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Development of low cost pulmonary surfactants composed of a mixture of lipids or lipids–peptides using higher aliphatic alcohol or soy lecithin

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ABSTRACT

The artificial pulmonary surfactant composition in the present study is characterized by a lipid mixture system composed of higher aliphatic alcohol, egg yolk phosphatidylcholine (egg PC), soy lecithin and higher aliphatic acid as the major components or a peptide–lipid mixture system composed of a combination of the lipid mixture system to which a peptide is added. Three peptides with amphiphilic surface-staying, membrane spanning, and both properties were designed and synthesized. The evaluation of pulmonary surfactant assay was performed by a hysteresis curve drawn upon the measurement for the surface tension–area curve with the Wilhelmy surface tensometer in vitro and the recovery of lung compliance for the pulmonary surfactant-deficient rat models in vivo. Lipid-mixture systems composed of octadecanol or soy lecithins containing no peptide were favorable hysteresis curves as compared with commercially available Surfacten®, but were not prominent. The peptide–lipid mixture systems composed of a combination of the lipid mixture of alkyl alcohol or soy lecithin to which peptides designed were added were desirable hysteresis curves similar to Surfacten® and amphiphilic Hel 13-5 peptide–lipids mixture systems were much more effective than the lipid mixture system. Particularly, the recovery of lung compliance treated with hydrogenated soy lecithin–fractionated soy lecithin PC70–palmitic acid–peptide Hel 13-5 (40:40:17.5:2.5, w/w) was comparable to that with Surfacten®. Because the artificial pulmonary surfactant compositions of this study can be prepared at lower costs, they are useful for the treatment of respiratory distress syndrome and acute respiratory distress syndrome as well as for inflammatory pulmonary diseases, dyspnea caused by asthma, etc.

1. Introduction

The pulmonary surfactant is a lipid–protein complex produced by the alveolar cells and secreted there from, which is a substance essential for the maintenance of life and which plays a role for the pulmonary function by reducing the surface tension of the alveoli [1]. Fujuwara et al. have reported about effect of the artificial pulmonary surfactant on human respiratory distress syndrome (RDS) in the newborn infant and succeeded for the first time in the world in the development of an agent for curing RDS ("Surfacten®") [2], which is prepared by extracting the active pulmonary surfactant components from the bovine lung. The incidence of acute respiratory distress syndrome (ARDS) in adults is also reported that the administration of large amounts of the artificial pulmonary surfactant at the early stage of ARDS can improve the pulmonary functions and minimizing the damages of the lung reducing the mortality up to 20% [3].

It is also known that the pulmonary surfactant substance is secreted from the bronchus, and the substance is considered to play a role as an expectorant by preventing the block of the peripheral airway. The pulmonary surfactant is expected to apply to various diseases that require improvements in respiratory disorders

Abbreviations: ARDS, acute respiratory distress syndrome; DPPC, dipalmitoyl-L-α-phosphatidylcholine; egg PC, egg yolk phosphatidylcholine; egg PG, egg yolk phosphatidylglycerol; OD, *n*-1-octadecanol; PA, palmitic acid; PE, phosphatidylethanolamine; PI, phosphatidylinositol; RDS, respiratory distress syndrome; SP-B and C, surfactant protein B and C.

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Hel 13-5 : NH_2-KLLKLLLKLWLKLLKLLL-COOH

P_{24} : AcNH-$K_2GL_{24}K_2A$-$CONH_2$

Hel 7-11-P_{24} : AcNH-KKLKKLLKKWKKLLKKLKG$_3$$K_2L_{24}K_2$ACONH$_2$

KL4 : NH_2-KLLLLKLLLLKLLLLKLLLLK-COOH

Fig. 1. Primary structure of various synthetic pulmonary surfactant peptides.

because, for example, the inhalation of the pulmonary surfactant can relieve a fit of allergy-induced asthma [4,5]. Thus, there is an increasing necessity of the pulmonary surfactant for the application not only to RDS and ARDS but also to inflammatory pulmonary diseases.

From the above, many medical doctors involved in the treatment of respiratory diseases have pointed out the possibilities of application of the pulmonary surfactant to many different kinds of pulmonary diseases; however, the pulmonary surfactant is very expensive that the application of Surfacten® to RDS only is currently covered by the health insurance in Japan. A further issue is that Surfacten® is a bovine protein preparation so that it still has the possibility of infection due to its antigenicity and unknown antigenicity and the problem with bovine spongiform encephalopathy (BSE) for example still remains unsolved. In this sense, the development of an artificial pulmonary surfactant that can be prepared at lower costs and has no side effects has been demanded.

Relating to this, the artificial synthetic surfactant (non-animal-derived pulmonary surfactant) has been also developed [6], which includes two categories: one is protein (or peptide)-free synthetic surfactants (Exosurf®, etc.) and the other is protein (peptide)-containing synthetic surfactants [7]. The peptide-containing surfactant that is currently under clinically available is a lipid–peptide complex (Surfaxin®) composed of 21 amino acids (KL4) [8,9]. More specifically, it is composed of phospholipids dipalmitoyl-L-α-phosphatidylcholine (DPPC) and PG, a synthetic peptide KL4 having a particular amino sequence, and an aliphatic acid [10]. However, because these surfactants are still expensive, the application to various lung diseases is limited.

It is needed to form a stable single molecular membrane and a bimolecular membrane in order to consider the mechanism of transcription of the pulmonary surfactant activity. Further, in order to prevent a collapse of the lung at the time of the compression of the lung, the presence of the phospholipids [11], particularly DPPC, is considered to be essential. Further, a small amount of PG is also considered to be need. However, the conventional artificial pulmonary surfactant composition using DPPC and PG has the demerits that the scope of application is limited because DPPC and PG are so expensive that the resulting artificial pulmonary surfactant composition results in an expensive one. Therefore, we considered in the present study the uses of an alternative material for the DPPC and PG. As an alternative material of PC, a phospholipid such as, for example, egg yolk phosphatidylcholine (egg PC) extracted and purified from relatively less expensive egg yolk lecithin or soy lecithin. Fractional soy lecithin from crude soy oils is commercially available at lower costs. As an alternative material to be used for the DPPC having a saturated alkyl chain, there may be used, for example, a saturated higher alcohol (e.g., *n*-1-octadecanol, OD) and hydrogenated lecithin prepared by hydrogenating the unsaturated aliphatic chain of the fractional soy lecithin. As the acidic component, there may be used a higher aliphatic acid (e.g., palmitic acid (PA), stearic acid, etc.) which has been frequently used as the lipid for the conventional artificial pulmonary surfactant. In addition, a neutral lipid (e.g., triacylglycerol, cholesterol (Ch), etc.) may also be used.

On the other hand, the proteins contained in the artificial pulmonary surfactant composition are considered to play a catalyzing action in order to permit the phospholipids to smoothly achieve the transduction between the single molecular membrane and the bimolecular membrane [12]. Systematic review of clinical trial has also shown that non-protein-containing synthetic surfactants are less effective than animal-derived or peptide-containing products. Therefore the peptides are added to the above-mentioned lipid mixture system as needed. As preferred examples of the artificial pulmonary surfactant peptides to be used for the present study are shown in Fig. 1.

The artificial pulmonary surfactant compositions according to the present study were prepared by admixing the above lipids with each other or the above lipids with the peptide or peptides at a predetermined rate. The artificial pulmonary surfactant compositions were assessed for their surfactant activity by using a surface tension–area diagram. For comparative purposes, the measurements were conducted as a control for Exosurf® composed of the lipid system only and containing no peptide, which is to be used as a medicine for treating respiratory disorders, Surfaxin® containing DPPC as a major ingredient and a synthetic peptide consisting of lysine (K) and leucine (L), and Surfacten®.

2. Materials and methods

2.1. Materials

As materials there were used the following: egg PC, a phospholipid purified from egg yolk (Avanti Polar Lipids, Inc.); egg PG (Sigma); egg yolk lecithin; and other lipids as well as reagents (Wako Pure Chemical Industries, Japan). Soy lecithin: hydrogenated soy lecithin ("SLP White H"), fractionated soy lecithin 70 (fractional lecithin SLP-PC70), hydrogenated soy lecithin 70 H (prepared by hydrogenating soy lecithin SLP-PC70) were purchased from True Lecithin Mfg. Co., Ltd. (Mie, Japan). The peptides were synthesized using an automatic synthesizer in the manner as described in literature [13].

As a control, Surfacten® was purchased from Mitsubishi Pharma Corp. (Tokyo, Japan). The lipids or lipid–peptide mixtures corresponding to Exosurf® and Surfaxin® were prepared each in our laboratory to yield a DPPC–1-hexadecanol–tyloxapol (84:16:0.25, w/w) system and a DPPC–egg PG–PA–KL4 (75:25:10:3, w/w) system, respectively.

2.2. Preparation of samples (lipid or peptide–lipid mixture)

The following synthetic peptide, lipid, aliphatic acid and alcohol were weighed each at a predetermined amount and dissolved in a chloroform/methanol solution. The peptide was weighed and added as needed to the above lipid mixture sample so as to give an appropriate concentration (w/w). Nitrogen gas was then blown into the above lipid mixture solution or the peptide-containing mixture solution and the resulting solution was dried under reduced pressure to evaporate the organic solvents thoroughly and form a film coating on the wall surface of the vessel. To the vessel was added physiological saline, and the solution was stirred to give a suspension of the film coating. The suspension was sonicated by the water-bath type sonicater and lyophilized. The

foam obtained was suspended again in physiological saline. The suspended solution obtained was used as a sample, since the pulmonary surfactant-deficient animals (rats) were not suffocated directly by administration of the solution. As a control, Surfacten® was used by suspending 120 mg in 4 ml of physiological saline in accordance with its manual for application to the living body.

2.3. Processes for formation experiments of surface tension–area diagram

The surface tension was measured with Acoma Wilhelmy Balance (Acoma Medical Industry Co., Ltd., Japan). A Teflon® water vessel (78 mm × 138 mm × 30 mm) was filled with physiological saline forming a closed liquid surface. The air–liquid interface of the liquid surface was developed with 20 μl of the lipid mixture and allowed to stand for 3 min to permit the lipid mixture to naturally spread thereon. A variation in the surface tension during this period of time was recorded as a surface-spreading rate with a platinum plate hanging vertically in the vessel. The single molecular membrane formed in 3 min was recorded as a surface area repeatedly spreading and compressing alternately at the speed of 3 min per cycle in the range from the maximum area of 45 cm^2 to the minimum area of 9 cm^2. The surface tension acting onto the platinum plate was converted into electrical signals with a force converter, and the electrical signals were automatically recorded continually with an X–Y recorder, together with the variation in the surface area. The recording was continued until no variation could be recognized any longer. The figures in the drawing are represented on the basis of the seventh cycle.

2.4. Pulmonary surfactant-deficient animal models

Adult Wister rats each having a body weight of approximately 500 g were anesthetized by injecting pentobarbital, 60 mg/kg, intraperitoneally. After tracheotomy, animals were supported on a pressure limited ventilator (Bear Cub Infant Ventilator, Bear Medical Systems, Riverside, CA) with a initial setting of frequency, 40 breaths/min, inspiratory time 0.5 s, positive end-expiratory pressure (PEEP) 4 cm H_2O, FiO_2 1.0. Throughout the experiment peak inspiratory pressure (PIP) was adjusted to maintain a tidal volume 9 ml/kg in each subject. After initial compliance of respiratory system measurement, the lung of each rat was lavaged with warm physiological saline to form a pulmonary surfactant-deficient model. Compliance (Crs) of respiratory system was calculated using the formula: Crs = (tidal volume)/(PIP − PEEP). As controls, we used three pulmonary surfactants: Surfacten® derived from the bovine pulmonary surfactant and extensively applied clinically, Surfaxin® and Exosurf®, and one (physiological saline, 2 ml) with no pulmonary surfactant administered. The method for the suspension and the amount of administration of each surfactant composition were followed in accordance with the method for the administration of Surfacten® to the living body. A suspension of 30 mg/ml was prepared by adding physiological saline, and 1 ml of the suspension was instilled via the tracheotomy. As a parameter for the effect of surfactant, recovery ratio of Crs was used. Crs was measured before lung lavaged, 30 min after lung lavaged but immediately before instillation of the surfactant. After that Crs were measured just after the surfactant treatment, 30, 60, 90, 120, 150 and 180 min. The lung lavage was carried out until a compliance of approximately 0.2 ml/cm H_2O to confirm the formation of pulmonary surfactant deficient model (Crs was approximately 0.6 ml/cm H_2O before the lung lavaged) After confirmation, the tests for treatments of various surfactants were then conducted by instillation each surfactant to a group of six or more rat models. After that recovery ratio of Crs was obtained using the formula: (recovery ratio of Crs) = (Crs after surfactant treatment)/(Crs after lung lavaged but before surfactant treatment).

Statistical analyses were done using Tukey–Kremer method and *p*-values of less than 0.05 were considered to indicate a statistically significant difference.

3. Results

3.1. Designed peptides

The pulmonary surfactant is composed of a complex of lipids and proteins. The proteins comprise four kinds of surfactant proteins (SPs), i.e., SP-A, SP-B, SP-C and SP-D, which amount to approximately 5% of the total weight [12]. The pulmonary surfactant proteins, SP-B and SP-C, which are of significance to the pulmonary surfactant activity, are different from each other in the mode of action to the membrane [12,14]. The surfactant protein SP-B lies on the membrane surface by partially penetrating hydrophobic parts while the surfactant protein SP-C is present penetrating through the membrane (transmembrane). The both surfactant proteins are considered to catalyze a transduction between the monolayer membrane and the bilayer membrane, which may occur on the air–liquid interface of the lung.

We have previously reported Hel series peptides, which are composed of 18 residues of Leu and Lys and have systematically varied hydrophobic–hydrophilic balance [13,15]. Among them, we chose an amphipathic α-helical peptide, Hel 13-5, composed of a lowly hydrophilic portion and a highly hydrophobic portion as the type, which stays on the membrane surface by penetrating hydrophobic portion into membrane (Fig. 1). As the type that penetrates through the membrane, we designed and synthesized a peptide, P_{24}, which composed 24 hydrophobic Leu residues with 2 hydrophilic Lys residues at the both N- and C-terminals and has enough length to span lipid bilayer [16]. We also designed and synthesized a peptides Hel 7-11-P_{24} having both properties staying at membrane surface (Hel 7-11) [13] and spanning through the membrane (P_{24}) [16]. On circular dichroism (CD) studies, peptides Hel 13-5 and P_{24} took α-helical structure in the presence of neutral and acidic lipid [13,16]. The peptide Hel 7-11-P_{24} also took highly α-helical structure in the presence of neutral (egg PC) and acidic {egg PC–egg PG (3:1)} liposomes (data not shown).

3.2. Surface tension–area diagrams

The surface tension of the pulmonary surfactant is characterized by a hysteresis curve drawn upon the measurement for the surface tension–area curve with the Wilhelmy surface tensometer [17]. Generally speaking, it is considered that a better pulmonary surfactant activity is achieved as the speed of a decrease of the surface tension at the time of compression is faster or as the capability of automatically spreading the surface tension is faster. In other words, the pulmonary surfactant activity is considered to be better as the area forming a hysteresis curve is greater or as a surface tension at the time of compression is smaller. As shown in Fig. 2, the minimal surface tension of Surfacten® at the time of compression was found to be 7 mN m^{-1} or the maximal surface tension thereof at the time of each spreading was found to be 45 mN m^{-1}.

Fig. 2a and b shows the results of the surface tension-area curves for the artificial pulmonary surfactant compositions having different kinds of lipid formulations as compared with Surfacten® as a control. As shown in Fig. 2a, no better hysteresis curves were obtained for the two-component systems: OD–PA (85:15), egg PC–PA (85:15) and OD–egg PC (40:60). A three-component composition, i.e., OD–egg PC–PA (35:40:25), gave a desirable hysteresis curve although its minimal surface tension (13 mN m^{-1}) is not com-

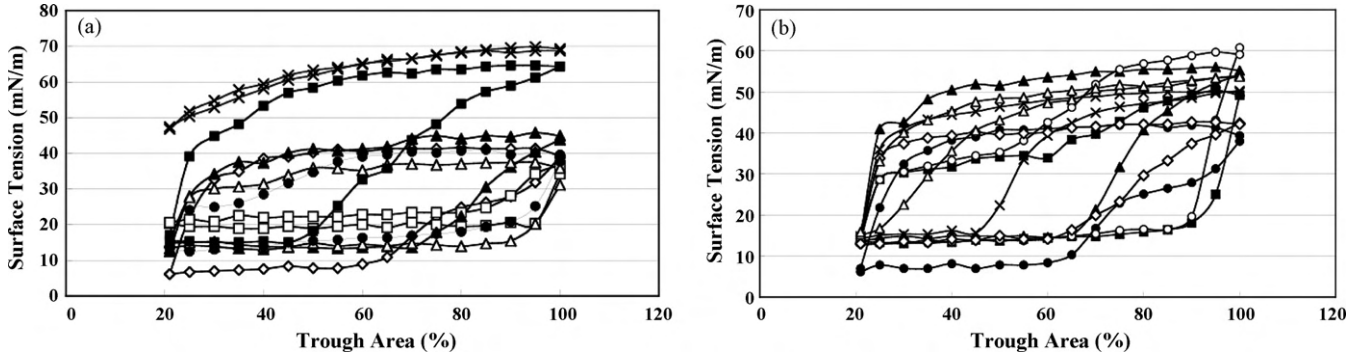

Fig. 2. The surface tension–area curves for the artificial pulmonary surfactant compositions having different kinds of lipids (a) and for the different composition ratios of OD–egg PC–PA mixture (b). (a) Surfacten® (◊); OD–PA (85:25, w/w) (□); OD–egg PC–PA (35:40:25) (▲); OD–egg yolk lecithin–PA (45:40:25) (△); egg PC–PA (85:15) (×); OD–egg PC–PA–Ch (30:35:25:10) (○); OD–egg PC–PA–TG (25:40:25:10) (■). (b) Surfacten® (●); OD–egg PC–PA (70:10:20) (■); (40:40:20) (▲); (10:70:20) (△); (80:10:10) (○); (40:50:10) (◊); and (10:80:10) (×).

parable to that of Surfacten®. No improvements were shown by the addition of cholesterol and saturated triacylglycerol (TG) to the OD–egg PC–PA composition or its mixture thereof.

As a good result was obtained for the OD–egg PC–PA composition, further experiments were conducted for the systems in which the compositions were modified (Fig. 2b). The systems where OD and egg PC were contained in more amounts gave no better results than Surfacten®. On the other hand, the systems that contained OD and egg PC at the amounts closer thereto showed good results. It was also found that the OD–egg PC–PA (40:40:20) system containing PA by 20% gave a surface tension pressure value at the time of expanding greater than the OD–egg PC–PA (40:50:10) system containing 10% of PA, resulting in a greater area drawn by the hysteresis curve.

Fig. 3 shows the results of lipid–peptide mixture systems. Better curves were generally obtained for the OD–egg PC–PA systems in which various peptides were added. For example, little difference was shown among peptides Hel 7-11-P_{24}, Hel 13-5 and KL4 contained in Surfaxin®. In the case of P_{24}, the surface tension increase in spreading process is slower than the other peptides, indicating that membrane spanning region alone in surfactant peptides (or proteins) is not enough to draw the preferable hysteresis curve.

Fig. 4 shows the results of the soy lecithin–lipid mixture system and the peptide mixture system thereof as well as Exosurf® and Surfaxin® as controls. It is interesting to note that the soy lipid mixture system (hydrogenated soy lecithin–fractionated soy lecithin 70–PA (40:40:20)) gave a favorable hysteresis curve as the OD–egg PC–PA system. It is also interesting to note that the system in which the peptide was added to a hydrogenated soy lecithin–fractionated soy lecithin 70–PA mixture system had a somewhat weaker spreading force at the time of expanding, but a decrease of the surface tension at the time of compressing was better than that of Surfacten®. It should be noted that the soy lecithin–lipid mixture system and the peptide mixture system containing hydrogenated fractionated soy lecithin 70H instead of hydrogenated soy lecithin were less favorable hysteresis curves than those containing hydrogenated soy lecithin. Neither Exosurf® nor Surfaxin® gave any curve like Surfacten®.

As noted above, some lipid mixture systems gave a hysteresis curve desirable as a pulmonary surfactant similar to that of Surfacten®. This reveals that the lipid mixture system alone can have a sufficiently high pulmonary surfactant activity even if no peptide or protein is contained. Among them, certain systems were found that the minimal value of the surfactant activity at the time of compressing was somewhat higher than Surfacten®, however, a more rapid decrease in the surface tension than Surfacten® was recognized.

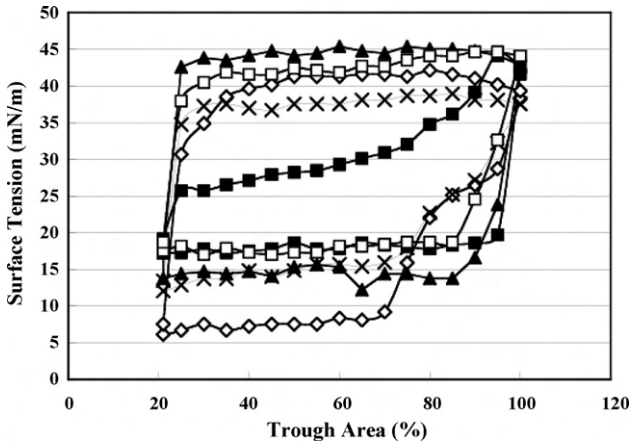

Fig. 3. The surface tension and the surface area curves in various lipid–peptide mixture systems. Surfacten® (◊); OD–egg PC–PA–P_{24} (35:40:20:5) (■); OD–egg PC–PA–Hel 7-11-P_{24} (35:40:20:5) (▲); OD–egg PC–PA–Hel 13-5 (35:40:20:5) (×); OD–egg PC–PA–KL4 (35:40:20:5) (□).

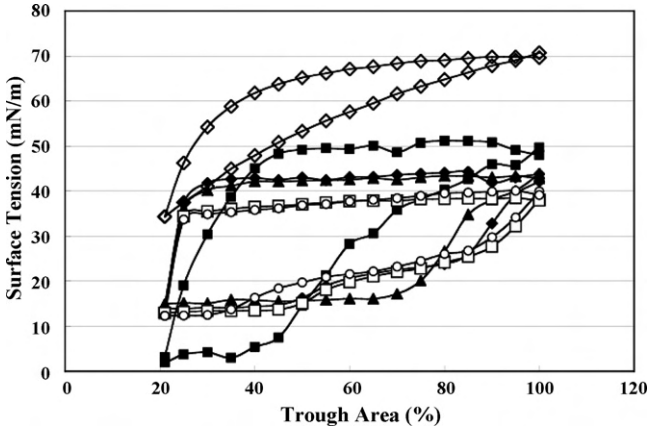

Fig. 4. The surface tension and the surface area curves in a soy lecithin–lipid mixture system and its peptide mixture system. Exosurf® (◊); Surfaxin® (■); hydrogenated soy lecithin–fractionated soy lecithin 70–PA (40:40:20) (♦); hydrogenated soy lecithin 70 H–fractionated soy lecithin 70–PA (40:40:20) (▲); hydrogenated soy lecithin–fractionated soy lecithin 70–Hel 13-5 (40:40:17.5:2.5) (○); hydrogenated soy lecithin PC70 H–fractionated soy lecithin 70–PA–Hel 13-5 (40:40:17.5:2.5) (□).

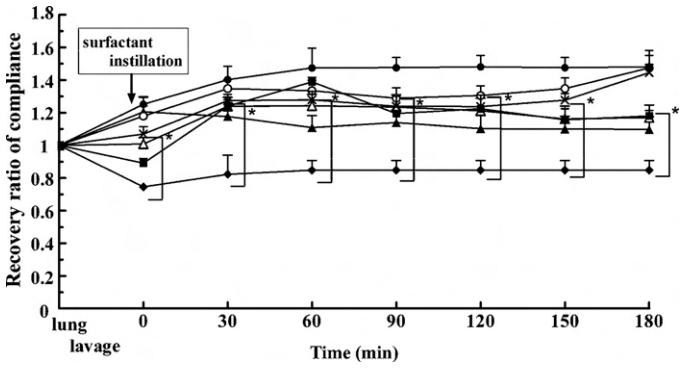

Fig. 5. Serial measurements of recovery ratio of compliance (Crs) of respiratory system are shown. Recovery ratio of Crs was calculated using the formula: (recovery ratio of Crs) = (Crs after surfactant treatment)/(Crs after lung lavage but before surfactant treatment). Where Crs was obtained using the formula: Crs = (tidal volume)/(PIP − PEEP).The data represents the mean ± S.D. $^{*}p < 0.05$ vs. Surfactant treatment group. Surfacten® (●); Exosurf® (♦); Surfaxin® (■); OD–egg PC–PA (35:40:25) (▲); hydrogenated soy lecithin–fractionated soy lecithin 70 (40:40:20) (△); OD–egg PC–PA–Hel 13-5 (35:40:20:5) (×); hydrogenated soy lecithin–fractionated soy lecithin 70–Hel 13-5 (40:40:17.5:2.5) (◯).

3.3. Recovery of the pulmonary function in surfactant-deficient animal models

A review has been made for the effects on the pulmonary functions using a pulmonary surfactant-deficient rat model formed by lung lavage of a rat weighing approximately 500 g with warm physiological saline [18]. The artificial pulmonary surfactant of the present study was instillation and measured for the prolongation of life and pulmonary compliance. Seven kinds of the pulmonary surfactant compositions were used for these experiments as follows: peptide-containing pulmonary surfactant systems (OD–egg PC–PA–peptide Hel 13-5 (35:40:20:5) and hydrogenated soy lecithin–soy lecithin PC70–PA–peptide Hel 13-5 (40:40:17.5:2.5)); pulmonary surfactant systems containing no peptide (OD–egg PC–PA (35:40:25) and hydrogenated soy lecithin–fractionated soy lecithin PC70–PA (40:40:20); Surfacten®; Surfaxin®; and Exosurf®. As a control in which no pulmonary surfactant was contained, 2 ml of physiological saline was used (Fig. 5).

It is to be noted herein, however, that the lung lavage caused to reduce the pulmonary compliance of all the rats used therefore to approximately 0.2 ml/cm H_2O. Further, it is noted that the pulmonary compliance of the rats in the group with no surfactant administered was continually reduced after lung lavage resulting to death of all rats within 1 h after administration.

For the rats in the group where Surfacten® containing the pulmonary surfactant protein was instilled, the recovery ratio of Crs just after the treatment elevated to 1.25 ± 0.041 (mean, S.D.), 30 min after the instillation and to 1.40 ± 0.081 thereafter, followed by maintaining the recovery ratio of Crs approximately 1.47 till 3 h when the experiment was finished. For the rats in the group where the pulmonary surfactant composition containing a peptide according to this study (hydrogenated soy lecithin–fractionated soy lecithin PC70–PA–peptide Hel 13-5 (40:40:17.5:2.5) was administered, recover ratio of Crs increased to 1.18 ± 0.062 immediately after administration, to 1.35 ± 0.055 within 30 min thereafter and to 1.33 ± 0.064 within 60 min thereafter, followed by reducing down to 1.29 ± 0.064 within 90 min and increasing again to 1.47 ± 0.11 in 3 h when the experiment was finished. It can further be noted that the recovery ratio of Crs in 150 min immediately after administration was recovered to some extent to a level somewhat poorer than Surfacten®, but recovered to a level equal thereto in 180 min thereafter. A comparison with Surfaxin® revealed that the artificial pulmonary surfactant composition of this study demonstrated better pulmonary compliances during all the measured period of time. The OD–egg PC–PA–Hel 13-5 composition demonstrated a somewhat weaker recovery of the pulmonary compliance as compared to the soy lipid system, but a better recovery than Surfaxin®.

Regarding the systems in which no peptide was contained, Exosurf® decreased the recovery ratio of Crs to 0.75 ± 0.018 immediately after its administration and sustained it at 0.82 ± 0.12 in 30 min thereafter and at 0.85 ± 0.060 in 60 min at sequential thereafter (Fig. 5). On the other hand, the OD–egg PC–PA composition of this study did not cause a decrease of the recovery ratio of Crs 1.20 ± 0.093 immediately after its administration, decreased to 1.18 ± 0.064 in 30 min thereafter and then reducing in 60 min, followed by increasing in 90 min and maintaining the compliance at approximately 1.10 till 3 h when the experiment was finished. It can be further noted that the pulmonary surfactant composition of this study which does not contain any peptide demonstrated better results than Exosurf® containing no peptide during all the measurement period of time and, further, that the recovery ratio of Crs in 3 h after administration was 0.85 ± 0.060. The system composed of the hydrogenated soy lecithin–soy lecithin PC70–PA demonstrated similar results.

From the results of the tests as described above in which the pulmonary surfactant-deficient rat models were used, the soy lipid–peptide mixture system showed somewhat weaker effects on improvements of the pulmonary functions for 150 min immediately after administration, but demonstrated the pulmonary functions as high as Surfacten® containing natural apoproteins derived from the bovine pulmonary surfactants in 180 min after administration. Furthermore, the test results showed better functions than Surfaxin® containing the synthetic peptide during the whole period of experimental time. In addition, the soy lipid mixture system containing no peptide demonstrated better functions than Exosurf® for the whole period of experimental time.

4. Discussion

The pulmonary surfactant present in the alveoli is composed of approximately 10% of proteins and lipid components consisting mainly of phospholipids [11,19]. The pulmonary surfactant contains the lipids, particularly a neutral phospholipid, i.e., PC which amounts to 80.5% of the total lipids; while PG which is an acidic lipid amounts to 9.1%, phosphatidylinositol (PI) to 2.6% and cholesterol to 7.3% [11]. In particular, it is to be noted that DPPC composed of saturated alkyl groups amounts to 47.7%, that is, approximately a half of the PC, and that this is considered to be a factor of preventing a collapse of the lung. It is rendered evident, however, that only the phospholipids including DPPC are not the major surfactant species in all animals [20]. In the present studies, we showed a mixture lipids of OD, egg PC, and PA can be applicable enough to the desirable hysteresis curve similar to Surfacten®. This indicates that saturated alcohol may be applicable as an artificial pulmonary surfactant material instead of DPPC. The recovery of the compliance of OD–egg PC–PA for the pulmonary surfactant-deficient rat models, was not prominent as compared to Surfacten®, which was firstly a little increase and then kept till 3 h. However, its recovery effect is better than Exosurf®. It is noted that same synthetic surfactant containing no protein as Pumactant® (Britania Pharmaceuticals Ltd., Redhill, UK), recently removed from the market, is composed of *n*-hexadecanol and DPPC.

It is interesting to note that the soy lipid mixture system (hydrogenated soy lecithin–fractionated soy lecithin 70–PA (40:40:20)) also gave a favorable hysteresis curve and moderate recovery of compliance for the pulmonary surfactant-deficient rat models. Hydrogenated soy lecithin used in the present studies is composed

of PC (~30 mol%), phosphatidylethanolamine (PE) (~25 mol%), PI (~15 mol%) and PA (~10 mol%) and fractionated soy lecithin is composed of PC (~70 mol%), PE (~13 mol%), PI (trace) and PA (~2 mol%) [21]. It is also reported that alkyl chains of phospholipids of soy lecithin are mainly C18 (~80%) [22]. These results indicate that stearoyl group in hydrogenated soy lecithin may work instead of palmitoyl group of DPPC. Although acidic PG was not included in soy lecithin, a relatively high amount of acidic PI may compensate it.

The peptide–lipid mixture systems composed of a combination of the lipid mixture of alkyl alcohol or soy lecithin to which peptides designed are added were desirable hysteresis curves similar to Surfacten®. Little difference hysteresis curves were generally obtained for the OD–egg PC–PA systems. In the case of KL4 contained in Surfaxin® was obtained the results similar to the other peptides. These results indicate that structural difference between membrane-spanning and surface-staying peptides is not important to show desirable hysteresis curves similar to Surfacten®. Appropriate hydrophobicity to penetrate lipid membranes might play an important factor in basic peptides. Thus we selected Hel 13-5 as a surfactant peptide, because it is more easily synthesized and purified than the other peptides.

Moreover, Hel 13-5-lipids mixture systems were much more effective than the lipid mixture system on the recovery of respiratory function for the pulmonary surfactant-deficient rat models. Particularly, the recovery of Crs of hydrogenated soy lecithin–fractionated soy lecithin PC70–PA–peptide Hel 13-5 (40:40:17.5:2.5) was comparable to the Surfacten®. The recovery ratio of Crs of OD–egg PC–PA–peptide Hel 13-5 (35:40:20:5) are better than lipid mixture system containing no peptide, but less than the soy lecithins–peptide system. These results indicate that amphiphilic peptides are important to show high activity of pulmonary surfactant. The phospholipid compositions of soy lecithin are also important because they contain various phospholipid species similar to natural source-artificial pulmonary surfactants.

As the artificial pulmonary surfactant compositions according to this study can be prepared at very lower costs as compared with Surfacten®, it can be applied not only to RDS and ARDS, but also to mitigating or preventing severe respiratory distress at the terminal stage of pulmonary cancers, etc., removing phlegm, relieving the symptom of respiratory difficulties caused by asthma and so on. Further, as the artificial pulmonary surfactant compositions of this study are composed of a completely man-made pulmonary surfactant that does not use any protein derived from a living thing such as the bovine lung, etc., as a raw material, it can be relieved from the problems with unknown infection, BSE, allergy, etc. In addition, the lipids to be used for the artificial pulmonary surfactant compositions according to this study are all authorized to be used for use as a medicine. Therefore, it is considered that no problem with toxicity will be caused to occur. From the above findings, the artificial pulmonary surfactant compositions of this study can be expected to be applying as a medicine for treating a wide variety of pulmonary diseases. As the artificial pulmonary surfactant composition according to the present studies can be prepared at lower costs, it has the possibility that it can be applied to a wide variety of diseases involving a surfactant deficiency or functional insufficiency, etc., that is, not only to RDS and ARDS, but also to the mitigation and prevention of disorders of respiratory functions caused by acute pneumonia, pulmonary cancers, asthma and so on as well as to the removal of phlegm caused by pneumonia, a cold and so on.

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