FORMULATION OF A PHYSIOLOGICALLY ACTIVE SYNTHETIC LUNG SURFACTANT WITH PROMISING THERAPEUTIC POTENTIAL IN HUMANS

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"Specially-manipulated" mixture of phospholipids (PL): dipalmitoylphosphatidylcholine (DPPC), phosphatidylglycerol (PG), and palmitic acid (PA) in appropriate ratios produced a synthetic lung surfactant, that possessed almost all the biophysical and physiological qualities of a native surfactant when tested in surfactant-depleted rats. On the basis of these findings it is proposed that further studies are needed to elucidate the chemical nature of the final products resulted from these mixtures. The absence of surfactant-related proteins (SP-A, SP-B, SP-C, SP-D) of bovine origin, make this mixture hypoallergenic, although the presence of some surfactant peptides have been shown to confer resistance to inactivation of surfactant function by serum factors, or oxygen radicals. As demonstrated previously, the addition of lysophosphatidylcholine (LPC) in minute quantity to the PL improved its biophysical properties significantly. It is conjectured that possible conversion of phospholipids to minute amounts of LPC during special processing resulted improvements in biophysical properties of the mixture. The immediate objective is to show the proportion of LPC formation, if any, in relation to other phospholipids in the final product which has has been shown to have interesting physiological properties. By utilizing the protective effects of liposome-encapsulated glutathione (GSH) in PL, the toxic effects of endogenous oxygen radicals or oxygen toxicity will be minimized further during treatment of premature infants, ARDS patients, or humans exposed to poisonous gases. Final improvements made in surfactant function of PL, and evaluation of its positive therapeutic effects in anesthetized-paralyzed, surfactant-depleted rats or primates, will qualify this product for clinical use.

BACKGROUND AND INTRODUCTION

Historical outline: Since the publications of Avery and Mead, Avery, on the surface properties of lung surfactant in relation to hyaline membrane disease [1,2], the main focus of investigators in RDS was on surfactant replacement

therapy [3-11]. After three decades of active research for understanding the biophysical, biochemical and physiological properties of lung surfactant system, now it seems possible that the surfactant deficient condition can be cured by a synthetic or a semisynthetic surfactants [5-11].

The Hayaline Membrane Disease (HMD) is a respiratory distress syndrome (RDS) ocurring at birth in some premature infants, and is the major cause of neonatal mortality and morbitity. Its pathological cause is linked with a deficiency in pulmonary surfactant, which acts as tensioactive material at the air-alveolar interface. The alveoli are stabilized at the air-water interface, and during expiration physiologically surfactant behaves as anti-atelectiasis and anti-edema agent. During inspiration, the poor surface tension allows not an easy distention of lungs, thus ventilator compliance becomes high. Therefore, the multiple atelectiasis and alveolar edema, secondary to surfactant deficiency in HMD infants considerably reduces the gas exchange. Treatments under high pressure of artificial ventilation, and intensive oxygen administration, instead of compensating these abnormalities, add complications due to production of reactive oxygen species (ROS).

After the successful trial of infants with RDS with exogenous bovine surfactant by Fujiwara, et al. [5], surfactant replacement therapy in neonatal RDS has been applied with different synthetic or semi-synthetic surfactants. Although statistical analyses have shown that the nations infant mortality has declined by about 6% in 1990, positive response to surfactant treatment is only 70% within 12-48 hours of treatment. The remaining 30% exhibit transient or no response, and usually progress towards chronic lung diseases or inflammation, in the form of bronchopulmonary displasia (BPD). The main cause of treatment failure is thought to be due to surfactant inactivation by leakage of plasma factors across damaged endothelium and epithelium into air spaces of the alveoli [12–14,26–28], possibly allergic reactions, or inflammations due to oxygen radicals[34]. Yet, the possible long term post-treatment failure due to the adverse effects of different components of surfactant proteins of bovine origin, has not been investigated thoroughly.

During the last decades, there has been a great emphasis on the role of surfactant-related proteins (SP-A, SP-B, SP-C, SP-D) for surfactant function: the rate of absorption and spreading of phospholipids at the air-liquid interface [15–19]; surfactant lipids without surfactant proteins adsorb poorly, or proteins confer a kind of resistance to inactivation by serum proteins or oxygen toxicity [22, 27].

EXPERIMENTAL DESIGN

Methods and material: DPPC, PC, PA, and LPC are purchased from Sigma Chemical Co. and solvents from Merck Chemical Co.

Chemical analyses: Liposome preparations containing DPPC, PC, PA (50*mg/ml*) in different ratios (w/w) were dispensed by an adaptation of modified technique of Tanaka, et al. [20]. Peroxidation index measurements were calculated from the absorbency ratio at 233*nm* and 215*nm* [35]. Liquid chro-

matography of DSPC, DPPC, etc. were assayed by reverse phase liquid chromatography using modification of reported methods [36]. Thin layer chromatography (TLC) was used for determination of LPC. palmitoyllysophosphatidylcholine (PLPC), stereoyllysophosphatidylcholine (SLPC) 2.5*mg/ml* chloroform and liposome were spotted (10*μl*) on a silica TLC plate (Kieselgel GF 254) and developed for 15*cm* with a mixture of chloroform-methanol-acetic acid-water (100:60:25:10, *v/v*). Samples were visualized with molybdenum blue [37].

Biophysical: Pulsating surfactometer was used at 37°C, using 2.5 *mg/ml* mixture of surfactant in saline, according to Enhorning [33].

Animal model assay: Response to treatment with different surfactant mixtures of anesthetized, paralysed rats, depleted of lung surfactant by bronchoalveolar lavage with 37°C normal saline were performed. The blood gases, blood pressure, and airway pressures were measured, while animals were breathing 100% oxygen [19].

TYPES OF SURFACTANTS USED CLINICALLY

At least eight types of surfactants are used in clinical trials around the world; two surfactant products, Exosurf Neonatal (Burroughs Wellcome Co.), and Survanta, (Abbott Laboratories) are currently marketed in the USA and other countries. Exosurf, is a synthetic protein-free mixture of DPPC, Hexadecanol (a 16-carbon alcohol), and tyloxapol (a polymer of a common laboratory detergent). Survanta is a modified formulation of the original Japanese bovine formulation of Fujiwara marketed in Japan as Surfacten (Tokyo Tanabe, Co.) with the name of TA surfactant in the USA. Both products are produced from organic extracts of minced cow lungs enriched with DPPC, palmitic acid and tripalmitin, they contain two of four surfactant proteins (SP-B and SP-C) essintial for optimal surfactant function.

Curosurf (Chiesi Farmaceutici, Parma, Italy, is a surfactant isolated from pig's lungs.

Surfactant containing all proteins are isolated from human amniotic fluids.

SLSE or Infasurf (Forest Labs. New York) Calf lung surfactant extract, Bovine surfactant.

ALEC (Artificial lung expanding compound) produced by a group in Great Britain) is a preparation containing only phosphatidylcholine and phosphatidylglycerol.

RATIONALE

Although there is ample scientific evidence in favour of superiority of surfactants containing surfactant-related proteins than those without proteins, physicians prefer to use products without foreign proteins. Synthetic surfactants that can mimic the biophysical properties of natural surfactant, but do not have the potential hazards of substances derived from animal sources with possible microbial or viral contaminants, naturally will be considered safer for therapy and are more affordable. Results from animal studies indicate neither

semisynthetic surfactant, Survanta (with SP-B and SP-C proteins) nor Exosurf (a protein-free surfactant) are as optimal as native surfactant [30,31]. Results from human studies show that almost 30% do not respond to surfactant treatment, and there is not enough knowledge of possible adverse effects of protein containing products, and there hase been no long term follow up studies during the post-treatment period. Thus, improvement in formulations and studies on possibly masked allergic reactions to protein-containing surfactants remains an important issue for investigation [23,25].

Although the main research on surfactant replacement models during the past decade has been the formulation and surfactant inactivation mechanisms, presently we are seriously concerned in developing a protein free surfactant. On the basis of experimental results of the present *in vitro* and *in vivo* studies of the protein-free mixture of surfactant PL, we have come to the conclusion that the choice of the particular phospholipids, with special processing techniques are important factors in the formulation of a safe and physiologically active surfactant. Thus, the immediate objective is to elucidate the chemical nature in the final product resulted from "special processing" of the mixture that has almost similar properties of the native surfactant.

THE ROLE OF PHOSPHOLIPID METABOLISM IN SURFACTANTS

PC remodeling mechanisms and interconversion of PC to LPC by possible hydrolysis like mechanisms *in vivo* or *in vitro* : The major components of lung surfactant are phospholipids, which account for 80-90% of surfactant by weight. Phosphatidylcholine (PC) is by far the most prevalent, accounting for 70-80% phospholipids. An unusually high percentage (60-70% of PC) is disaturated for the most part with dipalmitoylphospha-tidylcholine (DPPC), and contains palmitic acid (PA) as both of its fatty acids. Therefore DPPC must be responsible for the ability of surfactant to lower surface tension at air/liquid interface.

Experimentally induced septic shock in rats has been related to pulmonary disorders associated with altered phospholipid metabolism [32]. As a result of septic shock the ratio between PC and LPC is lowered both in lung tissues and in pulmonary surfactant; thus the average value of PC and LPC in normal lung tissue is (PC=50, LPC=2), and in surfactant (PC=78, LPC=0.5), when compared to septic shock lung tissues (PC=43, LPC=4). The surfactant from the septic shock-induced animals is (PC=67, LPC=3), seems to differ significantly [32]. Furthermore, the palmitic acid content of lung is significantly reduced and replaced mainly by arachidonic acid during shock, and consequently lead to the generation of potent mediators such as prostaglandins, thromboxane or slow reacting substances with the presence of reactive oxygen species (ROS).

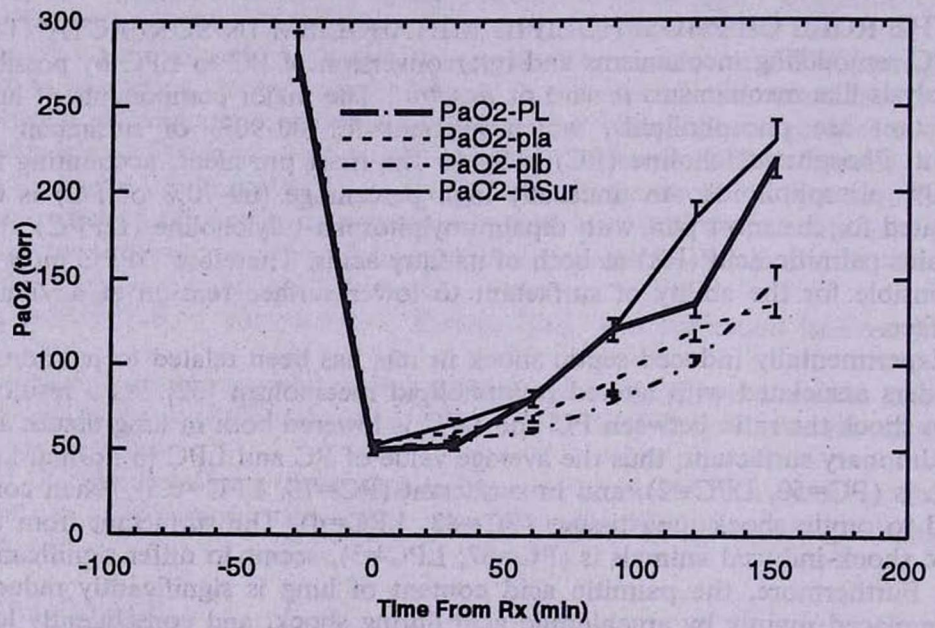
Lysophospholipids formed through the hydrolysis of parent major phospholipids by phospholipase A_2 are naturally occurring amphiphiles [32,32a,39]. Phospholipase A_2 takes part in a number of physiologically important cellular processes: inflammation, blood platelet aggregation and acute hypersensitivity.

these processes are initiated by the release of arachidonic acid from cell membranes which is catalysed by intracellular phospholipases. Because of their strong cytolytic properties, tissue levels of lysophospholipids are rigidly controlled in animals. Thus LPC is a catabolite of PC and an intermediate in one pathway of surfactant synthesis, and is the principal lysophospholipid identified in lungs, comprising only a small fraction of total phospholipids [32, 38,39]. It has also been demonstrated that most of the instilled LPC rapidly partitions into lipid fraction of the lung, and the principal metabolic fate of the labeled acyl group is incorporated into PC. It is possible that PC might be formed through direct reaction of the instilled LPC with endogenous acyl-CoA, a reaction catalyzed by LPC-Acyltransferase [39].

Therefore, it could be concluded from the above findings that LPC in minute quantities exerts improving effects on surfactant function of the phospholipid mixtures, as documented by in vitro studies (Fig. 1).

FIG. 1

Rats: Lipids with and without peptides



Generally, serum factors are shown to have inactivating effects on surfactants by increasing minimum surface tension of mixtures significantly, measured by pulsating bubble surfactometer. Yet the presence of surfactant-related proteins confer some kind of resistance to inactivation by serum [26,27,29]. The preliminary in vitro studies with "specially treated lipids" and lipids with

minute amount of LPC especially in the presence of <0.5 mM $CaCl_2$, have shown that they are more tolerant to inactivating effects of serum factors than the control mixtures.

RESULTS

Comparative biophysical studies, and blood oxygenation in surfactant-depleted rats, by instillation of mixtures of PL, pla (phospholipids containing SP-B and SP-C extract of bovine origin), plb (phospholipids with a segment of synthetic SP-B), and preparation of rabbit surfactant (RSur), the PL mixture proved to be almost as good as native RSur (FIG.1). Similarly, blood pressure, and airway pressure curves returned to almost normal levels after 200 minutes treatment of surfactant-depleted rats with LP mixture compared to other surfactants containing fragments of SP-B or native surfactant respectively (Fig. 2 and 3). Graphic presentation of sequential average arterial PaO_2, blood pressure (BP), and airway pressure measurements of total of 12 rats in each case were studied. The anesthetized and paralyzed rats were studied after depleting of surfactant by washing lungs with normal saline as reported earlier [19]. The measurements of PaO_2, BP and airway pressure (AirWP) were plotted against time in minutes in relationship to treatment (Rx) with that of rabbit surfactant.

FIG. 2

Rats: Lipids with and without peptides

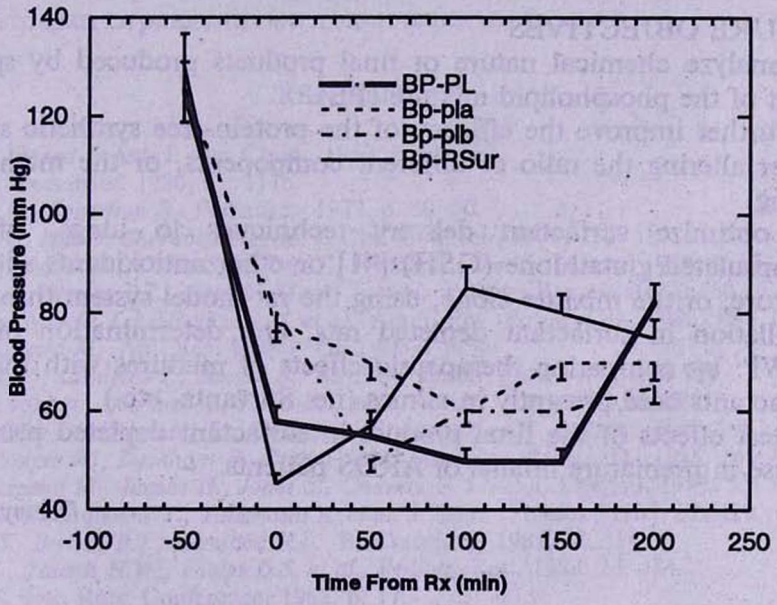
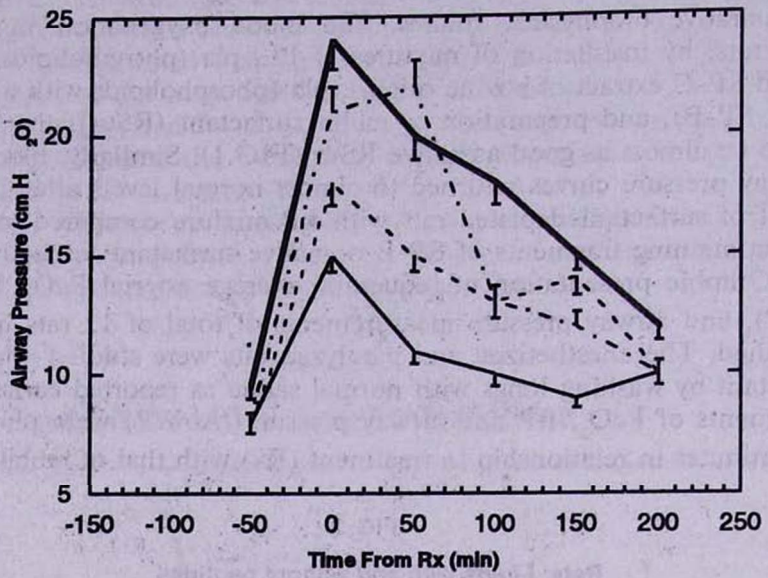


FIG. 3

Rats: Lipids with and without peptides



FUTURE OBJECTIVES

1. To analyze chemical nature of final products produced by special treatment of the phospholipid mixture (PL).
2. To further improve the efficacy of the protein-free synthetic surfactant by either altering the ratio of different components, or the method of processing.
3. To optimize surfactant delivery techniques to lungs, of liposome-encapsulated glutathione (GSH) [41] or other antioxidants with surfactant mixture, or the mixture alone, using the rat model system through tracheal instillation in surfactant depleted rats, and determination of paO_2, BP, AirWP: by comparing therapeutic effects of mixtures with that of known surfactants used presently in clinics (i.e. Survanta, etc.).
4. To test effects of the final product in surfactant depleted primates before its use in premature infants or ARDS patients.

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ԹԵՐԱՊԵՎՏԻԿ ԱԶԴԵՑՈՒԹՅՈՒՆ ՈՒՆԵՑՈՂ ՖԻԶԻՈԼՈԳԻԱՊԵՍ ԱԿՏԻՎ ՍԻՆԹԵՏԻԿ ԹՈՔԱՅԻՆ ՍՈՒՐՖԱԿՏԱՆՏ

Ջ.Դ. Ամիրխանյան

Հաստատվել է, որ ֆոսֆոլիպիդներ դիպալմիտոիլֆոսֆատիդիլխոլինի, ֆոսֆատիդիլխոլինի և պալմիտինային թթվի հատուկ եղանակով ստացված խառնուրդից պատրաստված սինթետիկ թոքային սուրֆակտանտը օժտված է բնական սուրֆակտանտի գրեթե բոլոր կենսաֆիզիկական և ֆիզոլոգիական հատկություններով: Հետագա հետազոտությունները՝ նրա տոքսիկ հատկությունների նվազեցման ուղությամբ թույլ կտան կիրառել այն կլինիկական պայմաններում անհաս նորածինների, շնչառական սուր դիստրեսով և գազային թունավորումներով հիվանդների բուժման ժամանակ:

ФИЗИОЛОГИЧЕСКИ АКТИВНЫЙ СИНТЕТИЧЕСКИЙ ЛЕГОЧНЫЙ СУРФАКТАНТ С ТЕРАПЕВТИЧЕСКИМ ДЕЙСТВИЕМ

Дж.Д.Амирханян

Представлен синтетический легочный сурфактант из смеси фосфолипидов – дипалмитоилфосфатидилхолина, фосфатидилхолина и пальмитиновой кислоты, обладающий почти всеми биофизическими и физиологическими качествами нативного сурфактанта.

Дальнейшие исследования позволят снизить его токсичность, что даст возможность применять его в клинических условиях при лечении недоношенных новорожденных, больных с острым респираторным дистрессом и острыми отравлениями токсическими газами.

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