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Inhibitory effect of meconium on pulmonary surfactant function tested in vitro using the stable microbubble test

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Abstract Meconium aspiration syndrome is related to mechanical obstruction of the airways and subsequent chemical pneumonitis. It has also been suggested that meconium causes inhibition of surfactant function. To assess its inhibitory effect on surfactant function in vitro, we used a stable microbubble (SM) test that was thought to reflect the adequacy of pulmonary surfactant. The mixtures were prepared by adding serial dilutions of human meconium to various concentrations of Surfactant-TA (Surfacten™). The SM count at each concentration of surfactant significantly increased with the increasing concentration of surfactant. This shows that the SM test closely reflects the quantified function of surfactant. When various concentrations of meconium were added to the surfactant concentration of 0.05 mg/ml and 0.25 mg/ml, the SM test results were decreased even at low concentrations of meconium. Also the increase in the meconium concentration caused a decrease in the SM test result, which was dependent on the surfactant and the meconium concentration, accordingly. These results suggest that meconium inhibits surfactant function.

Conclusion The stable microbubble test is an effective indirect method that tests the changes in surfactant quantity. In the in vitro experiment, we observed an inhibitory effect of meconium on the surfactant activity using the stable microbubble test.

Key words Meconium · Pulmonary surfactant · Stable microbubble test · Surfactant-meconium mixtures · Respiratory distress syndrome

Abbreviations MAS meconium aspiration syndrome · RDS respiratory distress syndrome · SM stable microbubble

Introduction

Meconium-stained amniotic fluid occurs in approximately 12% of live births [18]. In some cases, gasping before or during delivery can cause severe meconium aspiration syndrome (MAS) which is still a significant cause of morbidity and mortality, especially in term and post-term newborn infants. The effects of meconium on lung function include mechanical obstruction of the airways and subsequent chemical pneumonitis [32]. Recent studies have reported the interaction between pulmonary surfactant and human meconium as indicated by increasing minimum surface tension and is physical surface properties in vitro [3, 5, 9, 24, 29]. Based on this evidence, beneficial effects were noted after exogenous surfactant replacement therapy or saline lavage with surfactant in both experimental animal models [1, 20, 25, 26, 30, 31] and newborn

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infants [2, 4, 12, 14, 19, 21, 23] with respiratory distress due to MAS.

The in vitro methods used to assess the presence of pulmonary surfactant include biochemical measures of surfactant components, such as phospholipid or surfactant protein concentration, and biophysical measures of surfactant properties, such as surface tension measurement or stable microbubble (SM) count [6, 8, 16] and the shake test [10, 11, 13]. The inhibitory effect of meconium on surfactant function has been determined by the Wilhelmy balance or pulsating bubble surfactometer that measure surface tension in vitro, or by pulmonary function tests and microscopic studies of lung biopsies in vivo. The SM test is an alternative to measure biophysical properties of functioning surfactant and is a quick and reliable procedure used to make an early diagnosis of respiratory distress syndrome (RDS). However, the relationship between the meconium-induced inhibition of surfactant and the SM test have not yet been reported.

We observed the changes in the results of the SM test as we manipulated the surfactant concentration in vitro in order to establish the efficacy of the SM test by proving the existence of surfactant and reflecting the quantification of the surfactant by the SM test. We also performed the SM test on mixtures of meconium and surfactant at varying concentrations. Using this method, we attempted to demonstrate the inhibitory effect of meconium on surfactant by the decrease in the SM results. Since the SM test is an indirect method that represents the surfactant function, we performed our experiment to test the latest pathophysiological basis of MAS by observing the inhibitory effect of meconium on the physical properties of the surfactant.

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**Materials and methods**

**Preparation of surfactant and meconium**

The surfactant used in this study was Surfactant-TA (Surfactan™, Tokyo Tanabe, Tokyo, Japan), a reconstituted bovine surfactant [15] that has been used for the treatment and prevention of neonatal RDS in Japan [17, 22]. We used this surfactant because the “natural” surfactant shows a considerable variability of surface properties both in vitro and in vivo. Surfactant-TA containing 100 mg phospholipids per vial was dispersed in saline at a concentration of 25 mg/ml and was then serially diluted with saline. Samples of meconium collected from five healthy, term newborn infants were pooled and blended with a small amount of saline, lyophilised and kept at −70 °C; 1 g of the original pooled meconium corresponded to 0.3 g of lyophilized meconium. Before being mixed with surfactant, the lyophilized meconium was resuspended in saline by shaking for 1 h and the suspension was then serially diluted with saline. For the SM test, the meconium slurry was mechanically mixed with an equal volume of surfactant suspension.

**Stable microbubble test**

The SM test is based on the biophysical capacity of pulmonary surfactant to generate SMs that are 15 μm or less in diameter [27]. We used the SM test according to the method described by Chida et al. [6, 8, 16]. To generate bubbles, the uncentrifuged test sample was swirled to resuspend the sediment. A 40 μl aliquot (5 cm of the stem of a Pasteur pipette) was placed on a 50 mm × 35 mm microscope slide with a Fortuna (Hamburg, Germany) pipette. The drop was then sucked into and expelled from a Pasteur pipette (lumen diameter 1.0 mm; stem length 11.5 cm) in quick succession, 20 times in 6 s, with the pipette tip touching the slide vertically on the drop. The slide was then immediately inverted over a hollow slide so that a hanging drop was formed. After 4 min, the slide was examined under a microscope at a magnification of 10 × 10, using a 1 mm² field and a 15 μm scale (Tokyo Tanabe, Tokyo, Japan). Counts were made in five regions, in the four quadrants of and by the centre of the bubble field. All SMs <15 μm diameters were counted except those appearing only as black dots and those which were not circular. The mean count of SM/mm² of the five regions was then calculated. Investigators were blind to the nature of the meconium-surfactant mixtures throughout the study. This process was performed twice for each three subjects. In order to observe the changes in the SM test results with changing surfactant concentration, we performed the SM test on the saline-diluted surfactant at the concentrations of 0, 0.005, 0.01, 0.015, 0.02, 0.025, 0.03, 0.035, 0.04, 0.05, 0.06, 0.07, 0.09, 0.1, and 0.25 mg/ml. To test the effect of surfactant-meconium mixtures, the SM count was obtained at two surfactant concentrations (0.05 mg/ml and 0.25 mg/ml) each mixed with meconium concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, 10.0, and 50.0 mg/ml.

**Statistical analysis**

The relationship between the mean and the standard deviation of SMs and surfactant-meconium mixtures was calculated via the ANOVA test using SAS software Version 6.12 for Windows (SAS institute). The relationship was assessed by the regression analysis with correlation score. Statistical significance was assigned to P values <0.05.

**Results**

The SM test results at varying concentrations of surfactant are shown in Figure 1. When the surfactant concentrations were 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, and 0.25 mg/ml, the SM results were 5.8 ± 1.0, 28.5 ± 9.3, 59.5 ± 23.7, 87.8 ± 11.7, 124.0 ± 5.0, 142.0 ± 40.1, 182.3 ± 9.5, 208.8 ± 6.3, 239.0 ± 11.3, 287.5 ± 25.1, and 400 ± 78.1 respectively. The SM count at each concentration of surfactant significantly increased with the increasing concentration of surfactant. This shows that SM test closely reflects the quantified function of surfactant.

At the surfactant concentrations of 0.05 mg/ml and 0.25 mg/ml, the SM test results due to meconium concentration are shown in Figure 2. We observed a decrease in SM results even at low concentrations of meconium. The inhibitory effect of meconium on the surfactant function is shown in Figure 3. We observed a statistically significant decrease in SMs at a meconium concentration of 0.05 mg/ml and greater in 0.05 mg/ml of surfactant concentration and at a meconium concentration of 0.01 mg/ml and greater in 0.25 mg/ml surfactant concentration. These decrements were dose-dependant. The SM count at each concentration of meconium significantly decreased with the increasing concentration of meconium.