**PULMONARY DRUG DELIVERY**

# Exogenous Surfactant as a Pulmonary Delivery Vehicle for Budesonide In Vivo

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## Abstract

**Background** Lung inflammation is associated with many respiratory conditions. Consequently, anti-inflammatory medications, like glucocorticoids, have become mainstay intrapulmonary therapeutics. However, their effectiveness for treating inflammation occurring in the alveolar regions of the lung is limited by suboptimal delivery. To improve the pulmonary distribution of glucocorticoids, such as budesonide to distal regions of the lung, exogenous surfactant has been proposed as an ideal delivery vehicle for such therapies. It was therefore hypothesized that fortifying an exogenous surfactant (BLES) with budesonide would enhance efficacy for treating pulmonary inflammation in vivo.

**Methods** An intratracheal instillation of heat-killed bacteria was used to elicit an inflammatory response in the lungs of male and female rats. Thirty minutes after this initial instillation, either budesonide or BLES combined with budesonide was administered intratracheally. To evaluate the efficacy of surfactant delivery, various markers of inflammation were measured in the bronchoalveolar lavage and lung tissue.

**Results** Although budesonide exhibited anti-inflammatory effects when administered alone, delivery with BLES enhanced those effects by lowering the lavage neutrophil counts and myeloperoxidase activity in lung tissue. Combining budesonide with BLES was also shown to reduce several other pro-inflammatory mediators. These results were shown across both sexes, with no observed sex differences.

**Conclusion** Based on these findings, it was concluded that exogenous surfactant can enhance the delivery and efficacy of budesonide in vivo.

**Keywords** Exogenous surfactant · Glucocorticoids · Pulmonary inflammation · Drug delivery

## Introduction

Inflammation is associated with many respiratory conditions, including Asthma, Pneumonia, Bronchopulmonary Dysplasia, Chronic Obstructive Pulmonary Disease, and Acute Respiratory Distress Syndrome (ARDS). However, the effectiveness of anti-inflammatory medications, such as glucocorticoids, is location specific for these conditions in terms of airway (bronchi) or airspace (alveolar) involvement [1]. In Asthma, for example, inflammation is observed primarily in the small airways, which allows for a more direct delivery of therapeutics, as evidenced by the effectiveness of standard inhalers [1, 2]. On the other hand, in other conditions, such as ARDS and Pneumonia, inflammation occurs in the more distal, alveolar, regions of the lung, where the large surface area, and associated regions of alveolar edema or airway collapse may contribute to an inability of airway-delivered therapies to reach distal lung units, to provide effective anti-inflammatory functions [1, 3, 4]. In these clinical scenarios, alternative strategies are required to deliver therapeutic concentrations of anti-inflammatory medications to these peripheral sites within the lung.

One such approach, is with the use of exogenous surfactant as a delivery vehicle for glucocorticoids such as budesonide. Exogenous surfactant is a complex mixture of lipids and specialized proteins, usually obtained from natural sources such as cows or pigs [5]. The endogenous material, produced by type II alveolar cells in the lung, has been well studied and serves a vital biophysical role

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in reducing surface tension, thereby stabilizing the alveoli during normal breathing [6, 7]. The discovery of surfactant deficiency in preterm infants led to the development of exogenous surfactant therapy [8]. Given intratracheally, exogenous surfactant spreads throughout the lung, improving lung function and has resulted in significant reductions to infant mortality due to prematurity [8, 9]. It is suggested that the spreading properties of exogenous surfactant could improve glucocorticoid delivery to peripheral sites of inflammation in the lung. In support of this notion, exogenous surfactant has already been shown to enhance the delivery of glucocorticoids to remote sites using in vitro approaches [10, 11]. Similarly, in vivo studies, including those modeling ARDS, have observed improved drug distribution and anti-inflammatory effects for glucocorticoids delivered by a surfactant vehicle [12–14]. In addition, it has been shown that through these properties, exogenous surfactant can re-open collapsed airways, overcome regions of edema, and thereby efficiently spread to the deeper, more remote sites of inflammation even in an injured lung [6]. Together, this data highlights the potential for exogenous surfactant to provide these locally acting anti-inflammatory drugs' access to remote regions of the lung otherwise inaccessible to therapeutics.

When combined with the efficacy of glucocorticoids, the innate biophysical properties of surfactant suggest that utilizing exogenous surfactant as a vehicle for budesonide would improve its effectiveness for treating remote inflammation in the lung. It was therefore hypothesized that fortifying an exogenous surfactant with budesonide would enhance efficacy for treating pulmonary inflammation in vivo.

## Methods

### Reagents

Heat-killed bacteria (HKB) was created from a lab strain of *Pseudomonas aeruginosa* (ATCC 27853), purchased from Sigma-Aldrich (Oakville, ON, Canada). Using measurements of optical density, the bacteria were diluted in saline to $3 \times 10^7$ colony-forming units (CFU) per ml, before being heated at 90 °C for 15 min. A commercially available preparation of budesonide (0.5 mg/ml), suspended in deionized water, was obtained from AstraZeneca (Södertälje, Södermanland, Sweden). Bovine lipid extract surfactant (BLES) at 27 mg/ml phospholipid concentration was obtained from BLES Biochemicals (London, ON, Canada). Using saline; these preparations were combined and diluted to 10 mg/ml and 50 µg/ml for BLES and budesonide, respectively, with drug vehiculization being verified through the wet bridge transfer system as described previously [10].

### Animal Models and Treatments

All animal work was carried out in accordance with guidelines and regulations set forth by the Western University Council for Animal Care. For breeding, two adult male and seven adult female Wistar rats (250 g) were purchased from Charles River (St-Constant, QC, Canada). Acclimatization to the animal care facility and breeding were carried out as previously described [15]. Once pregnant, rats were housed individually and received standard chow. Immediately after birth the litters were culled to 10 pups in order to limit the effect of litter size on outcomes.

To initiate pulmonary inflammation, male or female offspring were weighed, anesthetized, and intratracheally instilled with 2 µl of HKB ($3 \times 10^6$ CFU/ml) or saline per gram of body weight at 25–35 days of age. In animals randomized to a treatment group, this first instillation was followed thirty minutes later by a second instillation of either budesonide (50 µg/ml) or BLES/budesonide (10 mg/ml; 50 µg/ml). To minimize the potential effects of any distinct litter, only 1 or 2 animals per litter were randomized to any individual experimental group. Animals were monitored for 6 h following instillation, before being euthanized by intraperitoneal injection of sodium pentobarbital and exsanguination, by severing the descending aorta. After this, a bronchoalveolar lavage (BAL) was performed as previously described [16], before the lungs were excised, divided into four pieces and snap frozen in liquid nitrogen to be stored at − 80 °C.

### Outcomes

Inflammatory cell counts and differential cell analysis of the lavage were done as previously described [16]. Briefly, lavage volume was recorded and centrifuged at $150 \times g$ for 10 min to obtain a cell pellet. This pellet was resuspended and used for cell counting and differential cell analysis to obtain the number of inflammatory cells and neutrophils in the lavage fluid. Protein content of the lavage fluid was also measured using a Micro BCA protein assay kit from Pierce Biotechnology (Rockford, IL, USA), per manufacturer's instructions. A multiplexed immunoassay kit was utilized per manufacturer's instruction (R&D Systems, Minneapolis, MN) to measure the concentrations of TNF-α, IL-6, MIP-2, and GRO/KC. A Bio-Plex 200 readout system was utilized from Bio-Rad (Santa Rosa, CA, USA) and cytokine levels (pg/mL) were automatically calculated from standard curves using Bio-Plex Manager software (v. 4.1.1, Bio-Rad). An aliquot of the $150 \times g$ supernatant was also analyzed through a Duck-Chong phosphorous assay as previously described [17]. Briefly, the total amount of

surfactant in the lavage was determined through the measurement of phospholipid-phosphorus [18, 19]. The remainder of the supernatant was then centrifuged at 40,000×$g$ for 15 min to obtain a pellet of the active form of the surfactant, the large aggregates (LA). This resuspended pellet, as well as the 40,000×$g$ supernatant containing the small aggregates (SA), was also analyzed for phospholipid-phosphorus [18, 19].

Frozen lung tissue was utilized for myeloperoxidase (MPO) activity as previously described [20]. Briefly, pieces of frozen lung were weighed and then homogenized in 0.02 M potassium phosphate buffer (pH 6; Sigma-Aldrich) using a PT2100 homogenizer. Using a high-speed centrifuge, the lung homogenate was spun at 6000×$g$ for 20 min at 4 °C, with the resulting pellet being resuspended in 1% hexadecyltrimethylammonium hydroxide detergent solution. This mixture was then re-homogenized, before being sonicated at 4 °C and 30% amplitude for 10 s. The resulting preparation was spun at 13,000×$g$ for 10 min at 4 °C. The supernatant was then aliquoted into a 96-well plate at 2 mg/ml and mixed with an MPO cocktail containing 3,3′,5,5′-tetramethyl-benzidine. Hydrogen peroxide was then added to each well and the plate was incubated at 37 °C for 5 min. Sulfuric acid was used as a stop solution and the plate was read at 450 nm using an iMark plate reader (Bio-Rad). MPO activity (units/mg) was calculated from standard curves (Sigma-Aldrich).

## Statistical Analysis

All data points shown represent one male or female rat. Statistical significance was determined by two-way analysis of variance (ANOVA) followed by a Tukey–Kramer post hoc test to determine differences among experimental groups. Results were considered statistically significant with a $P$-value of less than 0.05.

## Results

Some of the overall characteristics of the experimental groups are shown in Table 1. Prior to the first instillation, body weights were found to be similar among the experimental groups. There were also no significant differences across experimental groups for the protein content of the BAL. The phospholipid composition of surfactant, including total surfactant, as well as the large aggregate and small aggregate subfractions, was significantly higher in male and female rats receiving BLES/budesonide compared to all other treatment groups.

To evaluate the anti-inflammatory effects of delivering budesonide with an exogenous surfactant in vivo, standard inflammatory markers were analyzed using the BAL and frozen lung tissue. The instillation of HKB resulted in a significantly higher number of inflammatory cells compared to saline (Fig. 1). The instillation of budesonide following the inflammatory insult of HKB did not have a significant effect on the number of inflammatory cells as compared to the saline or HKB instilled groups. Instillation of BLES/budesonide resulted in significantly lower numbers of
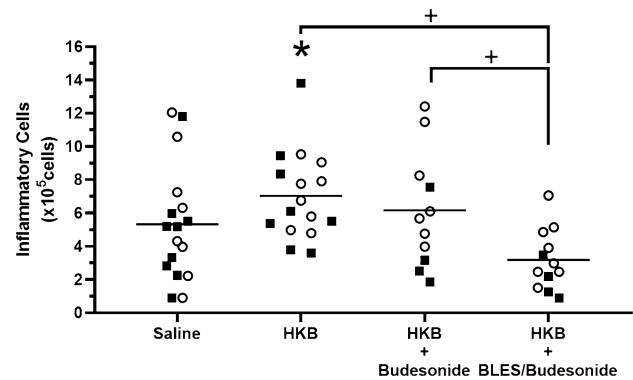


**Fig. 1** The effect of instilling budesonide or BLES/budesonide on the number of inflammatory cells in BAL for pediatric rats. Solid squares indicate female rats, open circles represent male rats. $n = 11$–17, *$p < 0.05$ vs saline, +$p < 0.05$

**Table 1** Baseline characteristics for male or female rats in each treatment group

|  | Saline | | HKB | | HKB + budesonide | | HKB + BLES/budesonide | |
|---|---|---|---|---|---|---|---|---|
|  | Males | Females | Males | Females | Males | Females | Males | Females |
| Number of animals | 7 | 9 | 8 | 9 | 7 | 9 | 8 | 9 |
| Body weight | 93.8 ± 8.2 | 88.7 ± 11.8 | 93.3 ± 6.9 | 85.1 ± 4.7 | 93.0 ± 11.4 | 87.3 ± 8.5 | 95.1 ± 13.2 | 86.7 ± 8.5 |
| Protein | 23.8 ± 10.7 | 19.6 ± 9.2 | 19.2 ± 7.7 | 21.7 ± 5.7 | 20.1 ± 6.3 | 17.7 ± 7.9 | 21.6 ± 6.2 | 23.9 ± 9.9 |
| Total surfactant (mg/kg BW) | 10.3 ± 2.0 | 12.0 ± 2.2 | 10.7 ± 1.7 | 11.5 ± 1.9 | 10.8 ± 2.1 | 11.0 ± 2.2 | 16.6* ± 2.8 | 17.2* ± 3.1 |
| Large aggregates (mg/kg BW) | 6.1 ± 1.3 | 6.9 ± 1.2 | 6.3 ± 1.2 | 7.0 ± 1.3 | 8.2 ± 2.3 | 7.4 ± 2.1 | 9.7* ± 2.3 | 10.9* ± 3.4 |
| Small aggregates (mg/kg BW) | 4.4 ± 0.9 | 5.1 ± 1.1 | 4.7 ± 1.2 | 3.8 ± 0.9 | 3.1 ± 1.4 | 3.9 ± 1.5 | 7.0* ± 2.8 | 6.1* ± 1.9 |

*$p < 0.05$ vs saline

inflammatory cells compared to the HKB and budesonide groups. Differential cell counts in the group receiving saline revealed that the majority of the obtained cells were macrophages. This analysis further revealed that administration of HKB resulted in a significantly higher number of neutrophils ($2.94 \pm 1.06$ for males and $2.12 \pm 0.93$ for females) compared to saline ($0.40 \pm 0.48$ for males and $0.11 \pm 0.03$ for females), but that both budesonide ($1.94 \pm 0.99$ for males and $1.49 \pm 0.93$ for females) and BLES/budesonide ($0.37 \pm 0.27$ for males and $0.35 \pm 0.20$ for females) had significantly lower neutrophil counts compared to HKB (Fig. 2a). Additionally, the number of neutrophils was significantly lower in animals administered BLES/budesonide compared to those given budesonide alone. The instillation of HKB ($9.14 \pm 1.13$ for males and $9.01 \pm 2.32$ for females) or HKB followed by budesonide ($7.52 \pm 2.04$ for males and $9.54 \pm 1.94$ for females) also resulted in significantly higher MPO activity compared to saline ($4.37 \pm 1.02$ for males and $3.71 \pm 1.68$ for females; Fig. 2b). However, only the animals instilled with BLES/budesonide ($5.32 \pm 1.75$ for males

and $4.01 \pm 0.96$ for females) had significantly lower MPO activity compared HKB or budesonide groups. In a separate cohort of animals, the effect of HKB with or without a second administration of BLES (10 mg/ml) was tested. In these animals, the number of neutrophils in the lavage was $3.39 \pm 0.47$ ($n=3$) for the HKB group and $3.25 \pm 0.36$ ($n=4$) for the HKB plus BLES group. Additionally, MPO activity for the HKB group was $10.16 \pm 1.41$ ($n=3$), while HKB plus BLES group had $9.77 \pm 1.06$ ($n=4$). For both measurements, the values were not statistically significant and were similar to values in the HKB group of the first cohort.

The instillation of HKB or HKB followed by budesonide was also shown to result in significantly higher levels of all pro-inflammatory cytokines tested compared to the saline group (Fig. 3a–d). Compared to animals administered HKB, those receiving a second instillation of BLES/budesonide showed significantly lower IL-6 and TNF-α concentrations (Fig. 3a, b). Furthermore, the BLES/budesonide group showed significantly lower concentrations of TNF-α and GRO/KC than the budesonide group (Fig. 3b, c). Although the BLES/budesonide group showed lower levels of MIP-2 and GRO/KC relative to HKB and budesonide groups, the levels were still significantly higher than the saline group (Fig. 3c, d).

## Discussion

This study tested the hypothesis that fortifying an exogenous surfactant preparation, BLES, with budesonide would enhance the efficacy for treating pulmonary inflammation in vivo. Overall, our results supported this hypothesis. Specifically, BLES was shown to enhance the anti-inflammatory effects of budesonide in a rat model of lung inflammation by reducing the number of neutrophils, as well as the concentrations of a several pro-inflammatory mediators in the BAL. Furthermore, combining budesonide with BLES was also shown to be beneficial for reducing MPO activity in the lung tissue. It should also be noted that these results were displayed across both sexes. Based on these observations, it is concluded that utilizing exogenous surfactant as a pulmonary vehicle for budesonide enhanced its ability to treat lung inflammation.

To address our hypothesis, we utilized an experimental in vivo model, where HKB was instilled into the lungs of young rats. The rationale for the use of young rats was practical in nature to limit animal usage, as these animals were also utilized for a separate experiment (see disclosures). The HKB solution contains a mixture of bacterial components, including lipopolysaccharides, that caused a rapid inflammatory response as evidenced by the significant increases in neutrophil counts, MPO activity and inflammatory cytokine concentrations as compared to animals not receiving HKB.
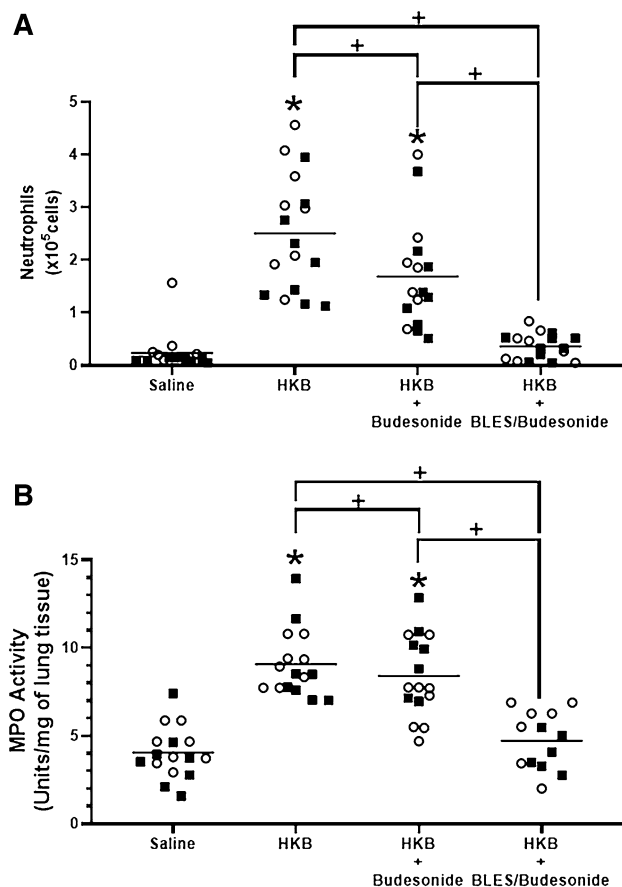


Fig. 2 The effect of instilling budesonide or BLES/budesonide on the **a** number of neutrophils in the BAL and **b** MPO activity in the lung tissue of pediatric rats. Solid squares indicate female rats, open circles represent male rats. $n=10$–$17$, $*p<0.05$ vs saline, $^+p<0.05$
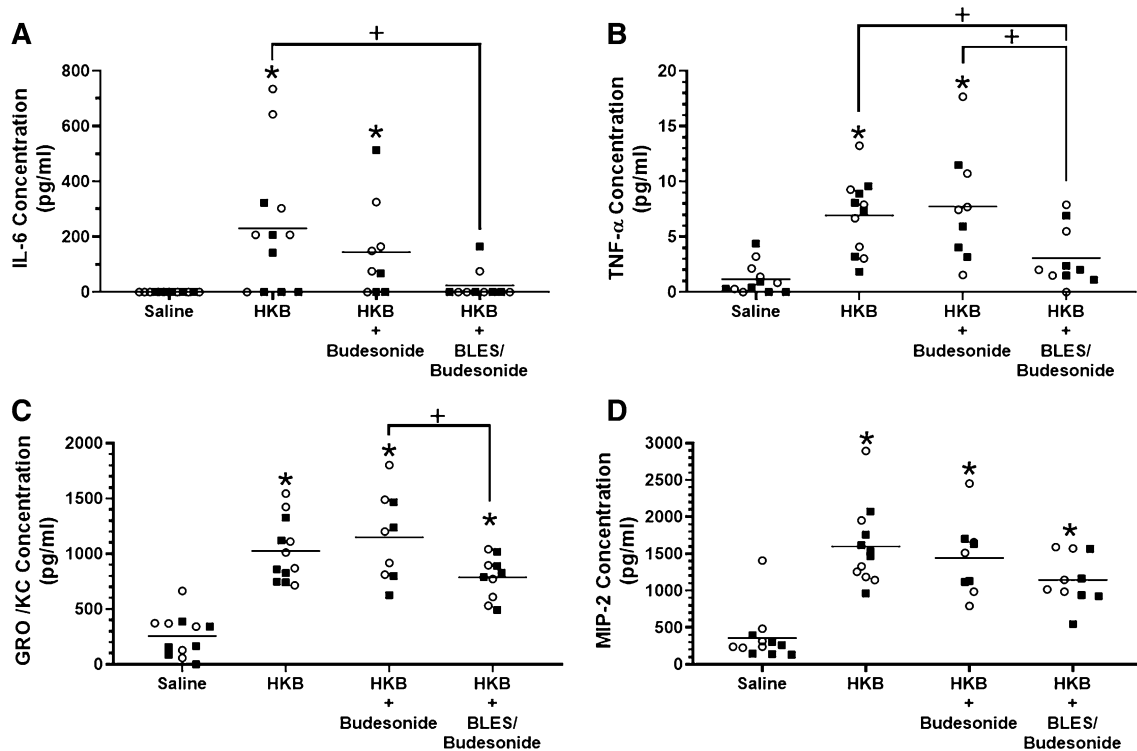
**Fig. 3** The effect of instilling budesonide or BLES/budesonide on pro-inflammatory cytokine concentrations in the BAL: **a** IL-6 or **b** TNF-α, **c** GRO/KC, and **d** MIP-2. Solid squares indicate female rats, open circles represent male rats. $n = 9$–$12$, $*p < 0.05$ vs saline, $+p < 0.05$

The experimental treatment tested was BLES/budesonide for which intratracheal instillation was confirmed via the increased surfactant levels in the BAL fluid following the experiment. It should be noted that our experimental design did not include a BLES only treatment. However, an additional experiment with 7 pups revealed that BLES alone did not impact the inflammatory response induced by the instillation of HKB. Based on these considerations, we deem the experimental approach as an appropriate test of our hypothesis.

An important aspect of this study was the evaluation of a potential glucocorticoid-based treatment strategy in both males and females. Although the objective of this manuscript was not to understand the underlying pathways leading to potential sex differences, numerous studies have demonstrated the role of sex in patient sensitivity to glucocorticoid treatment [21–25]. Unfortunately, these previous studies have also been inconsistent with respect to their findings. For example, when developing guides to predict responsiveness among asthmatic children, both Wu et al. (2017) and Galant et al. (2014) found that the female sex was associated with a higher likelihood of responsiveness to inhaled glucocorticoid therapy [21, 22]. On the contrary, some clinical trials and epidemiological studies have observed beneficial effects for daily glucocorticoid treatment in males, but not females [23, 24]. For the current model of pulmonary

inflammation, the instillation of HKB was found to result in a similar inflammatory response among males and females. Moreover, no sex differences were found for the responsiveness of rats to either of the glucocorticoid treatments. Since sex hormones have been shown to play such an essential role in inflammatory responses, this lack of differences may be related to the young, sexually immature age of the animals [26]. Despite these findings, the extensive role sex hormones play in modulating inflammatory pathways combined with the variability shown in human studies suggests that sex must be considered when evaluating new glucocorticoid-based treatment strategies [27].

Several previous studies have explored different aspects of utilizing an exogenous surfactant as a drug delivery vehicle for glucocorticoids, such as budesonide [6, 11–14]. For example, numerous in vitro studies have characterized the successful incorporation of budesonide into exogenous surfactants, demonstrated their ability to transport budesonide across air–liquid interfaces, and even showcased their ability to improve the drug's anti-inflammatory effects at a distal site, without interfering with the biophysical function of surfactant [6, 10, 11]. In animal experiments, recent data have demonstrated that intratracheally instilling budesonide with an exogenous surfactant enhanced its biodistribution within the lung [13]. Moreover, a rabbit model of meconium aspiration illustrated how the prophylactic administration

of surfactant and budesonide could alleviate inflammation more effectively than budesonide or surfactant alone [12]. Our study adds to these previous observations by comparing the therapeutic effects of budesonide delivered by surfactant to budesonide or surfactant alone, when they are administered after a broad inflammatory insult. The current study also investigates these therapeutic effects across both male and female animals, as well as expanding beyond the general markers of inflammation normally analyzed for this treatment strategy. Specifically, it uses outcomes such as MPO activity, neutrophil counts, and chemokine concentrations to focus on neutrophilic inflammation, which have been suggested to be a critical aspect of disease progression for ARDS [28] When combined with this previous data, our study further supports the use of exogenous surfactant as a delivery vehicle for budesonide in the treatment of pulmonary inflammation.

From a clinical standpoint, this study builds on previous work in the neonatal population. Specifically, it adds to previous clinical studies that explored exogenous surfactant or glucocorticoids as preventative treatments for poor pulmonary outcomes and respiratory conditions like Bronchopulmonary Dysplasia [29, 30]. For example, there are a number of clinical trials which have found that administering surfactant multiple times or using it as a vehicle for budesonide may reduce the risk of Bronchopulmonary Dysplasia [31, 32]. Similarly, there have also been clinical trials that have found intratracheal instillations of budesonide, with a surfactant vehicle helped to prevent the development of chronic lung disease among preterm infants [33]. The current manuscript expands these prophylactic approaches in premature lungs, by demonstrating anti-inflammatory effects of this treatment strategy, subsequent to the pulmonary inflammation, in both males and females.

To extrapolate our data to the clinical arena, there are a variety of respiratory conditions that may benefit from an anti-inflammatory exogenous surfactant; however, its potential for treating ARDS is of particular interest. Over the course of 2020, ARDS has become a well-known syndrome as it is the critical pulmonary complication resulting from severe acute respiratory syndrome coronavirus-2 infections known as COVID-19. However, even before the emergence of COVID-19, ARDS was the most common cause of death in the ICU, with no effective pharmacological therapies available [34–37]. Importantly, it has been shown that disease severity and progression are directly associated with the accumulation of neutrophils into the alveolar space [38, 39], and many aspects of the pathophysiology of ARDS, such as edema formation and surfactant dysfunction, are consequences of excessive inflammation in the lung [3]. This has provided a strong rationale for glucocorticoid-based treatments, as evident by numerous clinical trials for ARDS and an ongoing trial

for COVID-19 patients [40–42]. Unfortunately, to date, these highly effective anti-inflammatory medications have failed to prevent ARDS or show mortality benefits [41, 42]. One interpretation of this data is that the efficacy of the glucocorticoids is limited by suboptimal drug delivery. Based on our data, it is tempting to speculate that exogenous surfactant as a delivery vehicle will allow glucocorticoids to become an effective treatment option for ARDS.

It should be noted that there are several limitations to our study. First, this study only explored the benefits of one surfactant-glucocorticoid preparation. The improvements observed for budesonide when administered with an exogenous surfactant suggest therapeutic value in exploring a similar approach for other glucocorticoids or anti-inflammatory medications. To this end, our lab intends to perform more elaborate in vivo studies with multiple commercially available glucocorticoids, like dexamethasone and hydrocortisone, to further explore the benefits of surfactant delivery. Secondly, although our study shows clear benefits for treating lung inflammation with a surfactant-budesonide preparation, it is important to understand the limitations of our model and the extensive subsequent research that would be needed to translate this therapy to the clinical setting for ARDS. For example, based on the results of a recent study with this model, four main cytokines were selected to be measured [43]. However, there are a wide array of cytokines and inflammatory mediators increased in ARDS patients, not to mention the numerous other patient outcomes that have been shown to be important in disease progression [44, 45]. Moreover, our model of pulmonary inflammation did not imitate the pulmonary edema or airway collapse observed in many respiratory conditions. The current study did measure protein content in the BAL; however, its unchanging level across treatment groups suggests that a stronger stimulus is required to disrupt the alveolar capillary barrier. There is strong scientific evidence that exogenous surfactant can overcome regions of edema and airway collapse; however, future studies will be needed to evaluate this treatment strategy under inhibitory conditions and determine its efficacy for other important outcomes [3, 46].

In conclusion, this paper demonstrates that the use of exogenous surfactant as a delivery vehicle for budesonide can make it more effective for treating lung inflammation. Further, we propose that this novel treatment strategy can overcome the delivery challenges associated with respiratory conditions like ARDS and treat the neutrophilic inflammation underlying the disease. With no effective pharmacological options currently available for this condition, direct delivery with exogenous surfactant offers an intriguing method for mainstay medications to start effectively treating this devastating disease.

## Compliance with Ethical Standards

**Conflict of interest** The authors declare that they have no conflicts of interest.

**Research Involving Human and Animal Rights** Data reported in the manuscript on the response of male and female rats administered only saline or HKB are also utilized in another manuscript as control animals to examine the effect of fetal growth restriction on inflammation (https://doi.org/10.1139/cjpp-2020-0431). Animals were fully randomized for both studies.

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