COLCHICINE INHIBITS ELEVATIONS IN BOTH ALVEOLAR-CAPILLARY MEMBRANE PERMEABILITY AND LAVAGE SURFACTANT AFTER EXPOSURE OF THE RAT TO PHOSGENE

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Running Head: $COCl_2$, permeability, and surfactant

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Abstract. Colchicine inhibits elevations in both alveolar-capillary membrane permeability and lavage surfactant after exposure of the rat to phosgene. Ghio, A. J. and Hatch, G. E. (1991) Toxicol. Appl. Pharmacol. , - .

Colchicine inhibits neutrophil incursion into the lung after $COCl_2$ exposure and reduces lung injury as reflected by lavage protein. This may indicate either a decrement in damage to the alveolar-capillary membrane or a reduction in the accumulation of lavage surfactant after $COCl_2$ exposure. We measured changes in the permeability of the alveolar-capillary membrane and lavage surfactant concentrations after $COCl_2$ exposure and the influence of colchicine on both. Rats were treated with either colchicine 1.0 mg/kg or saline IP thirty minutes prior to $COCl_2$ 0.5 ppm X 60 minutes and air. To measure permeability, ten microcuries of ^{125}I bovine serum albumin in a volume of 0.30 ml were injected via the tail vein immediately and one day after exposure. After two hours, radioactivity of blood and lavage fluid was measured. Lavage surfactant was quantified as the total phosphorous immediately and one day after exposure. The two sources of protein showed very different responses to phosgene. Permeability was elevated immediately but returned to normal values one day after inhalation. Values of total phospholipid in the lavage fluid showed no immediate differences between phosgene and air exposed rats. However, significant increases were observed one day after $COCl_2$ inhalation. Colchicine inhibited both the immediate rise in the permeability index and the elevation of total phospholipid one day after exposure to phosgene.

Introduction.

Phosgene ($COCl_2$) is a highly toxic gas first used as an agent of warfare in 1915 and later widely applied industrially in the synthesis of isocyanates, polycarbonates, polyurethanes, acid chlorides, dye intermediates, and pesticides (Babad and Zeiler, 1973) As a result, it is estimated that a considerable number of workers are at risk of its exposure (National Institute for Occupational Safety and Health, 1976). Inhalation of a significant concentration X time product of phosgene is followed by a non-cardiogenic pulmonary edema and death after a clinical latent phase whose duration is inversely proportional to the extent of the exposure.

The mechanism of phosgene toxicity is not known but hypothesized to result from its low solubility in water (Nash and Pattle, 1971) and its capacity to rapidly acylate many different organic compounds (Potts et al, 1949). Its hydrophobic character allows entry into the lower respiratory tract where it can react with several functional groups (amino, hydrazino, sulfhydryl, and hydroxyl) of biological importance (Gerard, 1948). These reactions are assumed to result in immediate structural damage to the alveoloar-capillary membrane permitting plasma to enter the interstitium and alveoli. The ability of the lung to clear this fluid will be exceeded and edema results. This will become clinically apparent with continued accumulation and is the terminal event in most instances of death (Bruner and Coman, 1945).

Similar to the ozone and nitrogen dioxide, phosgene exposure induces a neutrophil influx (Currie et al, 1987; DeNicola et al, 1979; Seltzer et al, 1986). Recruited neutrophils further damage to the lung tissues through release of endogenous

oxidants and proteases. Inhibition of this neutrophil influx by cyclophosphamide, an inhibitor of leukotriene B_4 (AA861), or colchicine reduces both injury and mortality after inhalation of $COCl_2$ (Ghio et al, 1991). Increases of lavage neutrophils and protein at 24 hours after exposure to phosgene 0.5 ppm X 60 minutes were suppressed with colchicine 1.0 mg/kg treatment given intraperitoneally (IP) thirty minutes prior to exposure. Colchicine inhibits microfilaments whose contractile state effects the permeability of epithelial cells (Bentzel et al, 1980; Welsh et al 1985). Consequently, inhibition of the rise in lavage protein by colchicine was considered to reflect a decrement in damage to the alveolar-capillary membrane and the resultant pulmonary edema after $COCl_2$ exposure (Guth et al, 1986). However, surfactant is also elevated after phosgene inhalation and its associated proteins account for some portion of the elevated protein concentrations observed in the lavage fluid (Frosolono and Currie, 1985). We measured changes in the permeability of the alveolar-capillary membrane and lavage surfactant concentrations after $COCl_2$ exposure and the influence of colchicine on both.

Methods.

Materials included colchicine from Eli Lilly and Company (Indianapolis, IN), halothane from Halocarbon Laboratories Inc. (Hackensack, NJ), and ^{125}I labelled bovine serum albumin from New England Nuclear (Cambridge, MA). All other reagents were from Sigma (St. Louis, MO).

Animals were housed in temperature- and humidity-controlled rooms and fed a standard diet (Ralston Purina Co., St. Louis, MO). Food and water were available ad

libitum except while in the exposure chambers. Sixty-day old, male Sprague-Dawley rats (Charles River Breeding Labs, Wilmington, MA) were treated with either colchicine 1.0 mg/kg or saline IP thirty minutes prior to the exposure. Exposures to $COCl_2$ 0.5 ppm X 60 minutes and air were accomplished as described previously (Ghio et al, 1991). The resulting groups included: phosgene exposed after saline IP, phosgene exposed after colchicine IP, air exposed after saline IP, and air exposed after colchicine IP.

Percent neutrophils and protein in the lavage fluid were quantified immediately (0 to 2 hours) and one day (24 to 26 hours) after exposure. A total of 48 animals were used (6 per group at the 2 times specified). Animals were anesthetized with halothane, exsanguinated, and lavaged with a volume of saline which equaled 90% of their total lung capacity (35 mg/kg body weight) (Takezawa et al, 1980). Two hundred microliters of lavage fluid were pelleted on a microscope slide using a cytocentrifuge (Shandon Southern Instruments, Inc., Sewickley, PA) at 300 g X 3 minutes, dried, and stained with a modified Wright's stain (Diff-Quick Stain, ASP, McGaw Park, IL). The percentage of neutrophils rather than an absolute cell number in the lavage fluid was used to measure neutrophil influx (Currie et al, 1987). Cell differential counts were enumerated by counting 500 cells and expressed as a percentage. The remainder of the lavage fluid was centrifuged at 700 x g for ten minutes to remove cells. Protein in the supernatant was determined using a modification of Lowry's method (Lowry et al, 1951).

Permeability measures can reflect changes in either transport of serum proteins through an ineffective barrier or epithelial hypersecretion. Albumin was used as a tracer because of its high molecular weight and low permeability. Measurements can be made

of the movement of the radiolabelled tracer from the blood to the airways after intravenous injection or the converse. The former route was used as a result of its increased sensitivity and decreased variability. Ten microcuries of ^{125}I bovine serum albumin in a volume of 0.30 ml were injected via the tail vein immediately (0 to 1 hour) and one day (24 to 25 hours) after exposure. Forty-eight animals were used (6 per group at the 2 times specified). A short time interval was used between the injection and sampling to minimize changes in permeability that can occur during the measurement. Two hours after injection, rats were anesthetized with inhaled halothane (2 to 5%). The thorax was opened, blood was sampled by cardiac puncture, the abdominal aorta was cut, and the lungs were lavaged. Radioactivity of blood and lavage fluid was measured with a gamma counter (Packard Gamma Counter, Packard Instruments, Downers Grove, IL). Transfer of the radiolabeled tracer across the alveolar-capillary membrane is quantified as radioactivity in the lavage fluid. Data is expressed as a permeability index:

$$\frac{\text{[Radioactivity in 1.0 ml of tracheal lavage fluid]}}{\text{[Radioactivity in 1.0 ml of blood]}} \times 100$$

Disaturated phosphatidylcholine and phosphatidylglycerol are two phospholipids found in high concentrations in surfactant. Together they serve as a nonspecific marker for surfactant. Lavage surfactant was measured as the total phosphorous immediately (0 to 2 hours) and 1 day (24 to 26 hours) after exposure. Rats were lavaged with saline (35 mg/kg of body weight), the fluid collected, and the instillation repeated four more times. Lipid was extracted from the accumulated fluid and phosphorous content was quantified (Bartlett, 1959). Phospholipid amounts were calculated assuming 4% phosphorous

content.

Data is expressed as mean values $\pm$ standard error. Analysis of variance is used to determine differences between multiple groups (Colten, 1974). Duncan's Multiple Range Testing was selected as a post-hoc test of significance (Duncan, 1955). Significance is assumed at $p < .05$.

Results.

Phosgene and colchicine effected neither the weight of the rat (284 $\pm$ 3 g) nor lavage return (79 $\pm$ 3%). Similar to ozone and nitrogen dioxide, a time-dependent accumulation of neutrophils was apparent after $COCl_2$ inhalation (Figure 1). This influx of neutrophils was not significant immediately but was one day following phosgene exposure. Unlike percent neutrophils, there were significant increases in the protein concentrations in the lavage fluid immediately after $COCl_2$ inhalation (Figure 2). Lavage protein remained elevated one day after phosgene. The lack of a significant neutrophil incursion immediately after phosgene exposure contrasts the elevation of protein in lavage fluid at this time and suggests a mechanism of tissue damage independent of inflammatory cells.

Recovery of intravenously ^{125}I-labelled serum albumin from the alveolar space provides a sensitive measure of injury to the alveolar-capillary membrane observed before morphologic changes of lung injury (Alpert et al, 1971). Comparable to ozone and nitrogen dioxide, the permeability index was elevated after $COCl_2$ inhalation (Alpert et al, 1971; Sherwin and Richters, 1971). Coinciding with the increase in lavage protein,

the permeability index was elevated immediately but returned to normal values one day after inhalation (Figure 3). This change in the permeability index was measured prior to significant increases in percent neutrophils in the lavage fluid. Correction of the index by one day provides supportive evidence for the success of repair processes in the lung.

Surfactant is a complex mixture of phospholipids and apoproteins. Proteins account for approximately 10% of its composition (Holm and Matalon, 1989). Values of total phospholipid in the lavage fluid showed no immediate differences between phosgene and air exposed rats (Figure 4). However, significant increases were observed one day after $COCl_2$ inhalation. The two sources of protein showed very different responses to phosgene exposure with permeability changes and surfactant elevations occurring immediately and one day after respectively.

Colchicine binds tubulin to disrupt formation and function of microtubules (Famey, 1988). It is widely distributed in the body but concentrates in leukocytes. Pre-treatment of animals with colchicine 1.0 mg/kg IP inhibited the neutrophil influx after phosgene (Figure 1). In addition, it prevented increases in lavage protein both immediately and one day after $COCl_2$ inhalation (Figure 2). Corresponding with these effects on protein, colchicine inhibited both the immediate rise in the permeability index (Figure 3) and the elevation of total phospholipid one day (Figure 4) after exposure to phosgene.

Discussion.

Our data indicates that 1) injury to the lung, reflected by a rise in lavage protein,

occurs within 0 to 2 hours after $COCl_2$ inhalation in rats and is not associated with a significant neutrophil influx, 2) increases in alveolar-capillary permeability occur within 1 to 3 hours after exposure to phosgene and corrects by 27 to 29 hours, 3) alveolar surfactant is not effected immediately but is also increased by 24 to 26 hours after $COCl_2$ inhalation, and 4) colchicine inhibits the neutrophil influx, increases in lavage protein and permeability, and elevations in surfactant after phosgene exposure.

The time-dependent incursion of neutrophils into the lung after $COCl_2$ exposure demonstrated by our investigation is consistent with other studies (Currie et al, 1987). Leukotriene B_4 has been identified as one possible mediator of this cellular influx (Assaad et al, 1990). These cells contribute to tissue injury one day after exposure as reflected by lavage proteins (Ghio et al, 1991). In this investigation, however, protein was significantly elevated immediately after phosgene exposure while the intrusion of neutrophils was not. This supports a mechanism of tissue damage by $COCl_2$ independent of neutrophils which might include direct acylation of membranes or generation of free radicals with subsequent membrane damage. Alternatively, sampling the lavage fluid may not reflect the influx of PMNs into the pulmonary interstitium.

Corresponding with the lavage protein, there was an increase in the transfer of radiolabelled bovine serum albumin immediately after phosgene exposure confirming a permeability defect in the alveolar-capillary membrane. The transport of this tracer supports other evidence of injury to the membrane. In addition to lavage protein, lung damage after phosgene has been measured as an increase in lung dry weight (Currie et al, 1987), lung wet weight to body weight ratio (Coman et al, 1947), lung water

(Frosolono and Pawlowski, 1977), and lung wet weight (Franch and Hatch, 1986). It is also reflected in abnormalities in pulmonary function including compliance (Rossing, 1964) and diffusing capacity (Long and Hatch, 1961). Finally, edema after $COCl_2$ inhalation can be directly observed histologically (Tobias, 1945; Bruner and Coman, 1945). The potential mechanisms of edema formation are multiple and include increases in the hydrostatic pressures of the pulmonary capillaries, disruption of respiratory epithelium, and increased endocytic transport of osmotically active molecules such as albumin. Phosgene exposure has not been observed to be associated with changes in vascular pressures (Gibbon et al, 1948; Kennedy et al,) and edema is unlikely to be hydrostatic in origin. The normal respiratory epithelium is an extremely efficient barrier to the movement of macromolecules and fluid (Richardson et al, 1976). Adjacent cells join apically to obliterate intercellular space and form tight junctions. A disruption of these junctions may follow exposure to phosgene and oxidant gases allowing transfer of macromolecules and solutes. It is also possible that $COCl_2$ inhalation increases endocytic transport of macromolecules which induce pulmonary edema through an increase in oncotic pressure. However, evidence supports a defective barrier with leakage of serum proteins as the primary mechanism of edema formation after phosgene exposure. Collection of edema fluid by inverting a $COCl_2$ exposed animal reveals a protein concentration approximating that of plasma (Cameron and Courtice, 1946). The electrophoretic profile of lavage proteins after exposure to other oxidant gases is similiar to that of serum proteins (Selgrade et al, 1981). After inhalation of other oxidant gases, freeze fracture techniques demonstrate a disruption a tight junctions which would allow

paracellular transfer of serum contents (Case et al, 1982; Boucher et al, 1980). Intravenous instillation of horseradish peroxidase after these exposures is followed by its paracellular movement supporting a disruption of the tight junctions (Gordon et al, 1983; Hulbert et al, 1981). Furthermore, morphologic studies after phosgene exposure directly illustrate damage to the epithelium providing evidence that an effective epithelial barrier has been disrupted (Diller, 1985). The result is a leakage of proteins from the serum probably through a paracellular pathway.

Lavage surfactant, as reflected by total phospholipid, was not elevated immediately but increased one day after $COCl_2$ inhalation. This elevation is consistent with previous observations not only after phosgene but also following exposures of the rat to other oxidant gases (Blank et al, 1978; Shelley et al, 1989). Surfactant phosphatidylcholine is synthesized by type II cells, transferred to vesicles which mature to lamellar bodies, and secreted to the alveolus (Jobe et al, 1988). The kinetics of this movement are slow and an increased release of surfactant is predicted to take hours. While the increased lavage protein immediately after phosgene exposure is the result of a permeability defect, elevated proteins one day later are associated with surfactant. However, as a result of the permeability index indicating the condition of the barrier during the two hours between injection and sampling only, some portion of the differences in proteins one day after exposure may still reflect an accumulation of serum molecules which have been transported through a damaged alveolar-capillary membrane.

Colchicine inhibited both the immediate increase in permeability and elevations of surfactant one day after $COCl_2$ inhalation. Effects of the medication on permeability

may be dependent on its inhibition of the cytoskeleton. Microfilaments function as cytoskeletal units responsible for cell contact. Oxidants increase the permeability of cultured epithelium through altering the cytoskeleton of the tight junctions allowing paracellular movement of solutes (Welsh et al, 1985; Diamond, 1974). Destabilization of actin by colchicine might inhibit such a detrimental reaction to phosgene and oxidant gases. Alternatively the effect of colchicine on permeability could be mediated by its inhibition of the neutrophil influx. Consequences of colchicine treatment on neutrophils include diminished motility, a decreased production of chemotactic factors, and inhibition of both extracellular release of proteases and bursts of respiratory metabolism (Malawista, 1975). Inflammatory cells do effect epithelial injury and increase permeability. Depletion of cells using cyclophosphamide inhibited increases in permeability after exposure to ozone (Bhalla and Daniels, 1991). A significant influx of neutrophils could be present in the interstitium mediating injury and increasing permeability immediately after $COCl_2$ exposure. However, this cellular incursion was not apparent either in lavage fluid or on histology at a time when pulmonary edema is initially observed after $COCl_2$ exposure (Diller et al, 1969).

Inhibition of elevations of surfactant may result from colchicine's effect on cell trafficking and secretion which require microtubular function (Kelly, 1990). Disruption of the microfilaments by colchicine would decrease delivery of surfactant to the apical surface and subsequently, its accumulation in the lower respiratory tract. This effect of colchicine might be of benefit in the treatment of pulmonary alveolar proteinosis although the doses employed in these studies cannot be approached in humans without

toxic effects.

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Legend

Figure 1. Percent neutrophils in lavage fluid of rats exposed to phosgene and treated with colchicine. *Phos* = phosgene exposed and injected with saline; *PhosCOLC* = phosgene exposed after treatment with colchicine; *Air* = air exposed and injected with saline; and *AirCOLC* = air exposed after treatment with colchicine. Differences in percent neutrophils among the 4 groups are not observed immediately ($F=1.87$; $p=.17$) but are present 24 hours after phosgene exposure ($F=6.17$; $p<.05$). Post-hoc testing indicates *Phos* to be greater than all other groups (+).

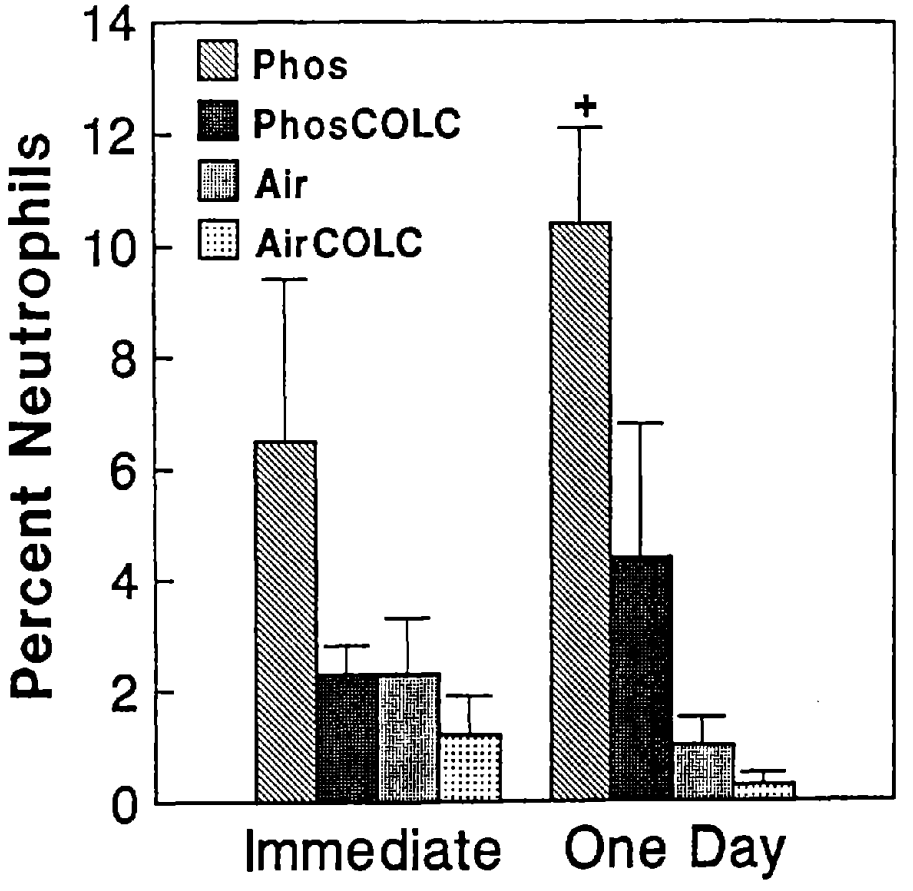
Figure 2. Protein in lavage fluid of rats exposed to phosgene and treated with colchicine. *Phos* = phosgene exposed and injected with saline; *PhosCOLC* = phosgene exposed after treatment with colchicine; *Air* = air exposed and injected with saline; and *AirCOLC* = air exposed after treatment with colchicine. Disparities in protein among the four groups are found immediately following phosgene exposure ($F= 4.63$; $p<.05$) with *Phos* being greater than all other groups. Similar differences are observed at 24 hours ($F=13.54$; $p<.05$) with *Phos* again being greater than all other groups (+).

Figure 3. Permeability indices of rats exposed to phosgene and treated with colchicine. *Phos* = phosgene exposed and injected with saline; *PhosCOLC* = phosgene exposed after treatment with colchicine; *Air* = air exposed and injected with saline; and *AirCOLC* = air exposed after treatment with colchicine. Differences in the permeability index among the four groups are detected immediately after phosgene exposure ($F=3.94$; $p<.05$) with

Phos being greater than all other groups (+). By 24 hours after phosgene inhalation, abnormalities in permeability have corrected with no differences between the groups ($F=0.48$; $p=.75$).

Figure 4. Total surfactant in the lungs of rats exposed to phosgene and treated with colchicine. *Phos* = phosgene exposed and injected with saline; *PhosCOLC* = phosgene exposed after treatment with colchicine; *Air* = air exposed and injected with saline; and *AirCOLC* = air exposed after treatment with colchicine. No differences in surfactant is observed among the four groups immediately after phosgene exposure ($F=0.69$; $p=.57$). By 24 hours after phosgene inhalation, surfactant is found to be significantly elevated in *Phos* (+) ($F=6.63$; $p<.05$).

Index Terms: colchicine; phosgene; respiratory distress syndrome, adult; surfactant; lung; rat.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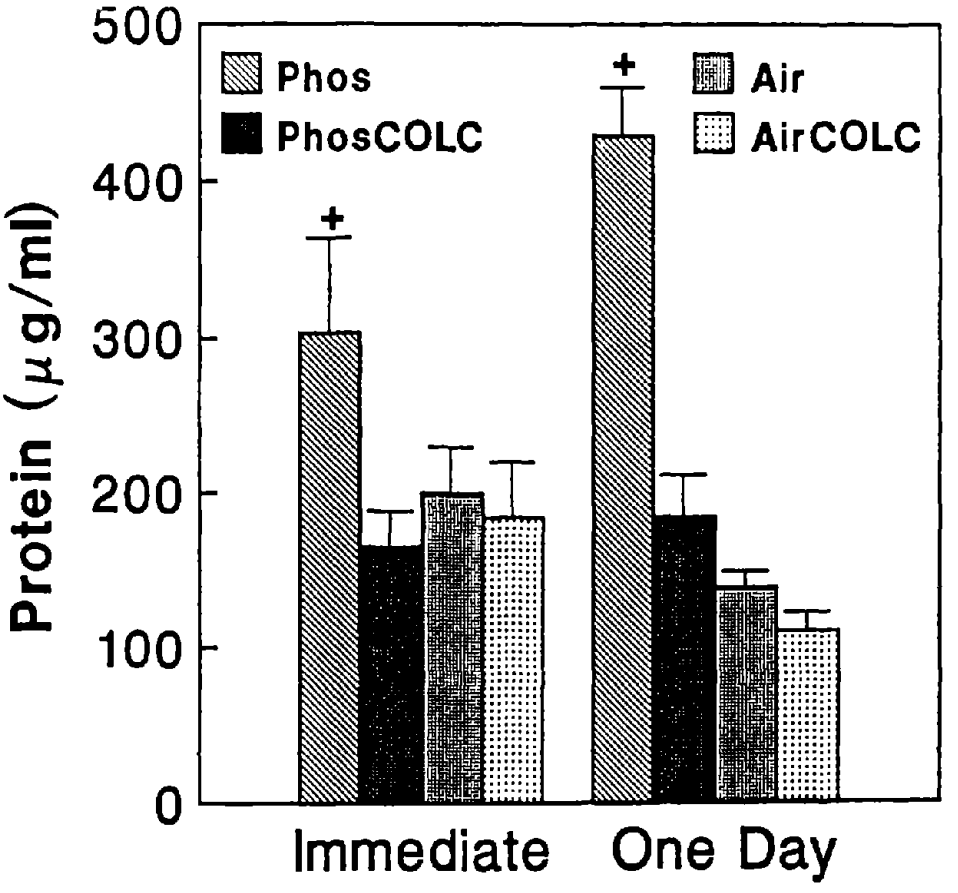
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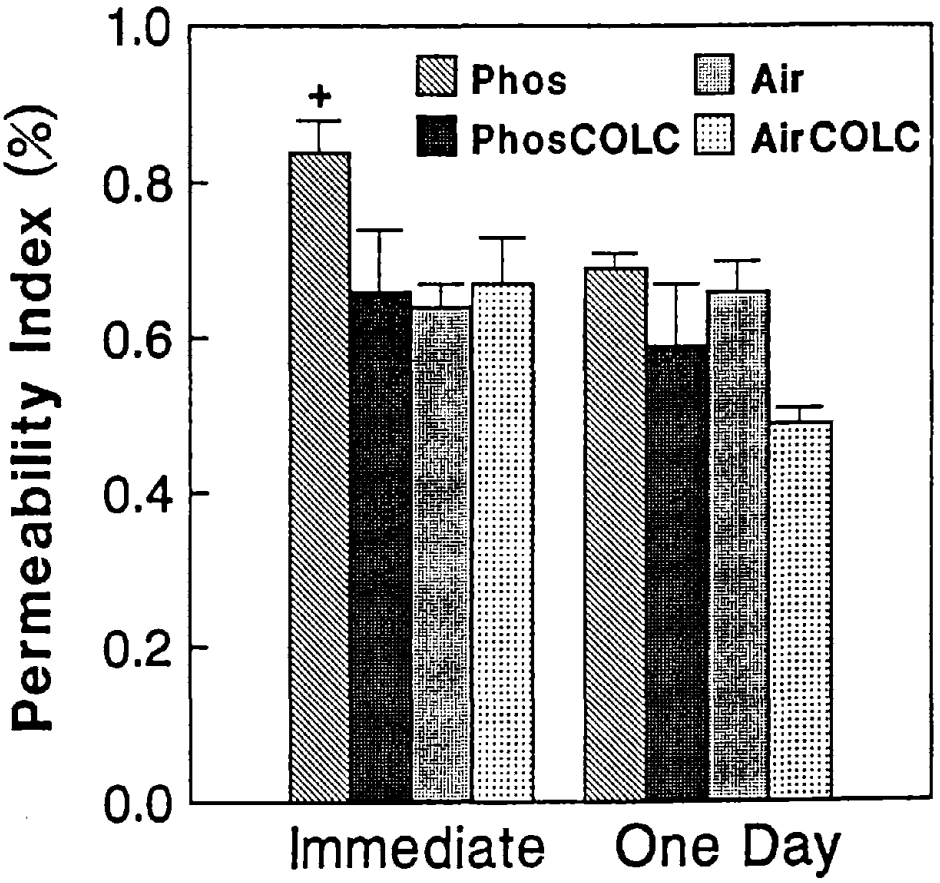
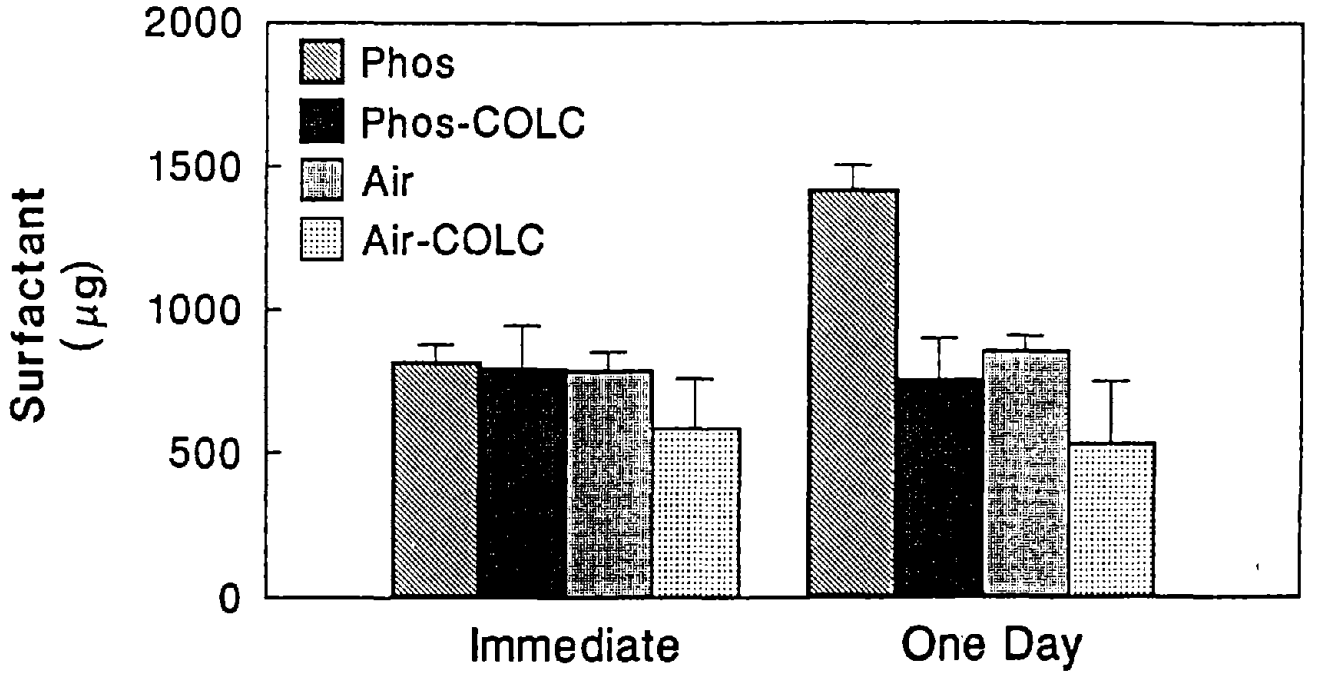
300
200
100
0
Protein (μg/ml)
Phos
PhosCOLC
Air
AirCOLC
+
+
Immediate
One Day


1.0
0.8
0.6
0.4
0.2
0.0
Permeability Index (%)
+
Phos
Air
PhosCOLC
AirCOLC
Immediate
One Day


2000
1500
1000
500
0
Surfactant
(μg)
Phos
Phos-COLC
Air
Air-COLC
Immediate
One Day