Notter et al 1986). In a study where a "dry" surfactant was deposited in the tracheal fluid of premature rabbits, a significant increase in distensibility of the lungs occurred, but when the same surfactant was sonicated in saline, stable liposomes resulted which trapped the lipids. It is suggested that these liposomes (wetted surfactant) release lipids at a low rate, influencing the rate and ease at which a surface-active layer is formed (Morley et al 1978). Both sonication and mechanical vortexing result in morphological forms of surfactant that are associated with rapid in vitro surface adsorption (Notter et al 1986). However, when equal doses of the same (natural) surfactant, prepared by either sonication (ice bath) or mechanical vortexing (at room temperature) are instilled into the trachea of an animal model, different results are obtained. Lambs that received both a high dose and a low dose of vortex-natural surfactant, and those treated with a high dose of sonicated natural surfactant, show clear improvement in blood oxygenation and lung compliance over that of control animals and animals treated with a low dose of sonicated surfactant (Notter et al 1985). Sonication at low levels may provide both a sub-population of microstructures (other than tubular myelin) capable of rapid

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adsorption (large thin-walled liposomes), and aggregates that adsorb poorly (Notter et al 1986). The present study utilized two mixing methods, probe sonication and mechanical vortexing, which may have impacted on the *in vivo* as well is the *in vitro* performance of the surfactant mixtures. Both steps were performed while the surfactant was reconstituted with distilled water.

Surface properties similar to that of natural surfactant can be obtained with a protein-free emulsion of synthetic lipids, provided that the lipids are first dissolved in soybean oil (or an analogous oil) before sonication (Grossmann and Larsson 1978). This procedure results in an emulsion, rather than suspension of stable liposomes in water (Robertson 1981).

The ability of sonicated mixtures of dipalmitoyl lecithin (DPL), in mixture with a variety of other phospholipids and natural surfactant to restore the pressure-volume (PV) characteristics of *adult excised rat lungs* have been studied (Ikegami et al 1979). The majority of mixtures improved PV- characteristics, but not to the same extend as natural surfactant. The mixture of DPL and unsaturated phosphatidyl glycerol (PG) restored TLC to 70% of the baseline value (at 5 cmH2O), whereas natural surfactant reached a level of 87% of the baseline TLC. In the same model, the commercially available synthetic surfactant (Exosurf) lacking unsaturated PG and / or proteins also performed poorly when assessed by measurements such as the restoration of PV mechanics, resulting in only a 10% improvement in PV levels compared to prelavage levels. The ability of Exosurf to restore lung mechanics was greatly enhanced by the addition of 1% protein (Hall et al 1992). In *excised rat lungs* total lung capacity (TLC) could not be improved by either nebulization nor instillation of synthetic surfactant

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(Ikegami et al 1977), whereas natural surfactant, obtained from calf lungs, produced a marked increase in percentage TLC after instillation, but not nebulization. This finding led the authors to conclude that the instillation of natural surfactant was the only method that restored the PV characteristics of surfactant-depleted lungs to normal. In *intact* animals, synthetic surfactant exhibits significantly less activity in terms of restoring lung mechanics to normal. Exosurf has less capacity to improve lung function than natural surfactant, in the ventilated preterm lamb (Cummings et al 1992). In saline-lavaged adult rabbits, natural surfactant replacement increases static lung compliance and FRC, but not dynamic compliance (Gommers et al 1993). To come to a conclusion, mixtures of natural surfactants have been more successful than synthetic formulations in restoring 'normal' physiology of the lung.

Addressing the issue of pulmonary mechanics in humans, i.e. ventilated preterm infants with RDS, Choukroun et al (1994) compared the effects of Exosurf and natural porcine surfactant on lung mechanics in ventilated preterm infants with RDS. Improvement over time in the Exosurf group was slow. In addition, the overall improvement in respiratory compliance was much less than that of the infants who received natural surfactant. In the majority of ventilated human infants dynamic respiratory compliance does not change over the first few hours after exogenous surfactant treatment, if mechanical ventilation is continued through breath sampling (Milner et al 1984, Davis et al 1988, Edberg et al 1990). However, some researchers report significant improvement in static respiratory compliance (measured by single breath occlusion) at 12 and 24 hours after fluidized Exosurf instillation, whereas no significant change in static compliance was observed after the instillation of dry artificial surfactant powder or artificial

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protein-free lung expanding component (ALEC, Britannia Pharmaceuticals, UK) (Milner et al 1983, Pfenninger et al 1992, Armsby et al 1992, Kelly et al 2000). Compliance changes after surfactant treatment has been attributed to an increase in functional residual capacity (FRC) reasoned to be brought about by either recruitment of new alveolar units or / and stabilization of airways and alveolar spaces during expiration (Edberg et al 1990, Goldsmith et al 1991).

In agreement with the findings of other studies, we could not demonstrate early restoration of dynamic pressure / volume relationships of the lungs after synthetic surfactant instillation, to its prelavage condition (Ikegami et al 1977). Dynamic respiratory compliance changed very little after surfactant treatment and no significant difference in the time profiles for dynamic compliance measurements could be demonstrated between the LPM-1 and Exosurf-treated groups. Following surfactant instillation, we found no correlation between dynamic respiratory compliance and the rapid improvement in oxygenation. More specifically, in the absence of FRC measurements, we could not demonstrate any significant relationship between the change in respiratory compliance and change in a/A ratio in the present study (p = ns, data not shown). An explanation for the lack of finding a significant change in dynamic respiratory compliance may be that mechanical ventilation produces high trans-pulmonary pressures, and that animals may be 'operating' on the flat upper portion of their lung pressure-volume curves. If inflation pressure and tidal volumes, or PEEP are not adjusted after surfactant instillation, any possible changes in pressure-volume dynamics of the lungs may be masked (Davis et al 1988). Muscle paralysis (employed in the present study), during mechanical ventilation, is another factor that may lower lung compliance (Bhutani et al 1988).

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Analysis of lung washings (broncho-alveolar lavage) permits qualitative and quantitative assessment of lung surfactant and alveolar and airway cells. The most commonly employed method for obtaining surfactant entails washing the broncho-alveolar spaces with saline. Three to five lavages result in the removal of more than 90 % of the extracellular forms of lung surfactant and permits one to compare the surfactant derived from the lavage of a specific animal with that of the human lung surfactant composition (Sanders 1982). The procedure is semi-quantitative since all of the fluid used to lavage the lungs cannot be recovered and electron microscopy of lavaged lungs demonstrates retained morphological forms of myelin in the airways (Sanders 1982). Physical analysis of the isolated surfactant is difficult and chemical analysis beyond measuring phospholipids and fatty acids is not always possible. In mammalian species, the lavage fluid contains approximately 93% lipid and 7% protein. The major phospholipid in the human lung and other mammalian species is dipalmitoylphosphatidylcholine (DPPC). The trend for the rabbit to have a considerably higher *BAL-phosphatidylcholine content* (PC) (~ 86%) than the human (~ 62%) (Sanders 1982, Rooney et al 1975, Harwood 1975) was confirmed in the present study (PC ~ 70 %). In a number of mammalian species the PC content vary between 81% (dogs) – 87 % (rats). The PC content of *minced lung washings* in cats (86%) and rats (87%) was shown to differ significantly from that of humans (80%), whilst the PC content of dog-lungs (81%) and that isolated from rabbit lungs (83%) were similar to that of humans. For all species (dog, cat, human, ox, pig, rat, rabbit, sheep) examined, palmitic acid (16:0) is the major fatty acid (54-87%), implying that the majority of the PC molecules are DPPC (Sanders 1982, Shelley et al 1984). Rabbit and rat lung surfactant PC, isolated from *minced lung washings* does not differ from the

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human in palmitic acid content, but differ in the content of palmitoleic acid (16:1) and 18-carbon fatty acids (Shelley et al 1984). In agreement with the findings of the aforementioned studies, the baseline *BAL fluid* of the animals in the present study contained similar levels of C16:0, C16:1 and 18-carbon fatty acids to that described by others (Rooney et al 1975, Harwood et al 1975). In contrast to some previous studies, we describe higher levels for phosphatidyldimethylethanolamine (PEA) (Rooney et al 1975, Harwood et al 1975). The PEA concentrations of the present study were however, similar to that reported by Fujiwara and coworkers 1970). An explanation for the high levels of PEA, may be that the lipid identified as PEA, was in fact partly phosphatidylglycerol (PG)-a lipid whose accurate identification was precluded for technical reasons (Sanders 1982).

Tracheal aspirate L/S ratios have been used as an estimate of lung surfactant maturity and have been correlated with the presence of respiratory distress syndrome and subsequent outcome, in preterm newborn infants (Kanto et al 1976). A low tracheal aspirate L/S ratio (<10) correlates with both a low incidence of RDS, whilst there is a significant agreement between the L/S and the static lung compliance of newborn infants with and without RDS (de Winter et al 1995). In the present study, after surfactant instillation, the PC/SM ratio, a reflection of the lecithin / sphingomyelin (L/S), decreased significantly in the TRE-group between the first and final lavage, but remained statistically unchanged in the animals treated with LPM-1 or saline. The change in ratio was mainly accounted for by a decrease in BAL-fluid PC content together with a rise in SM content. Although the difference within groups was only significant in the TRE-treated animals in which the mean PC/SM ratio fell to below 10, the PC/SM ratio decreased to below 10 in the saline group, while in the LPM-1 group it

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remained above 10. In contrast to the finding of a good correlation between the tracheal L/S ratio and a/A ratio or static lung compliance, in human infants, we found a poor correlation between the BAL-derived PC/SM ratio and indices reflecting oxygenation status (a/A ratio, OI), as well as between the PC/SM ratio and dynamic respiratory compliance, at the time of the final lavage. It is known that alveolar lung proteinaceous oedema fluid inactivates surfactant *in vivo* and that much of the lung's hysteresis (volume changes lagging behind transpulmonary changes) is related to surface phenomena, i.e. surfactant behaviour (Mead et al 1957, Berry et al 1986). Since we noted the presence of proteinaceous oedema fluid, hyaline membranes and fluid on lung histology and electronmicroscopy, in the airways of the majority of animals in the present study, we speculate that there must have been a degree of inhibition of surface tension-lowering properties of the lung surfactant by soluble proteins, despite the documented sufficient amounts of 'normal' surfactant phospholipids.

Neither the *in vivo* effects, nor the surface behaviour of a mixture of TRE / DPPC, have until now been studied in a surfactant-deficient model. Theoretically, the addition of TRE to DPPC improves the spreading of the LPM-1 surfactant at the air-liquid interface and / or stabilizes the monolayer upon incorporation. We have shown that the instillation of LPM-1 (containing DPPC, hexadecanol, tyloxapol, and trehalose) resulted in the formation of a slightly different type of epithelial lining fluid after lavage, when compared to the prelavage composition. The most pronounced changes occurred within the fatty acids, whilst the phosphatidylcholine values changed little. Palmitic acid concentrations (C16:0) (major fatty acid in BAL fluid) increased significantly, suggesting enrichment of the epithelial lining fluid ('monolayer') after instillation of LPM-1. This increase

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in C16:0 was concurrent with significant decreases in the percentage C16:1, C18:0, and C18:2.

How the changed epithelial lining fluid behaved *ultrastructurally*, at the *in vivo* air-fluid interface, in the present study is unclear. For instance, during the respiratory cycle, compression results in enrichment of the surface film with DPPC through the "squeezing-out" of more fluid molecules from the monolayer. The "squeezing-out" hypothesis, described for DPPC-PG mixtures, is however, controversial. Some authors believe that unsaturated phospholipids ('fluid' molecules) are driven out of the monolayer during expiration (van Golde et al 1994), whilst others challenge this (Rana et al 1993). Utilising ^{31}P NMR spectroscopy and infrared spectroscopy, Rana and co-workers (1993), demonstrated a complete lack of any surface re-organization of the monolayer at high surface pressure, and therefore no evidence to support the selective squeeze-out of phospholipids from the monolayer. An alternative hypothesis to explain the manner in which the mixture of DPPC-TRE operates at the air-water interface after instillation, could be an increased suspension of the DPPC-TRE liposomes at body temperature. It is speculated that more unfolded or "open" microstructures could allow rapid adsorption to the air-water interface (Notter et al 1986). Somewhat more difficult to explain is the effect of the 'hydration' state of DPPC on it's *in vivo* surface behaviour when TRE is added. Would this status of DPPC slow down its spread or not? Our *in vitro* results indicate that DPPC in mixture with TRE, adsorbs very slowly (hours). The poor *in vitro* performance of the surfactants is however, at odds with the good clinical response observed in the present study for improvement in oxygenation and percentage shunt, after its instillation. The discrepancy between a synthetic formulation's *in vitro*

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performance and its effect, once instilled into the trachea of an infant with respiratory distress syndrome, have also been reported by other researchers (Jobe 1993, Scarpelli et al 1992, Llyod et al 1999, Hall et al 1992).

The most prominent light microscopy findings of the lungs of animals in the present study included general lymphatic dilatation, congestion and lung polymorphonuclear infiltration, with no difference between study groups. Hyaline membranes were present in all surfactant groups, but significantly more so in the saline treated group. A prominent feature of the saline-lavaged rabbit model appears to be that of *lung neutrophil infiltration* associated with neutrophil activation (Lachmann et al 1980, Mccullock et al 1988, Sugiura et al 1994). The capillary bed of the lung harbors a large pool of polymorphonuclear cells that may be significantly affected by mechanical ventilation (Tremblay and Slutsky 1997). Recently, van Eeden and coworkers (2000) have illustrated that saline-lavage per sé causes lung leucocyte sequestration (van Eeden et al 2000). This sequestration was already evident ~ 30-45 minutes after completion of the final lavage in animals that did not receive ongoing ventilatory support (van Eeden et al 2000). This early lung leucocyte accummulation (after warm saline lavage) is a potential pro-inflammatory process that may or may not be aggravated by certain ventilatory patterns of support (Mccullock et al 1988, Sugiura et al 1994, Degraeuwe et al 1999). These neutrophil changes may contribute to progressive deterioration of lung function and surfactant activity through lung inflammation and fluid/protein leakage into airways (Sugiura et al 1994, Imai et al 1999).

In our first *in vivo* study, the presence of neutrophils in the lung interstitium as well as alveoli, was a common finding in all of the study groups towards the end

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of the study protocol. A significant increase in the BAL-fluid neutrophil count occurred in all animals, concurrent with a significant decrease in the BAL macrophage count. In agreement with previous studies that used the same model as that of the present study, no significant change occurred in the peripheral neutrophil count during the 3-hour study, suggesting recruitment of neutrophils from storage pools (Sugiura et al 1994). Interestingly, treatment with synthetic surfactant (LPM-1) did not have a significant effect on modifying the inflammatory response, since we found no significant difference in the BAL-derived cell counts between the LPM-1 and- saline groups. The author could not find studies that have systematically assessed changes in lung parenchymal and airway inflammatory cell accummulation after exogenous surfactant replacement in the saline lavaged adult rabbit. In fact, few studies address the role of surfactant replacement on lung inflammatory cell changes, and those that do, do not always support their conclusions with qualitative data (Zhao et al 2000). The question whether surfactant replacement would influence lung inflammatory cell infiltration and activation after lavage requires further exploration. Recently, Suwabe et al (1998) showed that alveolar neutrophils from bleomycin-induced neutrophilic alveolitis in hamsters, exhibited a lower tendency for adherence, and produced less superoxide after treated with bovine surfactant, when compared to controls. In the same experiment, liposomes of DPPC, gave similar results, thus suggesting that it is the lipid component that is involved in the inhibitory mechanism. Other researchers (Hayakawa et al 1989) previously showed that natural rabbit surfactant inhibited the oxidative burst of alveolar macrophages in infant rabbits. It appears therefore, that while on the one hand surfactant attenuates the lung inflammatory response in a beneficial way, while on the other

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hand, these 'beneficial' effects may be offset by a negative effect on these cells to perform their bactericidal functions, therefore indirectly, predisposing to lung infection. The aforementioned studies were performed with artificial natural or animal-derived surfactants, and it remains to be shown whether synthetic, protein-free surfactant, would produce similar immunomodulatory results *in vivo* and / or *in vitro*. In group B streptocci-infected premature rabbits, animals that were treated with a synthetic, protein-free surfactant (Exosurf), experienced a significantly lower growth of the bacteria in comparison to animals treated with either minced bovine surfactant (Survanta), porcine-derived surfactant (Curosurf), controls or rabbit surfactant (Sherman et al 1994). Intrapulmonary phagocytosis of the bacteria was not altered by any of the surfactants.

Information on how *natural surfactant* replacement influences adult rabbit lung morphology is available. However, the author could not find any information in the English literature that specifically addresses the electron microscopic appearance of the lung epithelium of a lavaged rabbit model, *studied after saline lavage and treatment with synthetic surfactant*. Lachmann et al (1980) showed in adult guinea pigs and adult rabbits (Lachmann et al 1982) that repeated lavage preferentially causes desquamation of bronchial epithelium and that alveolar epithelium is relatively spared (*light microscopy*). In the same study, animals ventilated for 2 hours after the lavage had more evidene of alveolar damage, including edema and hyaline membranes. These authors attributed the alveolar damage to the mechanical ventilation, and not the lavage procedure per se.

Progressive ventilation-induced lung injury is known to occur within 4-hours after the initiation of conventional ventilation in a saline-lavaged, surfactant-deficient

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adult rabbit model (Hamilton et al 1983, Sugiura et al 1994, Makhoul et al 1995). In a study that closely resembles the lung injury as well as the subsequent course of our study, Van Eeden et al (2000), assessed lung changes by light microscopy and *electron microscopy* in saline lavaged non-ventilated, and ventilated adult rabbits. In non-ventilated control rabbits, light microscopy of the lungs revealed congested alveolar walls and sequestration of leucocytes after saline-lavage (Van Eeden et al 2000). In the same study, electron microscopy of the type 2 cells showed loose interaction with mesenchymal and type 1 cells, reduced microvilli, lamellar bodies in the process of exocytosis and type 2 cells with fewer surfactant-containing lamellar bodies. In addition, type 1 cells displayed features typical of cell damage (membrane blebs, cytoplasmic vesicles and increased electron density). Cell surface microvilli are reduced, mithochondria dilated, and type 2 cells dislodged from their basement membranes. These findings refute the idea that saline lavage is not overtly injurious to the alveolar epithelial cells. Moreover, when saline lavage was followed by a 4-hour 'conventional' ventilation period, the morphological changes became more prominent. Both the study of Makhoul and coworkers (Makhoul et al 1995) and the present study show that these changes are translated into a deterioration in lung compliance and arterial oxygenation if surfactant are not replaced after lavage. In the present study epithelial damage was a consistent finding in all groups. As was the case with the study of Van Eeden et al (2000), the damage was more evident by electron microscopy examination and included hydropic changes, most readily observed in the mitochondria. The airspaces of study subjects showed the presence of oedema fluid. This luminal oedema appeared to be more prominent in the control group and LPM-2 group. Organellar debris, probably originating from

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lysis of epithelial cells, was present, despite treatment with synthetic surfactant. The electron microscopical appearance of the epithelial-lined substance ("hyaline membranes") in the present study showed a marked variability within groups as well as within the same case. The majority of cases showed a mix of membrane types with both granular and fibrillar materials present within the same membrane. In some cases there were layering of the membranes into distinct bands. In the *in vitro* setting, similar microstructural features have been described for a mixture of synthetic phospholipids (DPPC: egg-PG) (Notter et al 1986). When the overall light microscopy and electron microscopy findings of the present study are summarized it is evident that the instillation of synthetic surfactants in the present study resulted in milder morphological disease in comparison to that of the control group

It is becoming increasingly clear that the lavage procedure per sé does have significant effects on the alveolar epithelium, involving type 1 and type 2 cells. Whether these ultrastructural changes are related to lavage volume, number of lavage procedures, lavage-fluid temperature, or time allowed for stabilization after lavage (for saline to be reabsorbed), is at this stage unclear. There is a possibility that certain co-existng factors, acting through synergistic mechanisms, may have influenced lung morphological changes and other findings in the present study. These factors include the lung lavage procedure, lung circulatory changes, neutrophil activation, mechanical ventilator-induced biotrauma and the effects of hyperoxia. One hundred percent oxygen was used to ventilate all animals, regardless of arterial PaO_2. Theoretically, all animals were therefore equally at risk of oxygen toxicity, albeit some for slightly longer periods than others. Although the present study was not designed to address all of these

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specific questions, one may nevertheless draw conclusions from the available literature. With regard to the relationship between the administration of O_2 (hyperoxia) and pulmonary oxygen toxicity, it is clear that alveolar epithelial damage and lung microvascular injury manifests with increased permeability to solute only after 24 hours, in *spontaneously breathing* adult New Zealand rabbits (Matalon and Egan 1981). Despite these changes in function, there appears to be a delay before one identifies histological and morphological changes in the alveolar epithelial and capillary endothelial cells. Hyperoxia-exposed rabbits are able to maintain normal acid-base balance and arterial partial pressure of O_2 values to shortly before death. They then die from severe arterial hypoxemia that develops abruptly after 70 hours in oxygen. Concomittant to the increased permeability in alveolar epithelium, inflammatory cells accumulate in the alveolar capillaries and lung interstitium after 48 hours in O_2 (Nickerson et al 1981). In accordance with other studies (van Eeden et al 2000, Imai et al 1994), we found an increase in neutrophils in the broncho-alveolar lavage fluid and lung interstitium in all animals within 3-hours after inducing acute lung injury, followed by mechanical ventilation. Narimanbekov and Rozycki (1995) showed that when lung injury in rabbits is induced by lavage, and the animals subsequently exposed to either normal ventilation with room air or hyperventilation with 100% oxygen for 8 hours, animals receiving hyperventilation / hyperoxia showed significantly higher histological injury scores in comparison to the control animals. Administering recombinant Interleukin-1receptor antagonist after lavage, but before hyperventilation / hyperoxia significantly lowered lung inflammatory cells, elastase, and leakage of albumin compared to hyperoxia / hyperventilation, in the same study. During hyperoxia-injury, significant alterations in lung mechanics

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(compliance) also occurs, but only after 24-36 hours, suggesting, at least in part, changes in surface tension, i.e. lung surfactant at the alveolar air-fluid interface (Holm and Notter 1986, Mikawa et al 1995). With the aforementioned literature in mind and the fact that our ventilation protocol lasted for 3 hours, one may cautiously conclude that hyperoxia probably contributed to lung inflammatory cell influx, and therefore indirectly to damaging effects of mediators released by activated neutrophils, but contributed little, if any to the *structural changes* that occurred in the present study. It appears that the most likely explanation for the documented structural lung changes and lung inflammation findings in our study could be related to the initial lavage procedure, followed by 'conventional' ventilation with normal tidal volume delivery.

The primary function of lung surfactant is to form monolayers at the alveolus air-water interface that are capable of lowering the normal surface tension (72 mN/m) to near zero (Longo et al 1993). After secretion into the alveolar space, surfactant adsorbs onto the alveolar air / liquid interface and lowers interfacial tension, thereby reducing the force tending to collapse alveolar units. Surface tension (γ) is a manifestation of surface energy (E) and can be equated under isothermal conditions. (Hills 1988). Both E and γ have the same dimensions. *E* is expressed in erg cm^{-1} and γ in dyne cm^{-1}. By definition, 1 erg is the work needed to move a force of 1 dyne by 1 cm, therefore, in SI system of units, 1 dyne / cm is 1 mN/m.

Many methods and 'surfactometers' are available to measure surface tension directly or indirectly. Some determine surfactant's static surface adsorption while others assess its effect on suface-tension behaviour during cyclic film compression. The ring detachment method of Du Noüy (Sekabunga et al 1969,

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Barrow and Hills 1979) measures a unique relationship at the critical point of detachment and surface tension as the peak pull per unit perimeter necessary to pull out the ring. In addition, it avoids contact-angle artefact, something that is a problem with the Wilhelmy balance studies. The maximum force (FR) needed to pull or 'pluck' the ring out of the surface, is related to (*L*), the total wetted perimeter (inside + outside) and surface tension (γ) as:

$F_R/L = \gamma$ (Barrow and Hills 1979)

As yet, no study has been reported on the *in vitro* surface activity of DPPC in a mixture with trehalose. Our *in vitro* study measured the biophysical activity of artificial surfactants containing 1,2-dipalmitoyl-*sn*-phosphatidylcholine (DPPC), with or without a non-reducing sugar (trehalose), and with or without hexadecanol, tyloxapol, and $CaCl_2$, by using the Du Noüy ring method. In addition, the surface tension-lowering features of DPPC in a mixture with various chemicals at body temperature and under hypothermic conditions were studied. The results of the study show that serial dilutions of DPPC in combination with the described chemicals lowered the equilibrium of surface tension in a concentration-dependent manner at both 20°C and 37°C. At both temperatures, surface films consisting only of synthetic DPPC or DPPC in combination with TRE showed markedly higher mean maximal surface tension values (65.6 and 70.4 dyn / cm, respectively) than those surface films consisting of mixtures of DPPC with other chemicals.

We found the surface tension of distilled water (without surfactant added), as measured with the ring method, to be 72.8 dyn / cm at 20ºC (Barrow and Hills 1979: 73.3 ± 0.2 dyn / cm:). In agreement with the findings of others, we found

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that an expanded film (a film that is allowed to spread) of DPPC-alone had little effect upon the surface tension of water, highlighting the lack of ideal surface properties of DPPC on its own (Barrow and Hills 1979). The addition of TRE to DPPC did not significantly influence surface properties of the mixture. Significantly lower surface tension values were obtained when tyloxapol and $CaCl_2$ were added to DPPC and TRE. Substituting TRE for hexadecanol (LPM-2 + $CaCl_2$), improved the surface tension lowering ability of the mixture in a stepwise manner. LPM-1 (on-site Exosurf) and LPM-2 performed similarly, reducing surface tension to between 20% and 30% in comparison to the mixture of DPPC + TRE. This is in accordance with the findings of Hall et al (1992), who reported that the adsorption of Exosurf was significantly better than DPPC alone (~ 45% reduction in surface tension after 20 min, in comparison to DPPC). Our findings show that the maximal surface tension is dependent on the concentration of surface-active molecules (DPPC in combination with other surface active substances), and is in agreement with that of others. This was true for the studies performed at 20°C and 37°C.

In trying to imitate the surface properties of natural surfactant, Grossmann and Larsson (1978) found high maximal surface tension values for synthetic DPPC (46.5 dyn / cm), utilizing the pulsating bubble technique. These authors were able to reduce minimum and maximum surface tension values when they supplemented DPPC with other synthetic phospholipids. Bangham et al (1979) and Morley et al (1978) added unsaturated phosphatidylglycerol to DPPC (3:7), and found that the surfactant monolayer rapidly spread to an equilibrium surface pressure of 42-47 mN / m, when placed on an aqueous surface at 37°C.

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Many factors influence the comparison of studies assessing *in vitro* surfactant activity. Variations in study designs, equipment utilized, different surfactants evaluated, varying concentrations of surfactant phospholipids, the presence or absence of surfactant apoproteins, the influence of electrolytes and varying ionic concentrations in sample buffers, and the pH-dependent activity of surfactant, complicate comparison between studies (Davies et al 1986, Efrati et al 1987, Amirkhanian and Merritt 1995). Furthermore, controversy with regard to the Wilhelmy surface balance and the Du Nuoy ring method exist. In their articles (Barrow and Hills 1979a, Barrow and Hills 1979b), the authors state that the Wilhelmy balance has been an unfortunate choice of instrument for studying DPPC films. Using DPPC films, the Du Noüy ring method gives higher readings than the Wilhelmy balance (Barrow and Hills 1979). As was shown by these authors, the surface tension, with the ring method, may be 50% greater than that recorded on the Wilhelmy balance (see discussion above). The difference was ascribed to the contact angle introduced by DPPC, which in turn, influences readings (registered as lower surface tension values) obtained by the Wilhelmy balance. In the absence of any surfactant, and in agreement with our findings, both methods give very similar readings for pure water (72 vs 73.2 dyn / cm) (Barrow and Hills 1979), i.e. there is no contact angle. Dynamic surface tension measurements are preferred over static measurements. It has been suggested thet the Du Noüy ring requires a large hypophase (> 50ml), that the ring-pull changes the surface area, and that leakage artifacts may influence readings (Notter and Finkelstein 1984). For various reasons, studies in which the effect of surfactant is determined on a 'static' surface area (maximal surface tension) may not be as informative as those that are performed under circumstances where the surface

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area is constantly changed in order to mimic spontaneous breathing activity. For instance, under static circumstances the very low minimum surface tension values (0-5 dyn / cm) (obtained by compressing the film or study-surface area to 80-85% of the trough area), that are usually required to define the *in vitro* effectiveness of a particular surfactant preparation, cannot be measured. At 100% of a trough area, a film of lung extracts of DPPC prepared from adult rabbit lung washings (Grossmann and Larsson 1978) has a surface tension of 25 dyn / cm (Galston and Shah 1966) and 27 dyn / cm (at 37°C) (DPPC concentration 8 mg/ml). Bernhard et al (2000) suggested that a good surfactant should result in an equilibrium surface tension of 25-28 mN/m after 10s adsorption, and a minimum surface tension of < 5 mN/m during cyclic compression (surfactant bubblemeter). Barrow and Hills (1979b) described surface tension values obtained by the ring method for various DPPC concentrations on an aqueous hypohase of Ringer solution as well as dog serum. When measured on Ringer solution at a 37.5% area change in DPPC films, DPPC lowers surface tension in a concentration dependent manner from 70.4 dyn / cm (at 0.08 μg DPPC cm^{-2}) to 38 dyn / cm (at 1.9 μg cm^{-2}) (Barrow and Hills 1979b). These values change with different surface areas. The dependency of the biophysical activity of surfactants on phopholipid and / or the presence of surfactant apoproteins, calcium concentrations in sample buffers, pH, and temperature, has been extensively studied (King et al 1981, Benson et al 1983, Holm et al 1996, Bernhard et al 2000, Palmblad et al 1999). For instance, the phospholipid concentration for natural surfactants (of porcine and bovine origin) has to be raised by 6-12 times, respectively, to 'match' the *in vitro* surface tension-lowering capabilities of native pocine or bovine surfactants (Bernhard et al 2000).

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The synthetic, protein-free, commercially available surfactant, Exosurf, only lowers surface tension to a minimum of 22 mN/m, when tested with a pulsating bubble surfactometer. Exosurf contains DPPC and additives, hexadecanol and tyloxapol, instead of the proteins found in natural surfactant. The tyloxapol and hexadecanol serves to enhance the preparation's interfacial adsorpsion characteristics and activity. In both adsorption and pulsating bubble experiments, the minimum surface tensions reported for Exosurf are almost identical to those obtained by tyloxapol alone (Hall et al 1992). It has been suggested that detergents such as tyloxapol could disrupt the structure of biological compounds in solution under *in vitro* conditions (as is seen with detergents in general), therefore limiting for instance Exosurf's ability to reach low surface tensions during compression (Hall et al 1992). Despite enriching Exosurf with nonphysiological calcium concentrations (> 2 mmol calcium chloride) or DPPC, poor surface activity is found (Bernhard et al 2000). The lowest minimum surface tension achieved for Exosurf was 49 mN/m at a concentration of 8 mg/ml. The same authors also showed that in the presence of surfactant protein (SP)-B / C and a physiological concentration of calcium, surface activity of surfactant formulations corresponds to the concentration of DPPC, i.e. the higher the DPPC concentration, the better the surface tension-lowering capacity is of the particular formulation. It should be noted that there remains a disparity between Exosurf's poor *in vitro* performance and studies of clinical benefit in premature infants with respiratory distress syndrome (Hall et al 1992, Soll and McQueen 1992). This disparity has been ascribed to factors unrelated to Exosurf's intrinsic properties (Scarpelli et al 1992), and may be due to its combination with endogenous surfactant proteins or incorporation into endogenous recycling pathways

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(Hall et al 1992). For instance, similar phospholipid compositions were found in the tracheal effluent of newborn infants over time after treatment with either a natural surfactant or Exosurf (Lloyd et al 1999). Using the captive bubble technique to analyze minimal surface tension of tracheal aspirates (effluents) from babies treated with Exosurf, showed reductions in minimum surface tension as soon as 12 hours after treatment (McMillan et al 1998). The delayed onset of action relative to what is found with natural surfactant is partly explained by the suggestion that Exosurf requires incorporation into the endogenous surfactant pathways before it can effectively function as a surfactant (Hall et al 1992).

By adding agents other than phospholipids or protein to DPPC, we were able to lower the maximal surface tension of a pure surface film of DPPC from ~65 dyn / cm to between 40 and 50 dyn / cm. This was achieved for films containing DPPC, hexadecanol, and tyloxapol (mimicking Exosurf), and those consisting of DPPC, hexadecanol, tyloxapol, and trehalose (LPM-1), as well as a mixture of DPPC, trehalose and $CaCl_2$. However, the demonstration of a reduced *in vitro* activity of LPM-1 and LPM-2, similarly to that described for Exosurf (Hall et al 1992), in comparison to that of natural surfactants, reflects the 'ineffectiveness' of additives such as: hexadecanol, tyoxapol, and trehalose, in substituting for hydrophobic surfactant proteins. Exosurf reaches minimum surface tension values only after 20 minutes of adsorption, whereas natural surfactants (Survanta and Infasurf; of calf lung origin) reach equilibrium surface tension values within 5 minutes (Hall et al 1992). When Exosurf is supplemented with 1% by weight purified bovine SP-B / C, the resultant formulation adsorps more rapidly and, in addition, lowers surface tension to very low levels (Hall et al 1992).

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In vitro temperature has significant effects on surfactant activity. Both in our own study, and in that of others, an increase in temperature to 37°C was associated with lower surface tension readings than at 20°C (Cary and Rideal 1925). The aforementioned authors described a linear decrease in surface tension with increasing temperature up to a point at which the crystalline acid melts. They found that the slope over a temperature range between 10°C and 70°C were the same for saturated and unsaturated acids, esters, phenols, ethers, and ureas. In general, at any given temperature, the shorter the chain, the lower the surface tension. When the temperature is raised above 42°C, the minimum surface tension rises above 15 dyn / cm. With cooling, the surface tension usually decreases.

A factor that complicates the interpretation of our *in vitro* data on the surface tension lowering ability of the various surfactant mixtures, are the fact that distilled water was used as hypophase. It is known that surface tension rises if surfactant extract is *prepared with* distilled water (Tierney and Johnson 1965) and that the surface properties of bovine lung-derived surfactant are *pH dependent in distilled water* (Davies et al 1986). To improve the performance of the surfactant in the lastmentioned study, the authors had to raise the pH of the solution, as well as the sodium chloride concentration. In another *in vitro* study, alkalinization of the subphase (pH > 7.4) significantly decreased the surface tension lowering of ability of Survanta (bovine lung-derived surfactant containing SP-B), but to a lesser degree than that observed for the protein-free synthetic surfactant (Exosurf) (Amirkhanian and Merritt 1995).

Optimal adsorption of lipid extract surfactant requires the presence of calcium ions in the subphase since divalent cations are known to increase the speed of

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surface adsorption and to stabilize surfactant films (Bernhard 2000, Benson et al 1984, Kobayashi et al 1983). Calcium ions, and to a lesser degree, magnesium ions, induce structural changes in lung surfactant as observed by electron microscopy (Benson et al 1984, Efrati et al 1987, Ridsdale et al 1999). The addition of calcium ions to surfactant formulations devoid of calcium causes tubular myelin formation, whereas removal of calcium ions by chelation with EDTA, reverses the changes. In the absence of calcium ions, the surfactant morphology includes vesicular and lamellar structures, similarly to what we found (Fig 3.6.6, 3.6.7). It has been shown that calcium-induced structural changes occur in SP-A. The change is related to the formation of a "closed-bouquet" conformation of the molecule's stem (Palaniyar et al 1998). This change is suggested to result in SP-A's headgroups interacting with the phospholipids in lung surfactant. One may speculate that the process in which *hydrophilic* surfactant protein (SP-A), cations and lipids interact is an 'active' process. Oelberg and Xu (2000) recently showed that, apart from lowering surface tension, lung surfactants, especially the *hydrophobic surfactant proteins* (SP-B / C) in intact surfactant, induce channel-mediated transport in artificial membranes. In combination with SP-B and calcium, SP-A changes multilamellar structures into tube-like bilayer structures called tubular myelin (Williams et al 1991). We speculate that the absence of tubular myelin from electron microscopy findings in the present study may partly be credited to the absence of calcium from the surfactant formulations tested *in vivo*. The effect of calcium in formulation with phospholipids has not been studied in the living animal or human.

Recently, Taeusch and coworkers (1999) investigated the *in vitro* behaviour of a number of simple sugars (5-10% solutions of glucose, mannose, galactose,

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maltose), polyethylene glycols, dextrans, and polyvinylpyrrolidones of various molecular weights dextrose, *mixed with a natural surfactant* (Survanta) in the presence of known inhibitors of surfactant function. In this study, the polymers in 1-10% concentrations enhanced the ability of the surfactant to lower surface tension in the presence of meconium, serum and lysophosphatidylcholine. As was acknowledged by the authors, the study was limited to one means of assessing surface activity of surfactant mixtures (pulsating bubble surfactant meter) and did not assess effects in lungs of living animals, or effects of limiting conditions, such as mixing, time, or pH. In another study, Kobayashi et al (1999), have reported similar findings using dextran with lipid-extract porcine surfactant. These authors reported enhanced adsorption rates and increased lung compliance of surfactant mixtures containing dextran in a premature rabbit model.

The feasibility of animal models in studying exogenous surfactant replacement requires further elucidation. A large number of animal models relevant for neonatal RDS are available and many studies have reaffirmed the usefulness of the lavaged adult rabbit lung model for testing the early effects of different surfactant preparations (Kresch et al 1996, Notter and Shapiro 1987). The adult rabbit model, subjected to bronchoalveolar lavage, is considered by some to be an'intermediate' model of surfactant deficiency. 'Intermediate', since the most relevant animal model for surfactant deficiency is regarded to be premature animals *in vivo* (Kresch et al 1996, Notter and Shapiro 1987). The advantage of *in vivo* lavaged lung models over excised lavaged lung models are that these studies are performed over hours, thus enabling the researcher to more accurately asses the physiological effects of exogenous replacement therapy. Following the *in vivo* lavage procedure, the lungs of animals exhibit physiological, functional

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and morphological surfactant-deficiency characteristics, i.e. a decrease in compliance and functional residual capacity, a drop in arterial oxygen partial pressure, and neutrophil influx, atelectasis, and hyaline membrane formation, as well as increased permeability measurements at the alveolar-capillary membrane level (Lewis and Jobe 1993, John et al 1997). The disadvantage for both the *ex vivo* and *in vivo* adult model is the use of mature animals. Theoretically, these animal's lungs may retain the capacity for surfactant regeneration over a study period, i.e. shorten the time course of recovery (Kresch et al 1996, Sandhar et al 1988). As was the case in the present study, the saline-lavaged adult rabbit appears to be a hemodynamically stable model. However, the subject of pulmonary vascular reactivity in this model requires further research.

In summary, supplementing a surfactant with recombinant surfactant protein A and / or SP-B / C is an expensive and daunting task in the absence of adequate resources and specialized laboratories. Because of limited resources, many newborn infants throughout the world are not only denied access to life-saving intensive care units, but also the benefit of exogenous surfactant replacement therapy. These infants could benefit from redirected care if simple non-invasive breathing support combined with an affordable, effective, but safe surfactant could be provided. Unfortunately, no such lung surfactant has emerged on the 'market' over the last 14 years. Easily formulated, inexpensive, effective and safe surfactants should be developed to treat surfactant deficient conditions in developing and poor countries. The issue remains that unmodified DPPC lacks the adsorption and re-spreading capabilities of an effective surfactant. Therefore, supplementing DPPC with an agent that has the ability to improve the surface activity of DPPC remains a goal. The goal of the present study was an attempt to

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develop such a surfactant, composed only of synthetic components that could be used for treating neonatal RDS, and possibly, ARDS. The surfactant formulation (LPM-1) included DPPC and a sugar, TRE, together with other spreading agents. The *in vivo* and *in vitro* performance of the LPM-1 was compared to that of a commercially available synthetic surfactant (Exosurf), a locally prepared 'Exosurf' (LPM-2), and saline. Too this end, we showed i*n vivo* clinical equivalent efficacy for LPM-1 and Exosurf, over and above that for LPM-2 (on-site Exosurf) and the saline treated group, in an adult surfactant-deficient animal model. However, the addition of TRE to the DPPC-hexadecanol-tyloxapol mixture failed to raise the *in vivo* activity of LPM-1 above that of Exosurf. Too address this and other remaining issues were beyond the scope of the present study. Future research priorities relating to LPM-1 could include efforts to improve the methodology involved in the preparation of the product, *in vitro* assessment of its surface behaviour under dynamic conditions, and the evaluation of LPM-1 in the treatment of RDS in a premature animal model. Studying surfactant under dynamic *in vitro* circumstances makes sense since physiological conditions such as body temperature, humidity and cycling rates equivalent to that of the human body can be simulated.

New insights into how different exogenous surfactants formulations may interact with animal models representing the immature developing lung, or the more matured, but injured lung, could yield formulations for treating specific lung conditions. For instance, the majority of preterm infants with RDS benefit from treatment with either natural or - synthetic postnatal surfactant. However, despite surfactant replacement therapy, ~ 30% or more of surviving infants of less than 1200 g birth weight or with gestations below 30 wk, develop a form of chronic

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obstructive lung disease (Stevenson et al 1998). Chronic lung disease (CLD) remains an important cause of mortality and morbidity (Schwartz et al 1994, Kinali et al 1999). There is increasing evidence that inflammation plays an important role in the pathogenesis of CLD (Jobe and Bancalari 2001). Future research could determine the possible 'protective' role of LPM-1 in lung white blood cell migration and activation and thus the ensuing inflammatory cascade associated with mechanical ventilation and oxygen therapy.

Another area of future research is the hypothermic preservation of lungs for transplantation. Improvement in lung preservation methods may prevent the post transplant impairment in lung surfactant function. The re-implantation response is a form of acute lung injury and deterioration in surfactant composition and / or function occurs after lung transplantation (Hohlfeld JM et al 1998). In rat lungs the major change, after 20 h of storage, appears to be in the SP-A (hydrophilic glycoprotein) concentration (Erasmus et al 1997). In the human, persistent impairment of surfactant biophysical properties (higher surface tension, increased small aggregates to larger aggregate ratios) after lung transplantation have been related to type 2 cell malfunction (Hohlfeld et al 1998). Moreover exogenous natural surfactant replacement therapy has been shown to improve lung function before reperfusion in a rat lung transplantation model (Boglioni et al 2001). The role of LPM-1 in graft storage / preservation in lung transplantation therefore needs to be investigated since TRE has been successfully used for canine lung graft storage (Bando et al 1994).

Through the incorporation of TRE into LPM-1, DPPC gained the unique characteristic of a lowered transition temperature through spacing of the acyl

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chains (Lee et al 1986). This feature, together with a possible thermoprotective role against lung injury could be assessed under hypothermic *in vivo* conditions. For instance, the role of LPM-1 as replacement therapy in ARDS, developing after drowning or near drowning, could be explored. LPM-1 may also prove to be a useful adjunct to endogenous lung surfactant during the treatment of experimental asphyxia neonatorum with hypothermia.

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APPENDIX A

Methodology:

a) Calibration of pressure transducers

Blood pressure: calibrated at the start of the study against a pressure monitor, in mmHg.

Ventilator, volume and compliance measurements:

In the first study, volume was derived (read-off) from the screen on the Dräger ventilator. This ventilator goes through an internal calibration mode when the ventilator is activated. Unfortunately, these displayed volumes were not cross-checked with a standardized volume-syringe. Compliance measurements were calibrated by using fixed ventilator settings (40 bpm, I:E ratio 1: 1.5, tidal volume 10 ml/kg) to ventilate a lung model, made from copper and filled with copper wool for isothermic gas compression. The model has a compliance of 2.3 ml/cmH2O. Ventilator pressure measurements were assessed against a water column. These calibrations were performed before the start of the study and were not repeated thereafter. Volume measurements were not routinely checked. The calibration of the Bicore CP-100, used in the second study is explained on page 55 of the thesis.

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b) Reproducibility of findings

The issue whether findings observed in living organisms can be reproduced with a high degree of accuracy, at least in the same species, is important. Since individual animals of the same species, as well as their physiological responses, do vary, reproducibility depends on how effective and meticulous the studied phenomenon is 'isolated' from other, i.e. environmental influences. In the present thesis the author aimed to minimize the effect of 'outside' influences by standardizing or controlling experiments and conditions as far as was possible. Animals were kept comfortable in species-adequate housing prior to their enrolment into the study. Once enrolled, and anaesthetized, ventilation parameters were kept constant throughout the study to ensure comparable lung ventilation. Hemodynamic variables, including mean blood pressure and the rectal temperature were maintained within acceptable ranges, as far as was possible. Lastly, we used a relative 'pure' line of animals, the New Zealand white rabbit, in an attempt to exclude inter-specie variability. The control of these factors allowed us to minimize physiological variation between individual animals.

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c) Breaths sampled during mechanical ventilation:

In the first study, *one* numerical value for volume and the difference between Peak inspiratory pressure (PIP) and PEEP were read-off the ventilator at the indicated sampling points (page 53). During the second study, the values (volumes, flow and compliance) determined by the Bicore CP-100 device represented the mean of 10 breaths.

d) Agreement between histologists

Two pathologists scored the light microscopy slides for each criterion on every section, i.e. the presence hyaline membranes, neutrophil infiltration, congestion, etc. Their findings were compared and in cases (slides) where there was poor correlation, a final score for the specific criterion was assigned by consensus. No formal inter-observer analysis was performed. According to the pathologists, a lack of agreement occurred in less than 10% of the total assessed slides.

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APPENDIX B

Initial determination of study size:

A pilot study was performed prior to the execution of the Thesis' randomized trial in order to determine a sample size. This pilot study (95 / 102) was completed during 1996. The experiment was designed to assess differences in the efficacy of a mixture of 'in-house' Exosurf and air as placebo. Surfactant deficiency was induced by repeated lung lavage and 2 groups of rabbits (n =4 per group) randomized to either surfactant or air-placebo. From this pilot study it was determined that a minimum of 5 rabbits should be included per group to detect a mean difference of 3 kPa in PaO2 between the placebo group and surfactant group, before and after treatment (alpha 0.05, power 0.80). By using the above-mentioned data the chance that a type 2 error would have significantly influenced findings, was minimized. In addition, the aim of the thesis was to test a **single** hypothesis, thereby probably contributing to a smaller (minimum) number of animals to be included.

Since a small number of animals were studied per group, the question remains whether differences in response to an effective therapy have been missed due to a type 2 error. According to the Statistician (Prof JS Maritz), a type 2 error cannot in the strictest sense be prevented. However, the probability of a type 2 error can be controlled by using

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larger sample sizes. Since a small study sample was used, nonparametric methods were used to ensure validity of the statistical analyses so far as the Type 1 error is concerned. This aided with the issue of Type 2 errors, because nonparametric methods are not so dependent on distributional assumptions.

A retrospective power analysis:

If we now use the PaO2 obtained 15 minutes after the instillation of LPM-1 and Exosurf, and then retrospectively determine a sample size at an alpha level of 0.05 and with a power of 0.8, the sample size to detect a significant difference in PaO2, 15 min after the instillation of surfactant, is 141 rabbits per group.