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In Vitro Effect of Meconium on the Physical Surface Properties and Morphology of Exogenous Pulmonary Surfactant

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The pathophysiology of meconium aspiration syndrome(MAS) is related to mechanical obstruction of the ariways and to chemical pneumonitis. Meconium is also suggested to cause functional deterioration of pulmonary surfactant. Recent studies have reported that meconium inhibits the physical surface properties of pulmonary surfactant, and that administration of exogenous surfactant may provide therapeutic benefits in animal models or infants with respiratory distress due to MAS. To assess the effects of meconium on physical surface properties, especially the changes on the air-liquid interface and hypophase of pulmonary surfactant in vitro, we studied the following findings ; a) the surface spreading rate(SSR) and the surface adsorption rate(SAR), b) the viscosity, c) the electron microscopic changes, on a series of mixtures with various concentrations of lyophilized human meconium and Surfactant-TA(Surfacten™). The human meconium has significantly increased the surface tension of SSR and the viscosity of pulmonary surfactant, but had decreased the surface pressure of SAR of surfactant, and changed the electron microscopic findings of surfactant. We have concluded that these findings support the concept that meconium-induced surfactant dysfunction may play a role in the pathophysiology of MAS.

Key Words : *Pulmonary surfactant, Meconium, Meconium aspiration syndrome, Electron microscopy*

INTROUDUCTION

Meconium staining of the amniotic fluid is a common problem occurring in 10 to 22% of all deliveries. Meconium aspiration syndrome(MAS) occurs in 2% of these deliveries and is still a significant cause of morbidity and mortality of the newborn infants(Desmond et al., 1957 ; Holtzman et al., 1989).

MAS results from a combination of acute airway obstruction, chemical pneumonitis, alveolar edema, increased pulmonary vascular and airway resistance, changes in compliance and functional residual capacity, and ventilation-perfusion abnormalities(Tyler et al.,1978 ; Tran et al., 1980 ; Wiswell and Bent, 1993).

Recent studies have reported that inhibitory effects of meconium on lowering minimum surface tension(ST) and physical surface properties of pulmonary surfactant, which suggests that meconium-induced secondary pulmonary surfactant dysfunction in the lung of the newborn may contribute to the pathophysiology of MAS, and that administration of exogenous surfactant or saline lavage with surfactant may provide therapeutic benefits in animal models or infants with respiratory distress due to MAS.

To assess the effects of meconium on physical

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surface properties, especially the changes on the air-liquid interface and hypophase of pulmonary surfactant *in vitro*, we studied the following findings ; a) the surface spreading rate(SSR) which representing the changes of surfactant on the air-liqiud interface and the surface adsorption rate(SAR) which representing the moving of surfactant from hypophase to interface with a modified Wilhelmy surface balance, b) the viscosity with a viscosimeter, c) the electron microscopic changes on a series of mixtures with various concentrations of lyophilized human meconium and Surfactant-TA(Surfacten™).

MATERIALS AND METHODS

Preparations of surfactant and meconium

The surfactant used in this study was Surfactant-TA (Surfacten™, Tokyo Tanabe Co., Japan), a reconstituted bovine surfactant that has been used for the treatment and prevention of respiratory distress syndrome(RDS) in Japan and Korea(Fujiwara, 1984 ; Fujiwara et al., 1990 ; Bae et al, 1993 ; Konish et al., 1994). Surfactant-TA, containing 120mg phospholipid/vial, was dispersed in saline at a concentration of 25mg/ml, and was then serially diluted with saline.

Samples of the first meconium were collected from 5 healthy, term newborn infants. The meconium was pooled, blended with a small amount of saline. It was then lyophilized and kept at -70℃. One gram of the original pooled meconium corresponded to 0.25 gram of lyophilized meconium. The lyophilized meconium was resuspended in saline, and then serially diluted with saline.

Measurements of physical surface properties

The physical surface properties(SSR, SAR, and viscosity) of surfactant and surfactant-meconium mixtures were measured. The methods used for measurement of each surface property are as follows :

a) Surface spreading rate(SSR) measurement

We used a modified Wilhelmy surface balance (Acoma, Tokyo, Japan) to measure ST of the SSR. The meconium was mixed with an equal volume of the surfactant suspension, by mechanical mixing, which resulted in the following concentrations : meconium 0, 1.0, 10.0mg/ml and surfactant 25mg/ml. Each unit indicates mg dry weight of meconium/ml, and mg phospholipids of surfactant/ml. Phospholipid phosphorus analyses were performed by Bartlett's method(Bartlett, 1959).

500 μg of surfactant phospholipid or mixtures of surfacatant and meconium in each different concentrations were placed on a clean surface of physiological saline in a teflon trough, with a surface area of 39.6cm^2 of Wilhelmy surface balance, and the ST was recorded with an X-Y recorder for 180 seconds.

b) Surface adsorption rate(SAR) measurement

We used a modified Wilhelmy surface balance to measure the surface pressure(SP) of the SAR by Notter's method(Notter et al., 1982) with our modification. 0.5mg of surfactant phospholipid or mixtures of surfactant and meconium in different concentrations were injected into the hypophase of an additional 7ml of 0.15 mol/L NaCl, which was stirred at 120rpm by a teflon stirring bar(10×4mm) in a round teflon dish(1cm deep, 4cm in diameter). We checked the ST of the SAR in the following concentrations : meconium 0, 1.0, and 10.0 mg/ml and surfactant 0.5mg/ml. We maintained the temperature of the hypophase and air at 37℃. The SAR was described as SP. The equation for the calculation of SP was : SP(mN/m) = 72 - ST.

c) Viscosity measurement

We used a viscosimeter to measure viscosity of surfactant and surfactant-meconium mixtures in the following concentrations ; surfactant 5mg/ml and meconium 0, 5.0, 10.0mg/ml.

Electron microscopic examination

We did the ultra-structural study at Surfactant-TA alone(concentration : 5mg/ml) and surfactant-meconium mixture(concentration : surfactant 5mg/ml, meconium 5 mg/ml). To each of 1.5ml volume of dispersed surfactant or mixture of surfactant and meconium, an equal volume of 2% tannic acid in 0.1M sodium phosphate buffer, pH 7.2 was added. After 30 minutes of fixation, the mixtures were washed 5 times in buffer and pelleted at 3,000 rpm for 10 minutes after each wash to remove the excess tannic acid, the supernatant being discarded. Each pellet was then fixed for 1 hour in 1% OSO_4 in 0.1 M sodium phosphate buffer, pH 7.2, and dehydrated in increasing concentrations of ethanol. Embedment had been placed in polybed 812, nadic methyl anhydrase, dodecenyl succinic anhydrase and DMP-30. Thin sections were stained with uranyl acetate, and lead citrate was used for observation and photography.

Statistical analysis

Multiple comparisons were made using the mean

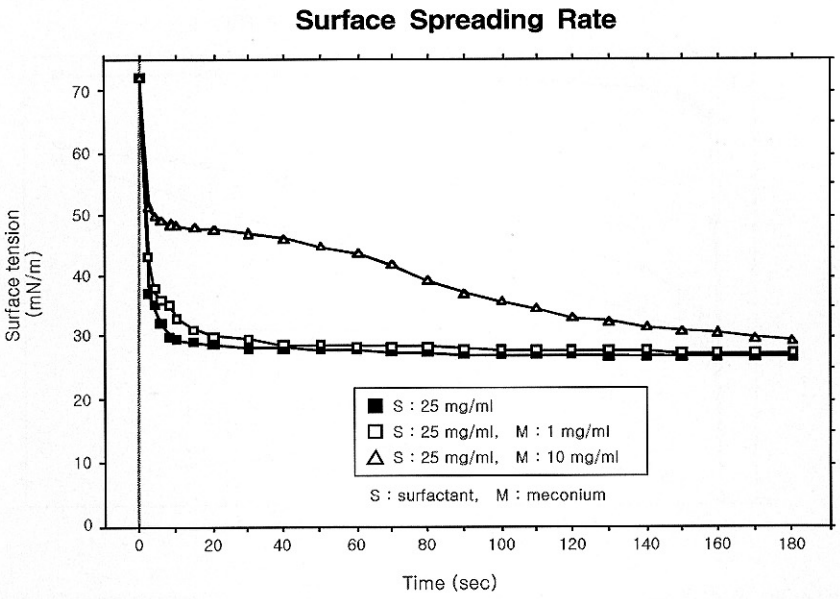

Fig. 1. The effect of various concentrations of meconium on the surface spreading rate(SSR) of pulmonary surfactant. When surfactant(Surfactant-TA) alone was placed on the surface, the surface tension(ST) was rapidly decreased to the equilibrium ST of less than 30 mN/m within 30 seconds. However, when mixture of the surfactant and meconium(10mg/ml) was examined, the STs of the SSR were significantly increased compared with those of surfactant used alone($p<0.01$).

and standard deviation of SSR(n=5), SAR(n=5) and viscosity(n=7) between surfactant and mixtures of surfactant and meconium, by employing Dunnett's method for analysis of variance(ANOVA), using Statview II software(Abscus Concepts, Inc., USA). Statistical significance was assigned to p values < 0.05.

RESULTS

Surface spreading rate

The effects of various concentrations of meconium on the SSR of the pulmonary surfactant were shown in Fig. 1 and Table 1. The ST at 30 seconds and equi-

Table 1. Surface spreading, adsorption, and viscosity of surfactant-alone and mixtures of surfactant and meconium

Concentration (mg/ml)		Spreading rate (mN/m)		Adsorption rate (mN/m)		Viscosity (D×T)
Surfactant	Meconium	Surface tension at 30 sec	Equilibrium surface tension at 3 min	Surface pressure at 10 min	Equilibrium surface pressure at 90 min	
25	0	28.2±0.1	27.0±0.0			
25	1	29.6±0.3	27.2±0.3			
25	10	47.8±1.8*	29.3±0.2*			
0.5	0			46.2±0.9	48.0±0.0	
0.5	1			25.8±0.6*	41.2±0.7*	
0.5	10			21.0±0.5*	38.4±0.4*	
5	0					6.04±0.09
5	5					7.35±0.19*
5	10					9.21±0.03*

Values are mean±S.D. : spreading and adsorption rate (n=5), viscosity : (n=7)
* $p<0.01$ vs surfactant-alone (meconium : 0 mg/ml) (ANOVA)
Surface pressure = 72-surface tension (mN/m)
D : depth, T : time

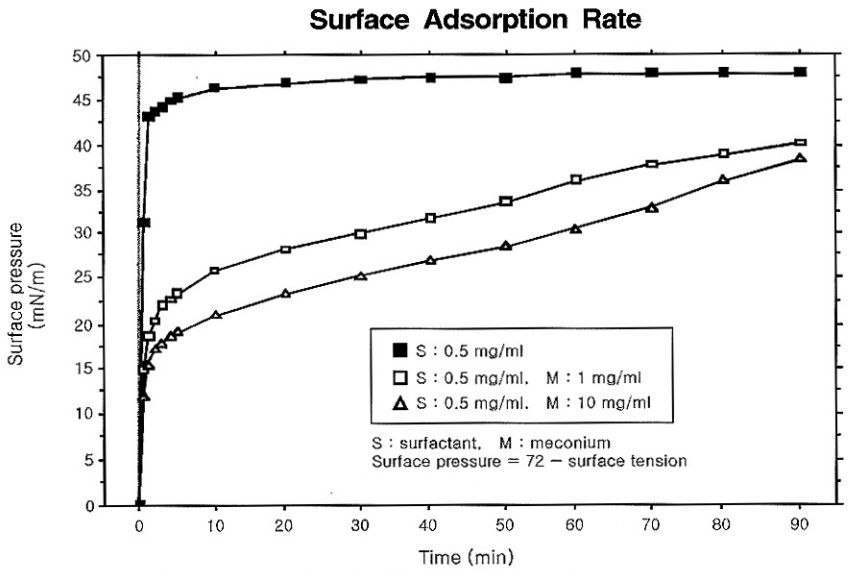

Fig. 2. The effect of various concentrations of meconium on the surface adsorption rate(SAR) of pulmonary surfactant. When surfactant(Surfactant-TA) alone was placed in hypophase, the surface pressure(SP) was rapidly increased to the equilibrium SP of more than 45mN/m within 10 minutes. However, when mixture of the surfactant and meconium was examined, the SPs of the SAR had been significantly decreased compared with those of surfactant-alone ($p<0.01$). Equation for calculation of SP was : SP (mN/m) = 72 - ST.

librium ST at 180 seconds of the SSR were 28.2±0.1, 27.0±0.0mN/m at 25mg/ml of surfactant(0mg/ml of meconium), 29.6±0.3, 27.2±0.3mN/m at the concentration of meconium 1mg/ml and surfactant 25mg/ml, and 47.8±1.8, 29.3±0.2mN/m at the concentration of meconium 10mg/ml and surfactant 25mg/ml, respectively. When surfactant-alone was placed on the surface, the ST rapidly decreased to the equilibrium ST of less than 30mN/m within 30 seconds, which means Surfactant-TA itself has good surface spreading activity when interfaced with air and liquid. But when mixture of the surfactant and meconium was examined(concentration of sample : surfactant 25mg/ml and meconium 10mg/ml), the STs of the SSR were significantly increased compared with those of surfactant - alone($p<0.01$) at 30 seconds.

Surface adsorption rate(SSR)

The effects of various concentrations of meconium on the SAR of pulmonary surfactant were shown in Fig. 2 and Table 1. The SP of the adsorption rate at 10 minutes and equilibrium SP at 90 minutes, were 46.2± 0.9, 48.0±0.0mN/m at 0.5mg/ml of surfactant-alone (0mg/ml of meconium), 25.8±0.6, 41.2±0.7mN/m at the concentration of meconium 1mg/ml and surfactant 0.5mg/ml, and 21.0±0.5, 38.4±0.4mN/m at the concentration of meconium 10mg/ml and surfactant 0.5 mg/ml, respectively. These findings revealed that Surfactant-TA showed good adsorptive activity and quickly adsorbed on the interface of air and liquid. When surfactant was placed in hypophase, the SP rapidly increased to the equilibrium SP of more than 45mN/m within 10 minutes. However, when we meaured those mixtures of surfactant and meconium(concentration of samples : surfactant 0.5mg/ml and meconium 1 and 10mg/ml respectively), the SPs of the SAR were significantly decreased compared with those of the surfactant-alone($p<0.01$, $p<0.01$).

Viscosity

The values of viscosity of surfactant-alone and changes in viscosity of the surfactant mixed with meconium, were shown in Fig. 3 and Table 1. Viscosities were 6.04±0.09 at 5mg/ml of surfactant-alone (0mg/ml of meconium), 7.35±0.19 at the concentration of meconium 5mg/ml and surfactant 5mg/ml, and 9.21±0.03 at the concentration of meconium 10mg/ml and surfactant 5mg/ml. Viscosities were significantly in-

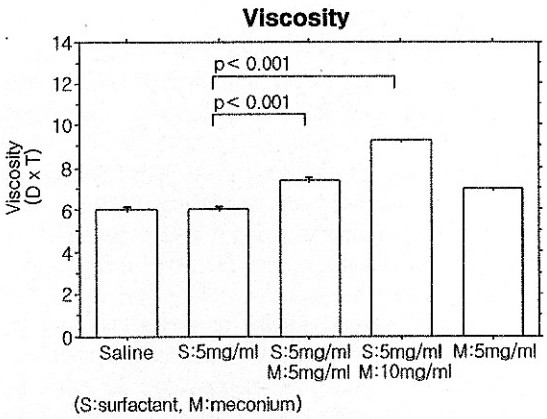

Fig. 3. The effect of various concentrations of meconium on the viscosity of pulmonary surfactant. Viscosities were significantly increased as a result of mixing surfactant with meconium, compared with that of surfactant used alone ($p<0.001$).

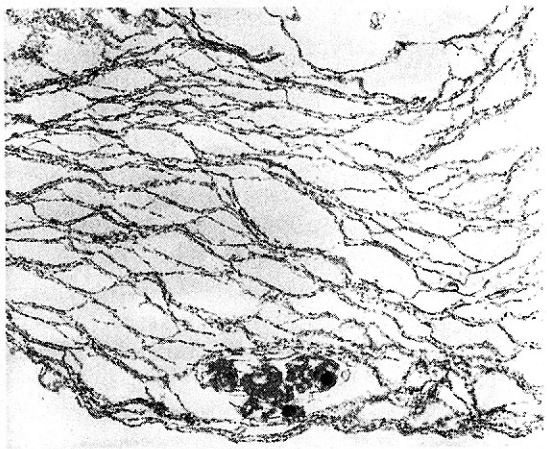

Fig. 4. The electron microscopic ultrastructure of the surfactant alone(Surfactant-TA, concentration : 5mg/ml) shows the characteristic lamellar rod like micelles with multiple open ends.(×8,000)

creased as results of mixing surfactant with meconium(concentration of samples : surfactant 5mg/ml and meconium 5 and 10mg/ml respectively) compared with that of surfactant-alone($p<0.001$, $p<0.001$).

Electron microscopic changes

Ultrastructure of the surfactant-TA (concentration : 5 mg/ml) was shown in Fig. 4. The changes in morphology of surfactant after mixing surfactant with meconium were shown in Figure 5. Figure 4 shows the characteristic morphology of Surfactant-TA, which revealed the lamellar rod-like micelles and multiple open-

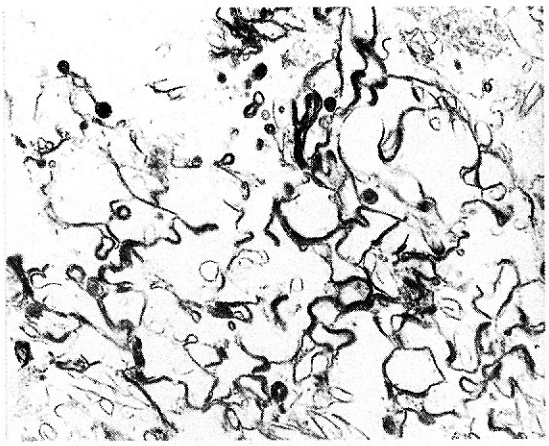

Fig. 5. After mixing surfactant with meconium(surfactant : 5mg/ml, meconium : 5mg/ml), the characteristic morphology of surfactant changed into the spherical lamellar structure with a loss at the open ends. (×8,000)

ends between ends of rod-like micelles at both sides. These characteristic morphologic findings were contributed to the good physical surface activity of Surfactant-TA. However, after mixing sufractant with meconium(concentration : surfactant 5mg/ml, meconium 5 mg/ml), the characteristic morphology of the sufractant changed from rod-like micelles into the spherical lamellar structure and thereby losing their open-ends at both sides of the surfactant rods(Fig. 5).

DISCUSSION

Meconium staining of amniotic fluid occurs in nearly 10~22 percent of all deliveries, as a response to fetal asphyxia. In some cases, gasping before or during delivery can cause severe MAS and this is still a major cause of morbidity and mortality mainly in term and post-term newborn infants(Desmond et al., 1957 ; Holtzman et al., 1989 ; Wiswell and Bent, 1993).

The effects of meconium on the lungs include mechanical obstruction of the airways, chemical pneumonitis, and subsequent respiratory distress in newborn. Tracheal suction, artificial ventilation i.e., high frequency ventilation, and extra-corporeal membrane oxygenation were recommended in severe cases of MAS in order to relieve the airway obstruction, ventilation-perfusion dysfunctions, and pulmonary hypertension(Tyler et al., 1978 ; Tran et al., 1980 ; Wiswell and Bent, 1993).

Recent studies, related to the interaction of pulmonary surfactant and human meconium, have reported an inhibitory effect of meconium on lowering minimum

ST and physical surface properties of pulmonary surfactant *in vitro*(Chen et al., 1985 ; Clark et al., 1987 ; Moses et al., 1991 ; Sun et al., 1993a ; Bae et al., in press) ; and also, the beneficial effects of the clinical use of exogenous surfactant replacement therapy or saline lavage with surfactant, in cases of experimental animal models(Paranka et al., 1992 ; Itakura et al., 1993 ; Sun et al., 1993b ; Al-Mateen et al., 1994 ; Ohama et al., 1994 ; Sun et al., 1994) and newborn infants(Auten et al., 1991 ; Davis et al., 1992 ; Blanke et al., 1993 ; Khammash et al., 1993 ; Lotze et al., 1993) with respiratory distress due to MAS.

Clark et al.(1987), Moses et al.(1991), Sun et al. (1993a) and Bae et al.(in press) have reported that meconium itself inhibits the ST-lowering properties *in vitro*. An analysis of the various components of meconium and its action, Clark et al.(1987) have demonstrated that the fatty acid components in meconium had increased the ST forces. They compared the inhibitory effects of a water-ethanol soluble fraction and a chloroform soluble fraction of meconium. Both phases had inhibitory effects, but the chloroform soluble fraction was shown to have a stronger inhibitory effect.

Paranka et al.(1992), Itakura et al.(1993), Sun et al. (1993b), Al-Mateen et al.(1994)., Ohama et al.(1994), and Sun et al.(1994) have reported that exogenous surfactant lavage or the administration of exogenous surfactant had improved oxygenation, lung function (lung-thoracic compliance, PCO_2) and morphology(alveolar volume density in histologic section) in piglets or rabbit models of MAS. These findings support the proposed new concepts that functional deterioration of surfactant is related to the pathophysiology of MAS. Auten et al.(1991), Davis et al.(1992), Blanke and Jorch (1993), Khammash et al.(1993), and Lotze et al.(1993), in their uncontrolled study of human infants with MAS, have demonstrated significant improvements in PaO_2 and the subsequent outcome after treatment with exogenous surfactant.

This study was designed to determine the changes of *in vitro* effects of meconium on surfactant in aspect to alterations of interactions of surfactant and meconium on the air-liquid interface and hypophase rather than measurement of minimum ST only. The results of this study revealed that meconium may cause significant inhibition of the SSR, SAR, viscosity, and changes the ultrastructural morphology of pulmonary surfactant *in vitro*.

Sasaki(1990) have reported the *in vitro* comparison of the surface properties(SSR, SAR, minimum and maximum ST) of five surfactant preparations [purified natural surfactant, crude natural surfactant lipid, Curosurf™, artificial lung expanding compound(ALEC), and Surfactant-TA]. Also Takahshi et al.(1994) reported the results of a similar study by using 3 surfactant preparations which were used in clinical fields for treatment of neonates with RDS in the world(Exosurf™, ALEC, and Surfactant-TA). They concluded that Surfactant-TA had the best *in vitro* surface active properties among exogenous surfactant preparations.

Fujiwara(1984) and Tanaka et al.(1986) have suggested the surface physical criteria for good artificial surfactant. According to their suggestions, Surfactant-TA showed the following surface activities : rapid spreading (less than 10 seconds to reach an equilibrium ST of 24 ~27mN/m), rapid adsorption (less than 1 minute to reach a ST of 27~30mN/m, which means SP of 42~45 mN/m) with a hypophase concentration of 50ug phospholipid/ml, and minimum ST less than 10mN/m with only 20~30% surface compression, and reproducible ST-area diagram with maximum ST of 27~30mN/m by using modified wilhelmy surface balance.

In this study, the results of the SSR and SAR of Surfactant-TA alone were very similar to Fujiwara's criteria for good surfactant(1984). But after mixing with meconium, the STs of SSR were significantly increased, and the SPs of SAR were significantly decreased. These alterations revealed that the meconium-contaminated surfactant does not have good enough exogenous surfactant activity compared with *in vitro* criteria of good exogenous surfactant. Therefore, meconium, in itself, might interfere the spreading action of surfactant on the interface of air and fluid, and interfere with the adsorptive action of surfactant from the hypophase of solution to the surface of air-fliud interface. These findings support that meconium-induced dysfunction of the surface physical properties of surfactant may contribute to one of the pathophysiologies of MAS.

The action mechanisms of meconium on the viscosity of surfactant are not well established yet. This study was carried out to determine the relationship between meconium-induced surfactant dysfunction and changes of viscosity of surfactant. In this study, the viscosity of mixture of surfactant and meconium was significantly increased than that of surfactant-alone. This finding supported that meconium, itself, inhibits the elastic function of bubble and changes of viscosity of surfactant solution, which also suggested that meconium contributed to a part of surfactant dysfunction *in vitro*.

Sasaki(1990) also reported the electron microscopic ultra-structures of five surfactant preparations(purified natural surfactant, crude natural surfactant lipid, Curosurf™, ALEC, and Surfactant-TA). Surfactant-TA had the best *in vitro* surface active properties and showed ultrastructure of large open-end, thin-walled, and lamellar rod-like micelles. However, other surfactant preparations showed spherical multi-lamellar bodies but few open-ends. Sasaki concluded that these structural differences between Surfactant-TA and other surfactants were correlated with the physico-chemical differences of them. These results revealed that ultrastructure of Surfactant-TA showed characteristic lamellar rod like micelles and multiple open-ends between the end of rod-like micelles at both sides. This characteristic morphology of Surfactant-TA had been changed from rod-like micelles into a spherical lamellar structure and lost the open-ends on both sides of the surfactant rods after mixing surfactant with meconium. These findings suggested that meconium-induced ultrastructural changes were correlated with inhibition of the physical surface properties of pulmonary surfactant treated with meconium.

On conclusion, the human meconium has significantly increased the ST of SSR and the viscosity of pulmonary surfactant, but had decreased the SP of SAR of surfactant, and changed the electron microscopic findings of surfactant. We have concluded that meconium causes significant inhibitions of the physical surface properties(meconium, itself, interferes with the spreading of surfactant on the air-liquid interface, the adsorption of surfactant from hypophase to interface and the viscosity of liquids) and change the morphology of surfactant. These findings support the concept that meconium-induced surfactant dysfunction may play a role in the pathophysiology of MAS.

According to the results of this study, the adverse effects of meconium on surfactant might be overcome by administration of large doses of exogenous surfactant, and exogenous surfactant theraphy or surfactant lavage may be beneficial in cases of newborn infants with respiratory distress due to MAS.

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