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Minced Calf Lung Surfactant Extract Peripheral Blood Mononuclear Cells to Release IFN-γ and TGF-β: A Regulation Response for Lung

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Abstract

BACKGROUND: Inflammatory reactions in pathophysiologic conditions of lung are a critical problem in the treatment process, which in some cases lead to death, particularly in neonate. Exogenous lung surfactant has been considered as a candidate to treatment of inflammation in the lungs.

OBJECTIVES: The aim of this study is to examine the efficacy of this substance in vivo and in vitro.

METHODS: Calf lung surfactant extract (CLSE) was obtained from freshly slaughtered calves' minced isolates. For in vivo study: the New Zealand white rabbits as appropriate animal model were treated with formulated CLSE, then peripheral blood mononuclear cells (PBMC) were collected and the level and gene expression of IL-10, IL-6, IL-1β, IFN-γ and TGF-β were assessed before and after surfactant treatment for 30 days. In vitro study: four different formulated drug concentrations were added to rabbit PBMC and cytokines level and gene expression were evaluated.

RESULTS: Our results indicate that IFN-γ and TGF-β increased at 24, 48 and 72 h which were statistically significant compared to baseline. While, IL-6 and IL-1β also started to decrease, IFN-γ and TGF-β increased due to surfactant therapy which reached its maximum expression after 7 days.

CONCLUSIONS: This study suggested that CLSE could contribute in reducing pathology effects of pro-inflammatory cytokines by inducing regulatory response in lung which can be used as auxiliary and protective drug in respiratory diseases.

KEYWORDS: Calf lung surfactants extract, cytokines level, exogenous lung surfactant, regulatory response

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Introduction

Surfactant is synthesized by pneumocyte type II cells in late months of pregnancy, stored in intracellular lamellar inclusion bodies, and released to the alveoli spaces (Bissinger & Carlson, 2006; Frerking, Günther, Seeger, & Pison, 2001; Paramothayan, 2018). Lung surfactant components including phospholipids, neutral lipids, and proteins line the mammalian lung and prevent atelectasis by reducing the surface tension at the air-alveolar interface (Notter, Wang, Egan, & Holm, 2002). There are several studies that mentioned exogenous lung surfactant can be used for the treatment of respiratory disorders (Baer, Souza, Pimentel, & Veldhuizen, 2019; Cai et al., 2011; Davidson et al., 2006; Han & Mallampalli, 2015), especially neonatal respiratory distress syndrome (Soll & Özek, 2009). The methods of obtaining natural surfactants differ from each other in two ways: extraction from BAL and water–salt extraction of minced lung.

T and B cells related cytokines and chemokines in lung, may possibly play a role in disease progression or healing (Akdis et al., 2016). IL-6 and IL-1-β, tend to promote fibro-proliferation, asthma and allergy (Akdis et al., 2016; Rincon & Irvin, 2012), whereas IFN-γ, TGF-β and IL-10 play a key role in healing (Akdis et al., 2016; Cypel et al., 2009; Dockstader, 2016; Pizzutto, Upham, Yerkovich, & Chang, 2015),

In patho-physiological conditions of lungs, production and release of IL-6 and IL1-β at high levels lead to development of disease. Among different pulmonary diseases, these cytokines are implicated in asthma, chronic bronchitis, chronic obstructive pulmonary disease (COPD), acute lung injury (Ramos-Payán et al.) and acute respiratory distress syndrome (ARDS), which can be ameliorated by using anti IL-1 and IL-6 drugs (Banchereau, 1994; Tanaka, Narazaki, & Kishimoto, 2016; Wispe et al., 1990; Zhang et al., 2015). Thus, pharmacological agents that can either suppress production of these cytokines or elevate modulatory may have potential therapeutic value against various respiratory diseases (Wispe et al., 1990).

Calf lung surfactants extract (CLSE) has been proven to be avaluable drug candidate for the treatment of the infant respiratory distress syndrome (RDS) and other respiratory disorders. However, effect of this surfactant on cytokines pattern is still unknown. In this study, calf minced lung surfactant extract's (CLSE) effect on cytokines profile (IL-1β, IL-6, IL-10, IFN-γ and TGF-β) of healthy rabbits was evaluated to find possible changes in trend of cytokines release, which may have potential therapeutic value against respiratory diseases.

Materials and Methods

Calf Lung Surfactant Isolation

Calf lung surfactant extract (CLSE) was obtained by water-salt extraction of freshly slaughtered calves' isolated minced lungs. Extracted fluids from the minced lungs were combined and debris was removed by centrifugation (Sigma, 3-30 K) at 1200×g for 15 min. Collected supernatant fluids were centrifuged at 20000×g for 30 min, and the obtained pellets were extracted by modified Bligh and Dyer (Bligh & Dyer, 1959). The organic phase was separated and concentrated using rotary evaporator for 30 min (IKA Co, rv10 digital). As a result, neutral lipids and cholesterol are removed from concentrated lipid extract by cold acetone precipitation. Briefly, cold acetone (-20 °C) was added to the extracted samples, vortex and incubated

overnight at -20 °C. It was formulated for the active pharmaceutical ingredients (API) by adding some DPPC and palmitic acid as excipients. Finally, a suspension was prepared by adding NaCl 0.9 %. The amount of endotoxin concentration in the finish product was measured by quality method (gel clot). The surface tension-reducing activity in these samples was then assessed using profile analysis tensiometer (PAT1, Sinterface Technology, Germany).

The different phospholipids classes of the formulated lung surfactant were determined by Hydrophilic Interaction Liquid Chromatography Coupled to Electrospray Ionization−Tandem Mass Spectrometry (HILIC-HPLC-ESI-MS/MS) by negative-ion mode and positive–ion mode. The formulated sample (concentration of the selected sample: 1 mg/mL) was injected to the column containing the mobile phase acetonitrile (A) and ammonium acetate 5mM (B).

Animal

Ten healthy male New Zealand white rabbits (Oryctolagus cuniculus), 2.5-3 kg body weight each were purchased from the Animal Sciences Laboratory of Pasteur Institute of Iran. Each rabbit was kept in separated standard cage with temperature 18-22 ºC, relative humidity 45-55%, 12-hour light, and 12-hour dark fed standard diet with ad libitum water intake.

The health of selected rabbits was approved by clinical examinations, hematology evaluation and radiology in two positions (lateral and antero-posterior). In addition, they were kept under adaptation period for 48 h prior to commencement of the procedure.

Ethics Statement

All experimental procedures involving animals were approved by the Ethics Committee of Faculty of Veterinary Medicine, University of Tehran and ethics aspect of the use of animals (reduction the used animals, refinement and the rehabilitation of housed animals) in present research was monitored by ethics committee. All methods in this study have been monitored and approved by Faculty of Veterinary Medicine, University of Tehran, Iran.

In Vitro and in Vivo Experimental Process

For in vivo, five rabbits were anesthetized with Xylazine 2% (5 mg/kg, Holland Interchemic Co) plus Ketamine (35 mg/kg, Trittau Co., Germany) via intramuscular injection to minimize pain, suffering and distress during procedure, then the tracheal tube was inserted into trachea. Following confirmation of the positioning by checking the end tidal CO2 measurement, broncho alveolar lavage catheter was inserted via tracheal tube, and the surfactant solution (4 ml/ kg BW) was infused into the lungs (Mokhber Dezfouli, Eftekhari, Heidari Sureshjani, Dehghan, & Dousti, 2017).

Blood samples were collected to isolate PBMC as described elsewhere (Hartmann, Emnéus, Wolff, & Jungersen, 2016). Then IL-10, IL-6, IFN-γ, TGF-β and IL-1β levels at 0h (before infusion of the surfactant) and for the following 24, 48, 72 h, 7 days, 14 days and 30 days after surfactant administration were evaluated.

In vitro evaluation: four different formulated surfactant concentrations (12.5, 25, 50 and 100mg phospholipid/ml) were added to PBMC cultures obtained from five rabbits (untreated with surfactant). After 24 h incubation at 37 oC, contents of the wells were centrifuged at 14000 rpm and 4 oC. Supernatants were collected to evaluate cytokines levels, and precipitated cells were collected for cytokines genes expression assessment

from both in vivo and in vitro group.

Cytokines Assay

Cell culture supernatants were harvested and analyzed for IL-10, IL-6, IFN-γ, TGF-β and IL-1-β cytokines by ELISA techniques with commercially available kits (My-Bio Source, Inc).

Gene Expression Assay

RNA was extracted from the isolated cells followed by the RNX-plus Cinna Gen kit (Tehran, Iran).

The reverse transcription process was conducted by Cinna-Gen First Strand cDNA synthesis kit using random hexamer primers (Tehran, Iran). Primer design was set up by ALLELE ID program. Then, primers were verified by NCBI BLAST NUCLEOTED. β–ACTIN was used as housekeeping gene. Finally, Real-Time PCR was used for gene expression analysis and cytokine quantification (Table 1). The crossing point or the cycle number was determined by the Light Cycler software (version 3.5) using the second derivative method. A melting curve analysis was performed to eliminate the possibility of non-specific amplification or primer-dimer formation (Ramos-Payán et al., 2003; Wispe et al., 1990).

Table 1. Primers design was set up by ALLELE ID program

Gene	Sequence of Primer
β – actin(References gene)	F: CCTCGCTCTCCACCTTCC R: TGCCAATCTCGTCTCGTTTC
IL-1 beta	F:TTGAAGAAGAACCCGTCCTCTG R:CTCATACGTGCCAGACAACACC
IL-10	F: GAGAACCACAGTCCAGCCAT R: CATGGCTTTGTAGACGCCTT
IFN-γ	F: TTCTTCAGCCTCACTCTCTCC R: TGTTGTCACTCTCCTCTTTCC
TGF -beta	F:CAGTGGAAAGACCCCACATCTC R:GACGCAGGCAGCAATTATCC
IL-6	F: CTACCGCTTTCCCCACTTCAG R:TCCTCAGCTCCTTGATGGTCTC

Statistical Analysis

The data were analyzed with repeated measures ANOVA using SPSS version 16.0 software; significance level was considered as P level less than 0.05. To evaluate the effect of drug administration, data were analyzed with Paired t-test and LSD Post Hoc test. To compare the gene expression at the level of five percent, t-test was applied. Data were analyzed based on Delta-Delta cT from the device and the REST2009 software. Normalization of data was conducted by b-actin as a references gene R or Efficiency: 1 or 100%. Comparison of the mean of gene expression at the level of five percent by t-test was performed between two varieties at different times.

Results

Quality Control of Formulated Drug

Different phospholipids classes based on molecular weight and retention time were determined by HPLC and HILIC-HPLC-ESI-MS/MS, the frequency of each class is mentioned in Table 2 and Fig. 1, 2. Based on obtained results the main isolated and detected phospholipids include phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositole and phosphatidylglycerole which were detected based on retention times. The highest amount of isolated phospholipids by HILIC-HPLC-ESI-MS/MS belongs to phosphatidylcholine (42.3 %), which is the main component of natural lung surfactant that can reduce surface tension.

Table 2. Different phospholipids classes and percentages based on molecular weight and retention time and determined by HILIC-HPLC-ESI-MS/MS

Phospholipids	(%) Frequency
PG	3.3%
PI	14.3%
PE+ PS	27.2%
LPE	3.1%
PC	42.3%
SM	9.7%

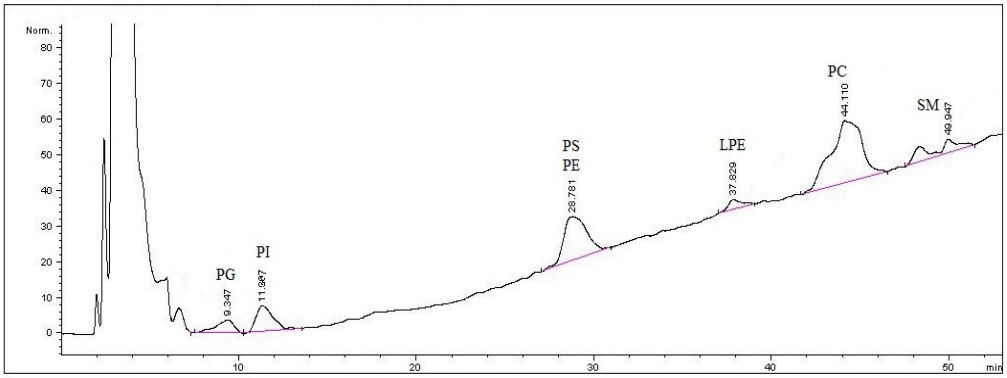

Figure 1. Different phospholipids classes based on molecular weight and retention time determined by HPLC.

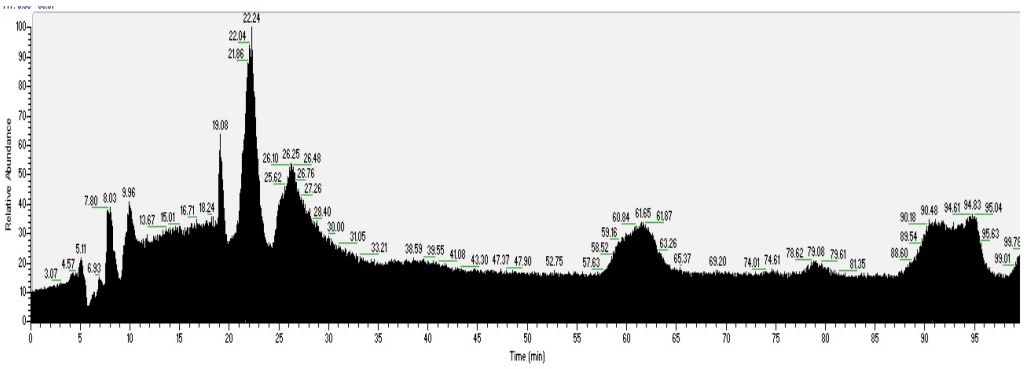

Figure 2. Different phospholipids classes based on molecular weight and retention time determined by HILIC-HPLC-ESI-MS/MS.

The results from profile analysis tensiometer in triplicate runs of the surface tension of samples was recorded 27. 13 ± 1.30 mN/m (Fig. 3), the obtained results of which belonged to natural surfactant surface tension in lungs.

In Vivo and In Vitro Evaluation of Cytokines Content in the Supernatant from PBMC Cells Culture

The in vivo results indicated that IFN-γ and TGF-β increase at 24, 48 and 72 h compared to launch hour (P=0.03and P=0.04). While, IL-6 and IL-1β level started to decrease over time in response to surfactant (P< 0.05). IL-6 and IL-1β concentrations in this experiment reached the minimum level at 24 h after challenge. However, IL-10 level did not change significantly. The in vitro assessment of these cytokines show IFN-γ, TGF-β and IL-10 increased in 25, 50 and 100mg surfactant concentration in comparison to 12.5mg (P=0.02, P=0.04 and P=001). While, IL-6 and IL-1β level started to decrease by increasing surfactant concentration (P<0.05). IL-6 and IL-1β concentrations in this experiment reached the minimum level at 100mg surfactant concentration.

Gene Expression of Cytokines Isolated from PBMC

The in vivo results showed that the gene expression of IL-6, IL-10 and IL-1β decreased (0.84 ±0.02 and 1.14 ±0.02) during the time after exposure to the surfactant 30 days after drug administration and reached its minimum expression (0.52 ± 0.02 and 0.46 ± 0.04).

The gene expression of IFN-γ and TGF-β increased due to surfactant therapy, reaching its maximum expression after 7 days. However, based on the present results, IFN-γ gene expression increased at 24 compared to 0 hour, (P =0.03). There are significant differences at the level of 5% at 24, 48, 72 h of the study.

The in vitro results showed that the gene expression of IFN-γ, TGF-β and IL-10 increased in 15, 25, 50 and 100mg surfactant concentration in comparison to 12.5mg (P= 0.04, P =0.03, P=0.04 and P=001). While, IL-6 and IL-1β level started to decrease by increasing surfactant concentration, (P< 0.05). IL-6 and IL-1β gene expression in this experiment reached the minimum level at 100mg surfactant concentration.

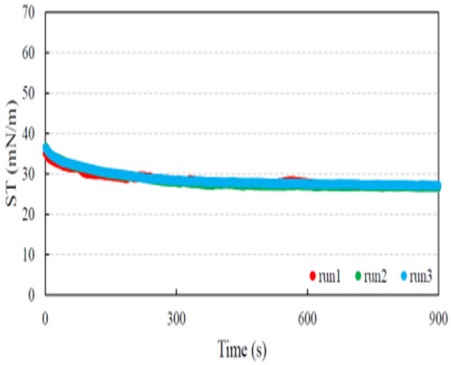

Figure 3. Extracted Surfactant surface tension analyzed by profile analysis tensiometer.

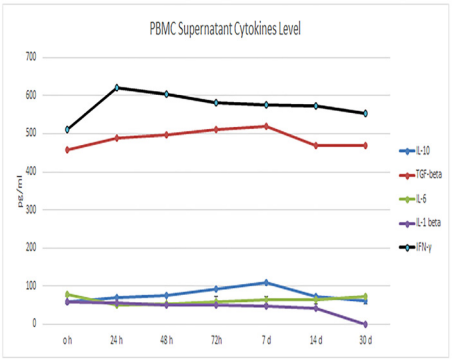

Figure 4. Trend of cytokines level in the supernatant from PBMCs culture isolated from treated rabbits. CLSE induce PBMCs to enhance IFN-γ (black trend line) and TGF-β (red trend line) secretion at 24, 48 and 72 hours after treatment. IL-1β, IL-6 and IL-10 secretion did not significant change in compare 0 hour.

Discussion

Lung encounters a countless number of particles and infectious agents daily, so the immune system should arrange a defense mechanism to determine whether or not to respond. Cytokines are one of the mediators for regulating the immune system (Appel & Jonsson, 2016). In this paper the effect of exogenous surfactant on the content and cytokines gene expression levels in healthy rabbits was evaluated as an appropriate animal model for the respiratory system.

Based on obtained results by HILIC-HPLC-ESI-MS/MS analysis on finished product, it became clear that the main component of formulated lung surfactant includes phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine that can play a crucial role in natural surfactant structure and reduce surface tension in cooperation with hydrophobic surfactant proteins (Notter et al., 2002). Moreover, in situ evaluation of extracted lung surfactant, by profile analysis tensiometer method (PAT1, Sinterface Technology, Germany) showed reduction in surface tension, our formulated sample was recorded 27. 13 ± 1.30 mN/m. The surface tension of water which covers glicocalex of alveolar cells is 72 mN/m that was used as control substance and natural surfactant in lungs adsorption on alveolar surface decreases the surface tension to 23 mN/m, which facilitates the work of breath and provides respiratory mechanics (Notter et al., 2002).

During severe septic conditions, lung surfactant may be lost which is related to pro-inflammatory cytokines activity specially IL-1β (Mukhopadhyay, Hoidal, & Mukherjee, 2006; Niederman & Fein, 1990). On the other hand, IFN-γ has anti-inflammatory aspect through inhibition of TNF-α and IL-1β, and administration of anti-IL-1 agents can ameliorate the pathogenesis effects of inflammation cases (Mühl & Pfeilschifter, 2003; Ouyang et al., 2000). Previous results showed that injection of Kinoret® with inhibition effect of IL-1 could considerably decrease the inflammation symptoms (Klimek, Sali, Rayavarapu, Van der Meulen, & Nagaraju, 2016).

In the present study, we found that rabbits injected with exogenous lung surfactant had a significant increase at the levels of IFN-γ and TGF-β in immune cells and decrease in IL-1β and IL-6. These findings were correlated with other previous studies, which confirmed the immune regulation and tissue repairing effect of IFN-γ and TGF-β (Chung, 2001; Sanjabi, Zenewicz, Kamanaka, & Flavell, 2009).

There are several therapeutic strategies for RDS among which exogenous surfactant therapy has good outcomes (Varvarigou et al., 2012). Based on the in vivo previous studies, pro-inflammatory cytokine profiles increased during initial phase of RDS, causing progression of disease (Hammoud, Raghupathy, Barakat, Eltomi, & Elsori, 2017; Polin & Carlo, 2014). However, these studies did not mention pleiotropic role of IFN-γ and TGF-β as pro-inflammatory and immune regulation cytokines (Fujio et al., 2016; Sanjabi et al., 2009). Additionally, our studies on gene cytokines expression were correlated with their secretion of these cytokines during time points and confirmed by effect of synthetic surfactant on immature lamb lung, causing decrease of inflammatory cytokines (Sato & Ikegami, 2012). As the previous study shows, IFN-γ stimulated SP-A expression in the H441 cell line, confirming its stimulatory effects on SP-A synthesis (Wispe et al., 1990), and triggered

anti-inflammatory effect of this protein on microbial pathogens (Reid, Clark, & Palaniyar, 2005).

However, TGF-β activation plays an important role in lung remodeling after mechanical ventilation in the acute lung injury induced by acid aspiration, which exogenous lung surfactant can ameliorate acute lung injury in adults, based on decreasing IL-1β, IL-6, IL-10 and TGF-β activation (Cabrera-Benitez et al., 2014). In addition, exogenous surfactant proteins in coordination with phospholipids can stimulate production and release of TNF-α, IL-1β, IL-6, and IL-8 by human type-II cells (Banchereau, 1994). So, there are contradictory results that clear the relationship between CLSE and directing the immune response. CLSE regulates the gene expression and production of cytokines profile that can induce anti-inflammatory properties, while in normal conditions they were inflammatory and harmful for lung. This concept came from in vitro study on assessment of CLSE concentrations and cytokines change. With increased CLSE concentration, IFN-γ and TGF-β and even IL-10 were increased.

In conclusion, this study suggests that CLSE increases the level of IFN-γ and TGF-β, and creation of the immune regulation response by decreasing IL-1β, IL-6 and IL-10 could contribute as drug candidate to reduce pathology effect of neonates with respiratory distress, particularly at the first 24 h of administration, which can be used as auxiliary and protective drug in respiratory inflammation diseases.

Research limitation

This study reports CLSE increases the level of TGF-β. There is evidence that reveals TGF-β is responsible for development of remodeling and fibrosis of different organs (Fernandez & Eickelberg, 2012). It is unclear whether the use of this drug candidate or continuing use of it can cause fibrosis or not.

• During in vivo study on rabbit, IL-10 level did not significantly change. While in vitro assessment shows IL-10 change. It means there are some uncontrolled factors which could effect on CLSE and cytokines production in in vivo.

• This study highlights more investigation on CLSE effect on Th17 cytokines as another inflammatory arm in healthy and infection phase.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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عصاره سورفکتانت ریوی گوساله از ریه خرد شده منجر به ترشح IFN-γ و TGF-β از سلول های تک هسته ای خون محیطی می شود: یک پاسخ تنظیمی برای ریه

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چکیده

زمینه مطالعه: واکنش های التهابی در شرایط پاتوفیزیولوژیک ریه یک مشکل مهم در فرایند درمان بوده و در برخی موارد باعث مرگ، به ویژه در نوزادان می‌شود. سورفکتانت اگزوژن ریه به عنوان یک کاندید برای درمان التهاب در ریه مورد توجه قرار گرفته است.

هدف: هدف از این مطالعه، بررسی اثربخشی این ماده در شرایط برون‌تنی و درون‌تنی است.

روش کار: عصاره سورفکتانت ریوی گوساله از ریه خرد شده گوساله‌های تازه کشتار شده جدا گردید. برای مطالعه برون تنی: خرگوش‌های سفید نیوزلندی به عنوان یک مدل مناسب حیوانی با عصاره سورفکتانت ریوی گوساله درمان شدند و سپس سلول‌های تک هسته‌ای خون محیطی جمع آوری و بیان ژن IFN-γ، IL-1β، IL-6، IL-10 و TGF-β قبل و بعد از درمان سورفکتانت به مدت ۳۰ روز ارزیابی شدند. در مطالعه درون تنی: چهار غلظت مختلف به سلول های تک هسته ای خون محیطی خرگوش اضافه شده و سطح سیتوکین ها و بیان ژن مورد ارزیابی قرار گرفتند.

نتایج: نتایج این پژوهش نشان داد که IFN-γ و TGF-β در ۲۴، ۴۸ و ۷۲ ساعت افزایش یافت که از نظر آماری معنی‌دار بود. در حالی که IL-6 و IL-1β روند نزولی را نشان داد، همچنین IFN-γ و TGF-β به دلیل درمان با سورفکتانت افزایش می یابد که بعد از ۷ روز به حداکثر بیان می رسد.

نتیجه گیری نهایی: این مطالعه نشان داد که عصاره سورفکتانت ریوی گوساله می‌تواند در کاهش اثرات پاتولوژی سیتوکاین‌های التهابی القاء کننده با ایجاد پاسخ تنظیمی در ریه به عنوان دارو کمکی و محافظ در بیماری‌های تنفسی مورد استفاده قرار گیرد، کمک کند.

واژه‌های کلیدی:

سورفکتانت اگزوژن ریه، پاسخ تنظیمی، سطح سایتوکین، عصاره سورفکتانت ریوی گوساله

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