

THE PATHOLOGICAL EFFECT OF THREE-METHYLINDOLE ON LUNGS
AND LUNG SURFACTANT COMPOSITION IN LAMBS

by

JOE A. KUBICEK

MVDr., University of Veterinary Medicine, Brno, 1967

A MASTER'S THESIS

submitted in partial fulfillment of the
requirements for the degree

MASTER OF SCIENCE

College of Veterinary Medicine

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1987

Approved by:


Major Professor

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ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my major professor, Dr. Jerome G. E. Vestweber, for the guidance and help.

Further, I would like to thank Dr. Horst W. Leipold, a member of my graduate committee, for the guidance and help.

My thanks also go to Dr. Larry M. Seitz, a member of my graduate committee, for the guidance and help.

Last but not least, I would like to thank Dr. William E. Klopfenstein for the guidance and help at the start of the project.

As well, I would like to express my thanks to the Department of Surgery and Medicine of KSU for funding of our project.

GENERAL INTRODUCTION

Three-methylindole (3MI=skatole), a ruminal metabolic product of tryptophan,^{1,2} produced by *Lactobacillus* species,³ causes acute pulmonary edema and emphysema in ruminants,⁴⁻⁷ and bronchiolitis in horses,⁸⁻¹⁰ when administered orally or intravenously.^{4,5} Three-methylindole is changed by mixed function oxidase (MFO) in lung cells into a reactive intermediate,^{3,8,10} which damages the lungs. This intermediate interacts with lecithin, which is present in cell membranes, resulting in membrane disruption of type I pneumocytes and Clara cells primarily.^{7,11} For this reason, necrotic bronchiolitis and alveoli void of type I pneumocytes are detectable in ruminants administered 3MI.^{3,7,9} To heal the resulting damage, bronchioles become epithelized, type II pneumocytes multiply and transform into type I cells.¹² Cell membranes of erythrocytes may also be damaged, and result in hemolyzed blood in the vasculature system, in urine, and inside pulmonary airways.^{3,5,13} Type II pneumocytes also produce lung surfactant,¹⁴ which is the lining material present in alveoli and airways. The surfactant composition may change, and its amount decreases in acute bovine pulmonary edema and emphysema.¹⁵

Three-methylindole was administered intravenously and intraperitoneally to lambs and adult sheep in this study to produce pneumotoxicity. Both lambs and sheep were killed at different intervals following administration of 3 MI. The major objectives were to study the sheep clinically, examine the lungs by gross and histopathological methods, and qualitatively determine surfactant composition.

GENERAL LITERATURE REVIEW

Acute bovine pulmonary edema and emphysema (ABPE), which is also called fog fever in Great Britain,^{2,3} usually occurs in beef cattle over 2 years of age.^{3,13} It is found commonly within 2 weeks after a change to a lush pasture, when preceded by a period of relative starvation.^{3,13} It has also been reported in dairy cattle, where it occurs rarely.^{3,16} ABPE is caused by a high tryptophan content in the lush pasture.^{2,3} Lactobacillus species in the rumen will produce 3MI (skatole) from tryptophan³ which is then absorbed from the fore-stomachs,^{1,17} and changed by the mixed function oxidase system (MFO) in the lungs into a reactive intermediate^{3,8,10} (imine methide of 3MI¹⁸). This compound then interacts with lecithin in cell membranes of type I pneumocytes and Clara cells, and disrupts them.^{7,11} The same disease can be reproduced experimentally by 3MI administration to ruminants orally or intravenously.⁴⁻⁶

Clinical Signs

Clinical signs are usually visible up to 12 hours following 3MI administration,^{4,5} and the onset is dependent on the dosage. Signs are detectable in adult ewes 1.5 to 2 hours following oral administration of 3 MI at 0.6 g/kg body weight (BW), or 3 to 4.5 hours following 0.2 g/kg BW.⁴ In cattle, ABPE was detected 6 to 12 hours following 0.2 g of 3MI/kg BW orally or 0.06 g/kg BW intravenously (IV).⁵ Early clinical signs consisted of muscular trembling, flared nostrils, irregular breathing, increased respiratory rate, dyspnea, kicking motions of hind limbs, and unproductive attempts at micturition.⁴⁻⁶ Cough was also observed in goats.⁶ Later, the

breathing became labored, the respiratory rate decreased, and an audible respiratory "grunt" developed.^{4,6} If the dosage of 3 MI was high (0.3 g/kg BW in goats orally or 0.6 g/kg BW in ewes orally), lateral recumbency, cyanosis of mucous membranes, frothy pinkish to straw-colored nasal and oral discharges occurred in 1.5 to 2 hours and death occurred 10 to 27 hours later in ewes, or goats died 5 to 11 hours later.^{4,6} Death commonly occurs by asphyxiation.⁶

Pulmonary function tests in goats, that were given 0.2 g of 3MI/kg BW orally, revealed a marked decrease in dynamic lung compliance, a moderate increase in airway resistance, hypoxemia, a sustained increase in respiratory frequency, a progressive decrease in tidal volume and alveolar ventilation, and an increased dead space to tidal volume ratio.²¹ A mild metabolic acidosis also developed.²¹ The tachypnea appears to be vagally mediated, because the respiratory rate decreased in horses administered 3MI after vagotomy.⁹

Gross Pathological Lesions

A post mortem examination revealed enlarged non-collapsed lungs which were firm to turgid and red.⁴⁻⁶ Severe pulmonary edema was predominant in acute stages,⁵ thickened alveolar septa and alveolar cell proliferation were found later.^{5,6} The airways were filled with froth, which was sometimes bloody.^{3,5} Hemorrhagic lesions were present in the larynx, trachea, and bronchi of animals dying from ABPE.^{3,19} The most severe lesions were found in ventral regions of the lungs. Focal pulmonary lesions were found in mild cases, while diffuse lesions were found with severe clinical cases of ABPE.² Pulmonary emphysema is believed to be caused by small doses of 3MI

given to goats, while larger doses (0.2, resp. 0.3 g/kg BW) cause pulmonary edema.⁶

The lesions in sheep are more variable than in cattle and goats, and sheep appear to require higher doses than the other two species before developing clinical signs.⁴

The severity of lesions produced by 3MI depends on the initial dosing, route of administration, animal's tolerance and age, and type of ration (more severe on hay with concentrate than on green forage).²² Repeated administration of 3MI in calves either leads to 3MI tolerance in which no lesions develop,²² or diffuse pulmonary fibrosis and alveolitis.²³

When 3MI is administered to calves 3 months of age, the disease syndrome is mild and the lesions are focal.²⁰ The authors concluded that calves are more resistant to pulmonary cytotoxicity than adults.²⁰

Histopathology

The microscopic lesions include alveolar and interstitial edema, focal hemorrhages and alveolar epithelial swelling.⁴⁻⁶ Alveolar spaces become filled with proteinaceous material,^{4,5,7} and mononuclear cells and cellular debris are found in bronchial trees.⁴ Epithelia lining cells in small bronchioles are slightly thickened and alveoli are filled with large mononuclear cells and neutrophils.⁴ Alveolar type I cells will degenerate⁷ and eosinophilic cellular debris is found in the alveoli.⁴ Alveolar hemorrhage can be present.⁵ The lining cells of the terminal bronchioles are usually necrotic⁵⁻⁷ and some bronchioles can become plugged with necrotic material.^{5,6}

Interlobular and subpleural septa are distorted by emphysema.⁵ In goats given a smaller dose of 3MI (0.1 g/kg BW), edema is less marked and a considerable proliferative cellular change is found.⁶ Alveolar walls are ruptured and form coalescing foci of emphysema.⁶

Another study in goats found that emphysema was secondary to labored breathing, but this was not a consistent feature in cattle.⁷ Cells present in alveoli are mostly neutrophils, but a few eosinophils and large mononuclear cells are also present.⁶ Eosinophillic hyaline membranes are found, lining some alveoli.^{3,6} In goats receiving a smaller dose of 3MI (0.1 g/kg BW intraruminally), proliferation of type II pneumocytes with frequent mitotic figures are seen.⁶ This also occurs in cattle.³ Alveolar epithelial hyperplasia of type II pneumocytes can result in up to 30 of these cells being found in an alveolus.¹² Intravascular hemolysis and hematuria are found in cattle following 3MI administration,⁵ and this can be explained by an interaction of 3MI intermediate with cell membranes, and resulting rupture.⁷ Lung lesions may correspond to the different vascular blood perfusion patterns of the lung lobules.²⁰ The most severe lung lesions probably develop in lung areas which are better perfused.²⁰

Electronmicroscopic Lesions

Changes found 4 to 8 hours following 3MI administration include extensive degeneration and necrosis of type I pneumocytes and bronchiolar epithelium.^{7,11} This results in interstitial and alveolar edema, which is followed by proliferation of type II pneumocytes.^{7,11} Type I pneumocytes and Clara cells also contain dilated endoplasmic reticulum, large cytoplasmic vesicles, and swollen mitochondria with

distorted cristae.^{7,11} Cell junctions are unaltered.⁷ Large vesicles are also found in both bronchiolar ciliated and nonciliated (=Clara) cells.^{7,11} Many type I pneumocytes, which are presumed to be shed into the alveolar spaces, have a diffuse increase in electron density and are separated from the basal lamina.^{7,11} Both interstitial and alveolar edema are very prominent: alveoli frequently contain erythrocytes.⁷ Type I pneumocytes also appear extremely swollen.¹¹

Twenty-four hours following the administration of 3MI both the alveolar and bronchiolar basal laminae are devoid of cellular lining and the alveolar edema fluid contains more fibrinous material.⁷

Forty-eight hours following administration of 3MI large areas of alveolar surface were still denuded, but some areas were partially covered by type II cells which contained free ribosomes.⁷ Occasional mitoses were also observed in type II cells, and smooth endoplasmic reticulum was still swollen.⁷

Seventy-two hours following 3MI administration in goats, a pathognomonic accumulation of glycogen alpha particles was observed in type II pneumocytes.²⁴ Defective lamellar bodies containing triglycerides instead of phospholipids were also observed.²⁴ These observations suggest an interference with surfactant production. Lysosomal particles and multivesicular bodies were also completely absent in type II cells.²⁴

Muscular pads of pulmonary veins were thicker 72 hours following 3MI administration in cattle, glycogen was accumulating in venous smooth muscle cells, myointimal cells were proliferating and the cytoplasm was protruding.²⁵ These findings suggest vasoconstriction

of the pulmonary veins. A migration of lymphocytes, platelets and erythrocytes through the vascular wall was also found in postcapillary venules.²⁵ A significant increase in the thickness of the media of pulmonary veins was also found in 3MI treated animals.²⁵

Microsequestration of rough endoplasmic reticulum was present in cattle 72 hours following 3MI administration.²⁶ A proteinaceous material was also stored in dilated cisternae of this reticulum.²⁶ Massive storage of glycogen particles was found in smooth muscle cells of the media.²⁶ These alterations may signify the early changes associated with pulmonary hypertension.²⁶

Biochemical Alterations

The reactive metabolite of 3MI is an imine methide adduct (radical), produced by the mixed function oxidase system in the lungs.^{3,10,18} Acute pulmonary edema and emphysema caused by 3MI administration can be prevented in goats by treatment with piperonyl butoxide, which inhibits the MFO system.¹⁰ Mixed function oxidase inducers, such as phenobarbital, will increase the severity of the disease in goats and ponies.^{8,10}

Cows which are given chlortetracycline orally several days before oral administration of tryptophan will not develop respiratory clinical signs because the conversion of tryptophan to 3MI is possibly prevented.²⁷ The polyether antibiotics, monensin or lasalocid, when fed before tryptophan administration, also prevented respiratory signs in cattle.²⁸⁻³¹ Hemoglobinemia and hemoglobinuria will still be present in cattle pretreated with monensin, and later treated with tryptophan, because tryptophan is converted into indole instead of 3MI

by ruminal microflora.^{29,30} Indole will cause hemolysis, hemoglobinuria and hemoglobinuric nephrosis, but it is not pneumotoxic.^{1,30,32} No evidence has been found for involvement of oxidative stress in the 3MI pneumotoxicity.³³ Superoxide, singlet oxygen, and hydroxyl radicals play no role in the lung damage caused by 3MI.³³

Lung surfactant produced by type II pneumocytes^{14,34} exerts both antiatelectatic and antiedematous properties.³⁴ It also acts in a nonspecific particle defense,^{34,35} as it enhances surface flux and enables bacteria precoat. Surfactant primarily enhances transport of particles from alveoli into terminal bronchioles³⁵ by a surface-pressure gradient. The precoat of bacteria with surfactant helps macrophages to kill staphylococci, while the absence of surfactant prevents the death of organisms following phagocytosis.³⁵ Surfactant also enhances phagocytosis in an opsonin deficient environment³⁶ and also helps dissolve oxygen, which is mediated by micelle formation.³⁷ Lung surfactant is catabolized by alveolar macrophages³⁴ and also recycled by absorption into type II pneumocytes.³⁸

Following tryptophan administration, the amount of surfactant on alveolar surfaces decreases significantly in cattle, but the surfactant composition does not change significantly.¹⁵ Molar percentages of palmitic, palmitoleic and oleic acids are not significantly different in the phosphatidylcholine fraction of treated animals as compared to control animals.¹⁵ The fatty acid composition is not affected by severity of response to the tryptophan administration, but pulmonary surfactant function is reduced in treated animals.¹⁵ Reduction of

surfactant activity and phospholipid content is greater in cows with lung edema.¹⁵

Numerous alveolar macrophages are found in underventilated lung areas connected with other diseases, where reactive overproduction of surfactant occurs.³⁹ Lamellar bodies do not spread out in these areas and act as a foreign material,³⁹ because they are phagocytized and transported away by macrophages.³⁹

The ratio of palmitic to stearic acids decreased following oral 3MI administration to cattle.⁴⁰ This suggests that the amount of palmitic acid in lung surfactant decreases, while the amounts of stearic and oleic acids increase.

Three-methylindole administration to goats results in depressed uptake and incorporation of ¹⁴C-choline into phosphatidylcholine and sphingomyelin in lung tissue slices in vitro.⁴¹ The authors concluded that choline kinase and choline transferases were rate limiting and played a role in the depressed synthesis of phosphatidylcholine.⁴¹

It was also reported that lamellar body structures in tissue slices from goats became disrupted by 3MI and contained neutral lipids instead of phospholipids.⁴² Indole will display the same effects on membranes as 3MI does, but it is not activated by the MFO system and that is why it does not cause lung injury.⁴² Choline uptake does not limit the phosphatidylcholine synthesis in the 3MI treated lung slices.⁴²

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PAPER 1

THE PATHOLOGICAL EFFECT OF THREE-METHYLINDOLE

ON LUNGS IN LAMBS

INTRODUCTION AND LITERATURE REVIEW

Three-methylindole (3MI=skatole), a ruminal metabolic product of tryptophan,^{1,2} produced by *Lactobacillus* species,³ causes acute pulmonary edema and emphysema in ruminants,⁴⁻⁷ and bronchiolitis in horses,⁸⁻¹⁰ when administered orally or intravenously.^{4,5} Three-methylindole is changed by mixed function oxidase (MFO) in lung cells into a reactive intermediate,^{3,8,10} which damages the lungs. This intermediate interacts with lecithin, which is present in cell membranes, resulting in membrane disruption of type I pneumocytes and Clara cells primarily.^{7,11} For this reason, necrotic bronchiolitis and alveoli void of type I pneumocytes are detectable in ruminants administered 3MI.^{3,7,9} To heal the resulting damage, bronchioles become epithelized, type II pneumocytes multiply and transform into type I cells.¹² Cell membranes of erythrocytes may also be damaged, and result in hemolyzed blood in the vasculature system, urine and inside pulmonary airways.^{3,5,13} Type II pneumocytes also produce lung surfactant,¹⁴ which is the lining material present in alveoli and airways. The surfactant composition may change, and its amount decreases in acute bovine pulmonary edema and emphysema.¹⁵

Signs of 3MI poisoning are detectable in sheep 1.5 to 2 hours following oral administration of 3MI at 0.6 g/kg BW, or 3 to 4.5 hours following 0.2 g/kg BW.⁴ Early clinical signs consist of muscular trembling, increased respiratory rate, dyspnea, kicking motions of hind limbs, and unproductive attempts at micturition.⁴⁻⁶ Later, the breathing becomes more labored, the respiratory rate decreases, and an audible respiratory "grunt" develops.^{4,6} If the dosage is high (0.6

g/kg BW in ewes orally), lateral recumbency, cyanosis of mucous membranes, frothy pinkish to straw colored nasal and oral discharges may occur 10 to 27 hours later in ewes.⁴ After death, caused by asphyxiation,⁶ the lungs remain inflated, and red.⁴⁻⁶ The pathological lesions are less pronounced in sheep, who seem to be more tolerant to 3MI than other ruminant species.⁴

Three-methylindole was administered intravenously and intraperitoneally to lambs and adult sheep in this study to produce pneumotoxicity. Both lambs and sheep were killed at 24 and 72 hours following administration of 3MI.

MATERIALS AND METHODS

All lambs were confined in an outdoor dry lot and brought into an indoor air conditioned pen at least 48 hours before experimentation.

Seventeen lambs, of cross and Suffolk breeding, 5 to 6 months old, weighing 24 to 62 kg, females and castrated males, were divided randomly into 4 groups.

Group 1 - Four lambs (#40, 46, 76, 123) the control group, received no drug administration. They were examined three times at 12 hour intervals, and killed after the last examination by giving an intramuscular injection of xylazine^a at a dosage of 0.22 ml/kg of body weight (BW). This was followed by an intravenous infusion of approximately 100 to 150 ml of saturated MgSO₄ in distilled water until death, and necropsy took place immediately.

^aRompun for horses, Miles Laboratories, Shawnee, KS

Group 2 - Five lambs (#6, 47, 49, 83, 84) received 3MI^b at a dosage of 0.03 g/kg BW and diluted in propylene glycol^c at a rate of 0.1 g/ml. One half of the dose was slowly administered Intravenously, and one half intraperitoneally^d to avoid immediate collapse and death. Lambs were killed 24 hours following the drug administration (method described in group 1), and necropsy took place immediately.

Group 3 - Four lambs (#4, 44, 48, 85) received 3MI at a dosage of 0.03 g/kg BW and diluted in propylene glycol at a rate of 0.1 g/ml. One half of the dose was administered slowly intravenously, one half intraperitoneally, as in group 2. Lambs were killed (method described in group 1) 72 hours following 3MI administration and necropsied immediately.

Group 4 - Four lambs (#80, 91, 93, 124) each received only 15 ml of propylene glycol intravenously. They were killed and necropsied immediately 24 hours following the administration.

Clinical Examination

Lambs were physically examined before and following the administration of 3MI or propylene glycol at 12-hour intervals until death. The first examination of the control group took place 24 hours before their death. Rectal temperature, heart rate and respiratory rates, eye and nasal discharges, type of respiration, presence of cough and auscultation sounds of the lungs and heart were examined.

^bThree-methylindole, Sigma Chemical Co., St. Louis, MO

^cPropylene glycol, Fisher Scientific, Fair Lawn, NJ

^dDr. J.E. Garst, KSU: personal communication, August 1986.

Necropsy and Histopathological Examinations

Lambs were necropsied immediately following death. The lungs, heart, liver, and the right kidney were the major organs grossly examined. Following gross necropsy examination, the left lung was severed from the remaining respiratory tract and the left bronchus was clamped with a hemostat.

Tissue sections for a histological examination were taken from the left lung. One slice, approximately 3 to 5 mm thick, was cut vertically through the middle region of the diaphragmatic lobe, and sections approximately 0.5 to 1 cm x 1 cm were cut from the laterodorsal, laterocentral, and lateroventral regions of the slice. Tissue sections of similar size were also taken from the liver just lateral to the gall bladder, and the right kidney was sectioned in the mid-region, and the interventricular septum of the heart was also sectioned. All tissue sections were placed in 4% buffered formalin as a fixative for at least 48 hours. All the tissue sections were embedded in paraffin, cut at 6 microns, and stained with hematoxylin eosin. The lung tissue sections were also stained with periodic acid Schiff, Van Gieson, and Giemsa stains.

Histopathological changes were then evaluated by light microscopy, with a special attention to the bronchi, bronchioles, alveoli, the interstitium, and the vasculature. The histopathological changes were evaluated and expressed as a percent of normal.

Three additional tissue sections (0.5 x 0.5 x 0.5 cm) were collected from the laterodorsal, laterocentral, and lateroventral areas of the left diaphragmatic lobe. These sections were frozen

Immediately in a mixture of freon 22 and dry ice at -70°C . All these tissue sections were transferred to a cryostat (-30°C), embedded in the OCT medium,^e and cut at 10 microns. After transferring the sections onto slides, they were stained histochemically for lung surfactant phospholipids.

A "tricomplex" method was used for staining lung phospholipids,¹⁶ after using Baker's method in the Dunnigan's modification,¹⁷ which turned out to be unreliable. The "tricomplex" method uses lead nitrate and potassium ferricyanide in formalin for staining while fixation takes place, and ammonium sulfide for developing the color. The procedure is the following:¹⁶ After air drying, slides with tissue sections are submerged in a mixture of 0.05 N $\text{Pb}(\text{NO}_3)_2$ and 0.05 N $\text{Fe}(\text{CN})_6$ in 4% unbuffered formalin for 2 min. They are then rinsed twice with distilled water, and the color is developed by placing them into 30 mM $(\text{NH}_4)_2\text{S}$. Lastly, the slides are washed in distilled water and mounted in glycerin jelly.

The histochemical staining process attaches lead ions to the phosphorus portion of the phospholipid molecule. Ammonium sulfide binds to the lead ion and changes it to lead sulfide, which is dark brown or black. The phospholipid film then becomes visible as a thin dark line at the circumference of the air bubbles trapped in the frozen lung tissue. Phospholipids present in the lung parenchyma are also stained by this method and become visible as dark brown to black spots inside the collapsed frozen tissue.

^eOptimal Cutting Temperature Medium, Miles Scientific, Naperville, NJ

RESULTS

Clinical Signs

The clinical findings are reported in Table 1. There were no significant differences of the respiratory rate between the control group and other 3 groups (<0.05). The increase in the respiratory rate was highest in the 72 hour group (mean 109.6/min), while the respiratory rate was lowest (86.9/min) in the control group.

The body temperature and the heart rate were not statistically analyzed.

Gross Pathology and Histopathology in Hematoxylin-eosin stain

1. Control Group - 4 lambs: All lambs had gross pathological changes: 3 lambs had a dilated heart, while 1 lamb had lobular pneumonia in the apical and ventral positions of the cranial and cardiac lobes, 1 lamb had fibrinous pneumonia. Microscopically, all lambs had varying degrees of peribronchitis and peribronchiolitis, and all lambs had interstitial pneumonia, which was characterized by mononuclear cells infiltrates of the interstitium. Pneumocytes, alveolar walls and vasculature were normal. The bronchi and bronchioles of 2 lambs were partially closed. No histopathological changes were found in tissue sections from the heart, kidney and liver.

2. Three-methylindole administration followed by necropsy examination in 24 hours - 5 lambs: Grossly, 1 lamb did not have pathological changes, 4 lambs had a dilated heart, and the lungs of 2 lambs did not collapse completely. One lamb had also lobular pneumonia grossly in both cranial and cardiac lobes. Microscopically,

all lambs had peribronchitis and peribronchiolitis. The bronchi and bronchioles of 4 lambs were partially closed. The alveolar walls and pneumocytes were normal. One lamb had intraalveolar hemorrhage in a few alveoli. In all lambs, the interstitium was expanded by the infiltrating mononuclear cells. Mild vasculitis was present in 2 lambs. No histopathological changes were found in tissue sections from the heart, kidney and liver.

3. Three-methylindone administration followed by necropsy examination in 72 hours - 4 lambs: Grossly, 2 lambs had a dilated heart, 1 lamb had fibrinous pleuritis, 1 lamb did not have gross pathological changes. Microscopically, all lambs had varying peribronchitis and peribronchiolitis. The bronchi and bronchioles of all 4 lambs were partially closed. The alveolar walls, pneumocytes and the vasculature were all normal. Multifocal alveolar edema was present in 2 lambs. The interstitial spaces of all lambs were wider, because of mononuclear cell infiltration. No histopathological changes were found in tissue sections from the heart, kidney and liver.

4. Propylene glycol administration followed by necropsy in 24 hours - 4 lambs: All four lambs had gross pathological changes which included a dilated heart, and the lungs of 2 lambs did not collapse. Microscopically, varying peribronchitis and peribronchiolitis were present in all 4 lambs. The alveolar walls, pneumocytes and the vasculature were all normal. The bronchi and bronchioles of 2 lambs were partially closed. Interstitial spaces of the lungs of all 4

lambs were wider, because of infiltrating mononuclear cells. No histopathological changes were found in tissue sections from the heart, kidney and liver.

Lung Histopathology - Special Stains

The periodic acid Schiff stained the lung cells and membranes containing carbohydrates, Van Gieson stained collagen and muscular elements. The Giemsa stained the lung structures and blood cells present.

No additional pathological changes were found with these three special stains between the control and experimental groups when compared mutually.

Phospholipid ("tricomplex") Stain

The freezing caused the lung structures to collapse, and where the freezing mixture was in a direct contact with the tissue, the phospholipids were removed, and could not be visualized. The air bubbles trapped in the collapsed tissue were lined with a solid, fine, dark brown to black line. The bronchi, bronchioles and blood vessels also contain phospholipids, which became visible as dark spots inside these structures. The dark, solid, fine line was present and distributed around the periphery of the air bubbles in the lungs from all lambs. Differences in the amount of surfactant between the control and experimental groups could not be detected.

DISCUSSION

We initially administered in our pilot studies 3MI orally as a drench, but we were concerned about 3MI ruminal metabolism, so we then

gave it intravenously by a slow infusion over several minutes. This method of administration resulted in immediate death of 1 lamb. She exhibited panting, collapsed in lateral recumbency, and died within a few minutes. Lung edema was found on necropsy examination, and probably was the cause of death. In this study, we gave one half of the calculated dosage of 3MI in propylene glycol intravenously, and one half intraperitoneally, to avoid sudden death. At a dosage of 30 mg of 3MI/kg BW, lambs did not develop clinical signs of toxicity and on necropsy we found no edema or emphysema. Young lambs may not be as sensitive to 3MI as adults; as described in young calves,²⁰ also they may not possess at a young age an enzymatic system for converting 3MI into a toxic reactive radical. In addition, sheep may not be as sensitive to 3MI as goats and cattle.⁴

We found only small differences in the respiratory rate between the control and 3MI treated groups (killed 24 and 72 hours later), and the differences were not statistically significant. The rectal temperature and the heart rate were not analyzed statistically, because some of the control animals also had an elevated rectal temperature above 40.0°C.^f The heart rate was easily influenced by the presence of the examining person, and could not be attributed solely to the disease process.

Neither gross nor histopathological examinations revealed lesions, which could be attributed to 3MI alone. The lungs from 2 lambs in the 24-hour group and the lungs from 3 lambs in the 72-hour

^fThe Merck Veterinary Manual, Rahway, NJ, 1979, p 1483.

group did not collapse completely on gross examination, but neither did the lungs from animals in the propylene glycol group. We found histopathological changes suggestive of chronic interstitial pneumonia in almost all lambs, which could have been caused by a viral agent. Failure of the lungs to collapse completely could have been due to interstitial pneumonia.

We did not find hematuria on necropsy examination, even though it has been described in cattle,⁵ following the administration of 60 mg of 3MI per kg BW IV.

The heart of most experimental sheep was dilated on post mortem examination. This was attributed to the $MgSO_4$ used for euthanasia, because Mg^{++} ions probably cause heart muscle relaxation.

The reason for special stains used for lung tissue sections was to find the amount of mucus in goblet cells, and the amount of hyalin (both by periodic acid Schiff), the amount of fibrous tissue, collagen, and muscle fibers (by Van Gieson), and to differentiate various blood cells present in the tissue from lung tissue cells (Giemsa).

The Giemsa stain proved to be the best for staining the diversified cells present in the lungs, and it also visualized red blood cells present in the alveolar capillaries, or in the alveoli. This stain also helped in the study of type II pneumocytes and helped to distinguish them from macrophages. The nuclei of type II pneumocytes stained light blue, while the nuclei of macrophages turned dark purple.

To stain lung tissue sections for phospholipids, we used Baker's method utilizing Nile blue A, the Dunnigan's modification.⁴³ This staining method turned out to be unreliable, because the dye faded from the stained tissues, plus it did not stain the surfactant film, but only stained the phospholipids present in the tissues.

The "tricomplex" phospholipid staining method visualized the phospholipid film, but we did not find any difference between the control and 3MI groups. Lung structure cannot be studied by this procedure, because alveoli are collapsed.

We were unable to evaluate the toxic effect of propylene glycol on the lungs, again because most animals were also suffering from interstitial pneumonia. It has been reported that propylene glycol will cause slight endothelial swelling of pulmonary blood vessels and variable infiltrates of some muscular arteries.⁵

SUMMARY

Seventeen lambs of Suffolk and cross breedings, 5 to 6 months old, females and castrated males, were divided randomly into 4 groups. Four control lambs in groups 1 were clinically examined 3 times within 24 hours, and killed without treatment. Five lambs in group 2 received 0.03 g of 3-methylindole (3MI) per kg of body weight, diluted in propylene glycol at the rate of 0.1 g/ml, intravenously and intraperitoneally, and were killed 24 hours later. Four lambs in group 3 received 0.03 g of 3MI/kg of body weight in propylene glycol at the rate of 0.1 g/kg of body weight intravenously and intraperitoneally, and were killed 72 hours later. Four lambs in

group 4 received 15 ml of propylene glycol each intravenously and were killed 24 hours later. All lambs in groups 2 to 4 were also examined clinically at 12 hour intervals as the lambs in group 1.

All lambs were necropsied, examined grossly, and their left lung, heart, right kidney and liver were examined histopathologically. The lung sections were also frozen in a mixture of freon 22 with dry ice, and stained for lung surfactant phospholipids.

No statistically significant clinical changes were found. Pathologically, all 17 lambs suffered from chronic interstitial pneumonia at a various degree, maybe caused by a virus. No typical lesions caused by 3MI were found in lambs. On the other hand, in our pilot study two adult ewes and one lamb, who collapsed during the 3MI infusion, showed typical changes and lesions of acute pulmonary edema and emphysema, caused by 3MI. Lung tissue sections stained for surfactant phospholipids also did not reveal any differences among the groups.

The dosage of 3MI was found to be below detectable toxicity levels for lambs and, in general, lambs are maybe more tolerant to 3MI than adults or other ruminants.

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APPENDIX:

Table 1: Rectal temperature, heart rate and respiratory rate of lambs receiving no drug administration, or 3-methylindole IV and IP, or propylene glycol IV, killed 24 hours later

| Time: | Hours | 0 | 12 | 24 | 0 | 12 | 24 | 0 | 12 | 24 |
|---|--------|--------------------|------|------|----------------|-----|-----|----------------------|-----|-----|
| Group | Lamb # | Rectal temperature | | | Heart rate/min | | | Respiratory rate/min | | |
| | | In °C | | | | | | | | |
| 1 Control Group - no drug administration | | | | | | | | | | |
| | 40 | 40.0 | 39.7 | 39.4 | 175 | 85 | 75 | 85 | 55 | 38 |
| | 46 | 40.3 | 40.6 | 40.8 | 205 | 185 | 185 | 105 | 150 | 100 |
| | 76 | 39.9 | 40.1 | 39.6 | 105 | 90 | 75 | 110 | 115 | 55 |
| | 123 | 39.9 | 39.7 | 39.9 | 180 | 120 | 165 | 92 | 100 | 58 |
| 2 Killed 24 hours after 3MI administration followed by necropsy | | | | | | | | | | |
| | 6 | 39.7 | 40.4 | 39.7 | 180 | 110 | 140 | 55 | 75 | 90 |
| | 47 | 40.1 | 39.9 | 39.6 | 200 | 140 | 145 | 62 | 70 | 83 |
| | 49 | 40.2 | 41.2 | 40.3 | 215 | 225 | 240 | 85 | 62 | 60 |
| | 83 | 39.7 | 39.4 | 39.9 | 110 | 195 | 105 | 64 | 140 | 115 |
| | 84 | 40.3 | 39.9 | 39.3 | 125 | 70 | 90 | 130 | 120 | 85 |
| 4 Propylene glycol followed by necropsy in 24 hours | | | | | | | | | | |
| | 80 | 40.3 | 40.6 | 40.1 | 165 | 200 | 195 | 125 | 130 | 115 |
| | 91 | 39.6 | 39.8 | 39.7 | 110 | 95 | 95 | 120 | 140 | 80 |
| | 93 | 39.6 | 40.1 | 39.8 | 195 | 185 | 210 | 38 | 86 | 60 |
| | 124 | 39.6 | 39.4 | 39.3 | 200 | 150 | 130 | 75 | 95 | 80 |

APPENDIX:

Table 2: Rectal temperature, heart rate and respiratory rate of lambs killed 72 hours after 3MI administration (IV and IP), followed by necropsy

| Time: | hours | 0 | 12 | 24 | 36 | 48 | 60 | 72 |
|--------------|---------------|--------------------------|------|------|------|------|------|------|
| <u>Group</u> | <u>Lamb #</u> | Rectal temperature in °C | | | | | | |
| 3 | 4 | 39.5 | 39.8 | 39.3 | 39.9 | 39.8 | 39.8 | 39.2 |
| | 44 | 39.8 | 40.0 | 39.9 | 40.1 | 39.6 | 40.2 | 39.8 |
| | 48 | 39.8 | 40.3 | 40.2 | 40.0 | 40.1 | 39.9 | 39.4 |
| | 85 | 39.3 | 39.9 | 40.1 | 39.7 | 39.8 | 39.7 | 39.2 |
| | | Heart rate per min | | | | | | |
| | 4 | 125 | 180 | 155 | 140 | 140 | 140 | 125 |
| | 44 | 110 | 155 | 110 | 102 | 115 | 102 | 90 |
| | 48 | 105 | 75 | 135 | 110 | 115 | 125 | 110 |
| | 85 | 135 | 100 | 160 | 125 | 105 | 110 | 100 |
| | | Respiratory rate per min | | | | | | |
| | 4 | 88 | 110 | 120 | 80 | 60 | 95 | 55 |
| | 44 | 84 | 98 | 120 | 75 | 75 | 75 | 70 |
| | 48 | 120 | 160 | 80 | 140 | 150 | 140 | 110 |
| | 85 | 125 | 125 | 85 | 160 | 135 | 145 | 70 |

APPENDIX:

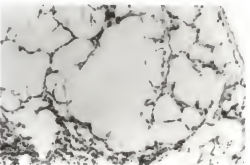
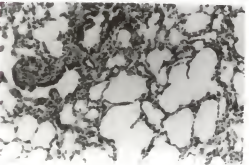
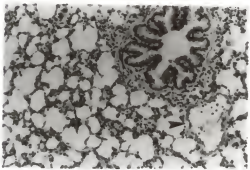
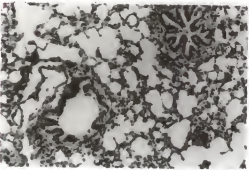
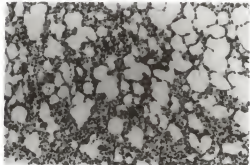
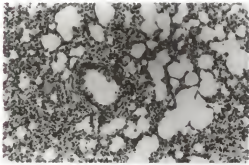
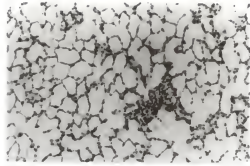
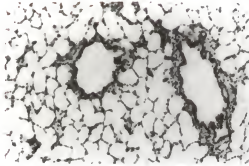
Table 3: Clinical signs of lambs receiving no drug administration,
3-methylindole IV and IP, or propylene glycol IV

| <u>Group</u> | <u>Lamb #</u> | <u>Changes found</u> |
|---|---------------|---|
| 1 (control) | 46 | Spontaneous cough at hour 0, mucous discharge from the right nostril from 0-24 hours |
| | 76 | Mucus in the left eye at 0 hours |
| 2 (3MI followed by necropsy in 24 hours) | 47 | Irregular breathing at 12 hours |
| | 49 | Hour 0: bilateral mucopurulent nasal discharge, respiratory rhonchus on auscultation Hours 12 and 24: irregular breathing |
| 3 (3MI followed by necropsy in 72 hours) | 4 | Hours 48 through 72: scanty to moderate seromucous bilateral nasal discharge |
| | 44 | Scanty to moderate seromucous bilateral nasal discharge found intermittently, mucopurulent eye discharge found at hour 60, irregular breathing found intermittently |
| | 48 | Irregular heart activity found intermittently (on first, second, fourth, fifth examinations) |
| | 85 | Bilateral seromucous nasal discharge found intermittently (on second and seventh examinations) |
| 4 (Propylene glycol followed by necropsy in 24 hours) | 80 | Shallow breathing at hours 0-24 |
| | 91 | Irregular heart activity at hours 0-24 |
| | 93 | Moderate seromucous bilateral nasal discharge present at hours 0-24 |
| | 124 | Hours 0 and 12: moderate seromucous discharge from the left nostril plus Hours 0 to 24: shallow breathing |

Figures on the facing page - Histological photo-
micrographs from lungs of some lambs:

| | |
|---|---|
| 1 | 2 |
| 3 | 4 |
| 5 | 6 |
| 7 | 8 |

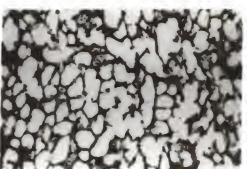
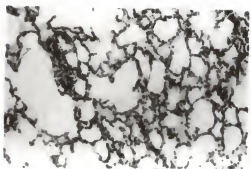
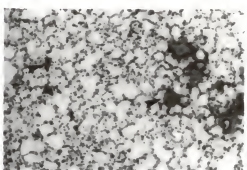
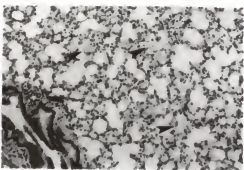
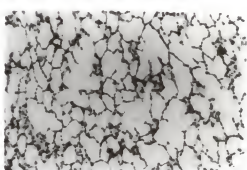
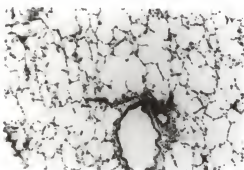
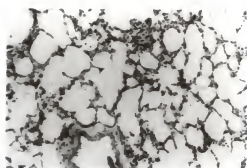
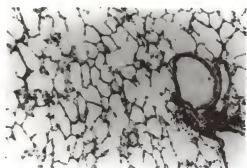
1. Lamb #40 - control group: normal lung
2. Lamb #40 - control group: some of the interstitial spaces are wider than normal.
3. & 4. #47 - killed 24 hours following 3MI administration: The alveoli are compressed by the expanded interstitium, which is several times wider than normal. Mononuclear cells infiltrate the interstitium. No alveolar edema is present.
5. Lamb #83 - killed 24 hours following 3MI administration: Note the closed bronchus in the upper portion of the photograph, mild peribronchitis is also present. The interstitial spaces are up to 3 times wider than normal. No lung edema or alveolar damage is present.
6. Lamb #83 - killed 24 hours following 3MI administration: A half-open bronchus is in the upper portion, the interstitium is up to 3 times wider than normal. An arrow marks a peribronchial artery.
7. Lamb #44 - killed 27 hours following 3MI administration: A half-open bronchus in the upper portion. Some of the interstitial spaces are wider, infiltrated with mononuclear cells. The alveoli on the right are expanded, and emphysema is present.
8. Lamb #44 - killed 72 hours following 3MI administration: Alveolar emphysema is present. Emphysema was present only in a small part of the histological section from the ventral lung region.



Figures on the facing page - Histological photo-
micrographs of some lambs and ewes:

| | |
|----|----|
| 9 | 10 |
| 11 | 12 |
| 13 | 14 |
| 15 | 16 |

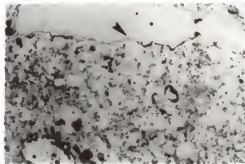
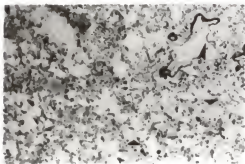
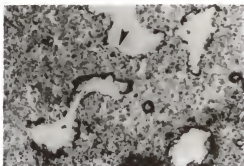
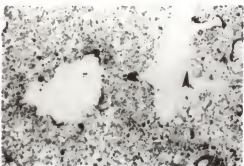
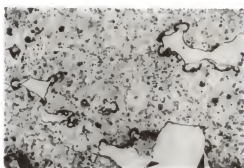
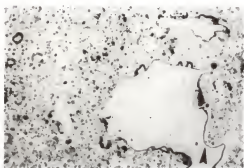
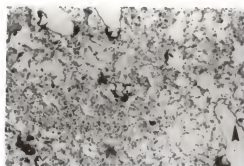
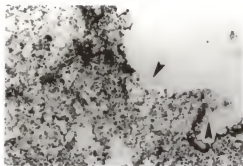
9. Lamb #48 - killed 72 hours following 3MI administration: Mostly a normal lung section, the alveolar cells cut diagonally seem to be more numerous than normal, when compared with Fig. 1 on the previous page.
10. Lamb #48 - killed 72 hours following 3MI administration: Some of the interstitial spaces are wider than normal, infiltrated with mononuclear cells.
11. & 12. Lamb #91 - killed 24 hours following propylene glycol administration: Normal lung sections.
13. Lamb #88 - died during an IV infusion of 3MI: Acute lung edema in the interstitium and also in the alveoli. Alveolar spaces are smaller. Cellular debris, red blood cells and mononuclear cells are present in the alveoli, three are marked with arrows.
14. Lamb #88 - died during an IV infusion of 3MI: Acute lung edema in the interstitium and alveoli. Alveoli containing hemolyzed and undamaged red blood cells are marked with arrows.
15. Ewe #86 - killed 24 hours following 3MI administration: Alveolar edema is present, some alveoli are emphysematous. The damage is less severe than in figures 13 and 14.
16. Ewe #92 - killed 72 hours following 3MI administration: The alveolar damage is nearly healed. The amount of alveolar fluid is minimal, some of the interstitial spaces are wider than normal. The slide was overstained.



Figures on facing page - Lung sections from
lambs stained for surfactant phospholipids:

| | |
|----|----|
| 17 | 18 |
| 19 | 20 |
| 21 | 22 |
| 23 | 24 |

17. & 18. Lamb #123 - control group: The surfactant film is visible as a fine dark line on the circumference of air bubbles, marked with two arrows. The lung structure collapsed and cannot be studied, although some alveolar spaces are still recognizable.
19. & 20. Lamb #47 - killed 24 hours following MI administration: The line marking the air bubbles is visible, marked by narrow arrows. Surfactant deficiency is not recognizable. Where surfactant was washed out in the staining process, the line disappears, marked with wide arrow in Fig. 20.
21. & 22. Lamb #44 - killed 72 hours following MI administration: The surfactant film is visible as a fine dark line, marking the air bubbles (arrows).
23. & 24. Lamb #91 - killed 24 hours following propylene glycol administration: The fine line marks the air bubbles (narrow arrows). The collapsed alveoli are more visible than in Fig. 18, and are marked with wide arrows in Fig. 23.



APPENDIX:

Gross and Histopathological Changes of Tissues

Stained with Hematoxylin-eosin

1. Control Group - no drug administration:

#40 - The only gross pathological change found was a dilated heart. Microscopically, approximately 33% of the bronchi and bronchioles were affected with peribronchitis and peribronchiolitis. The alveolar walls were normal, pneumocytes were also normal in size and number. Approximately 25% of interstitial spaces were 2 to 3 times wider than normal, because of mononuclear cell infiltration. The vasculature appeared normal. The alveoli were empty and of normal size.

#46 - The ventral 1/4 of the apical and middle lung lobes were consolidated on gross examination. The right ventricle of the heart was dilated. Approximately 50% of the bronchi and bronchioles were surrounded with peribronchitis and peribronchiolitis. The alveolar cells and walls appeared normal, but the alveoli were smaller, because 25 to 50% of the interstitial spaces were 3 times wider than normal due to mononuclear cell infiltration. Pneumocytes II were normal in size and number. The vasculature appeared normal. Lobular pneumonia was found microscopically on one tissue section. The cell types infiltrating the area were neutrophils, eosinophils, and mononuclear cells.

#76 - The only gross pathological change was a dilated heart. Microscopically, approximately 50% of the bronchi and bronchioles were

closed, and also approximately 50% of them were affected with peribronchitis and peribronchiolitis. The alveoli were smaller, but open, with some proteinaceous material present in approximately 10% of them. The alveolar walls were normal, pneumocytes II were also normal in size and number. Approximately 50% of the interstitial spaces were up to 5 times wider than normal. The vasculature appeared normal.

#123 - Grossly, fibrinous pneumonia was present in 75% of the caudal region of the right diaphragmatic lobe. Microscopically, 20% of the bronchi and bronchioles were closed. Peribronchitis and peribronchiolitis were present around approximately 50% of the bronchi and bronchioles. The alveoli were smaller, in some areas not distinct, but empty. The pneumocytes II appeared normal. Fifty percent of the interstitial spaces ranged from 2 to 5 times wider than normal. The vasculature appeared normal. Chronic interstitial pneumonia was present in 2 to 3 sections taken from the consolidated area of the right diaphragmatic lobe.

2. Group 2 - Lambs killed 24 hours following 3MI administration:

#6 - Grossly, the lungs were approximately twice normal size and the heart was very dilated. Microscopically, the bronchi and bronchioles were open, but approximately 50% of them were affected with peribronchitis and peribronchiolitis. The alveoli were empty, of normal size, and the pneumocytes II were normal in size and number. Multifocal hypercellularity due to mononuclear cell infiltration of the interstitium was found. The vasculature appeared normal.

#47 - Grossly, the only pathological change was a very dilated heart. Microscopically, approximately 20% of the bronchi and bronchioles were closed. Fifty percent of the bronchi and bronchioles were surrounded with peribronchitis and peribronchiolitis. Fifty percent of the alveoli were depressed by the expanded interstitium which was up to 5 times wider than normal because of mononuclear cell infiltration. The pneumocytes II were normal, the vasculature was also normal.

#49 - Grossly, approximately 25% of the left and right apical and cardiac lobes were consolidated. The lungs were also pale, with multiple dark 1 to 2 mm foci located on the surface. The lungs remained inflated approximately twice normal size. The heart was dilated. Microscopically, 50% of the bronchi and bronchioles were closed, and also approximately 50% of the bronchi and bronchioles were affected with peribronchitis and peribronchiolitis. Some of the alveoli contained red blood cells, while pneumocytes II were normal in size and number. The alveoli were depressed by the expanded interstitium of which approximately 50% were up to 5 times wider than normal. The vasculature was normal.

#83 - Grossly, the lungs remained approximately 50% larger than normal, and the heart was very dilated. Microscopically, 25% of the bronchi and bronchioles were closed, 50% of them were affected with peribronchitis and peribronchiolitis. The alveoli were empty but depressed by the expanded interstitium, up to 60% of which was 2 to 3 times wider than normal. This was due to mononuclear cell infiltration. The pneumocytes II were normal in size and number.

Approximately 30% of the interstitium was up to 3 times wider than normal which was due to the accumulated mononuclear cells. The vasculature was normal.

3. Group 3 - lambs killed 72 hours after 3MI administration:

#4 - Grossly, the ventral half of both lungs was affected with fibrinous pleuritis with multiple fibrin deposits on the pleural surface and adhesions to the chest wall. The lungs did not collapse completely, and the heart was dilated. Microscopically, 30% of the bronchi and bronchioles were closed and up to 75% of them were surrounded with peribronchitis and peribronchiolitis. The alveoli were depressed by the expanded interstitium. The pneumocytes II were normal in size and number. Multifocal alveolar edema was present on 1 slide. Thirty to 50% of the interstitial spaces were up to 3 times wider than normal which was caused by mononuclear cell infiltration. The vasculature appeared normal.

#44 - Grossly, the lungs did not collapse and remained 3 times larger than normal. Microscopically, up to 50% of the bronchi and bronchioles were closed, and 50% were affected with peribronchitis and peribronchiolitis. The alveoli were depressed by the expanded interstitium, up to 50% of interstitial spaces were 3 times wider than normal because of mononuclear cells infiltrating them. The pneumocytes II were normal in size and number. The vasculature was normal.

#48 - Grossly, the lungs remained inflated twice normal size, and the heart was dilated. Ecchymotic hemorrhages and coagulated blood

were present in the pericardial sac, under the epicardium, and in the perirenal tissue of the right kidney. Hemorrhage was also present in the left bronchial lymph node. We did not find the cause of the hemorrhages. Microscopically, 50% of the bronchi and bronchioles were closed, while peribronchitis and peribronchiolitis surrounded approximately 30% of them. All alveoli were empty except one slide (#3, from the ventral area), where alveolar edema was present. The pneumocytes II were normal in size and number. Fifty percent of the alveolar spaces were up to 5 times wider than normal because mononuclear cells infiltrated them. The vasculature was normal.

#85 - No gross lesions were present. Microscopically, 20% of the bronchi and bronchioles were closed, while 30% of them were affected with peribronchitis and peribronchiolitis. The pneumocytes II were normal in size and number. The alveoli were open but depressed by the expanded interstitium, 50% of which was 3 times wider than normal because of mononuclear cell infiltration. The vasculature was normal.

4. Group 4 - Propylene glycol, killed after 24 hours:

#80 - Grossly, the heart was very dilated. Microscopically, peribronchitis and peribronchiolitis were present around 50% of the bronchi and bronchioles. The alveoli were empty and of normal size. The pneumocytes II were normal in size and number. About 25% of the interstitial spaces were 3 times wider than normal. The vasculature was normal.

#91 - Grossly, the lungs remained inflated approximately twice normal size. The heart was dilated. Microscopically, 50% of the

bronchi and bronchioles were surrounded with peribronchitis and peribronchiolitis. The alveoli remained empty and of normal size. Also, the pneumocytes II were normal. Fifty percent of the interstitial spaces were 3 times wider than normal because mononuclear cells infiltrated them. The vasculature was normal.

#93 - Grossly, the lungs remained inflated twice normal size and the heart was dilated. Microscopically, 30% of the bronchi and bronchioles were closed and 50% of them were accompanied by peribronchitis and peribronchiolitis. The alveoli were empty, of normal size, and the pneumocytes II were normal. Fifty percent of the interstitial spaces were 3 times wider than normal. The vasculature was normal.

#124 - Grossly, only the heart was dilated. Microscopically, 50% of the bronchi and bronchioles were closed, while 30% of them were affected with peribronchitis and peribronchiolitis. The alveoli were empty but depressed by the interstitium because about 75% of the interstitial spaces were 5 times wider than normal. This was caused by the infiltrating mononuclear cells. The pneumocytes II were normal in size and number. The vasculature was normal.

APPENDIX:

MATERIALS AND METHODS

One female Suffolk lamb and two adult Suffolk ewes are included in our study. All three animals were examined clinically at 12-hour intervals, the first examination took place immediately before the 3MI administration.

Lamb #88 - weighed 33 kg and received 30 mg of 3MI/kg BW in 15 ml of propylene glycol IV, but she died at the end of the infusion. A post mortem and histopathological examinations were done.

Ewe #86 - weighed 78 kg and received 20 mg of 3MI/kg BW, diluted in 15 ml of propylene glycol IV. She was killed 24 hours later with 20 ml of N-gamma-hydroxybutyramide with methylene-bis and tetracaine with dimethylformamide in distilled water,⁹ and a gross and histopathological examinations were done.

Ewe #92 - weighed 92 kg and received 20 mg of 3 MI/kg BW in 20 ml of propylene glycol IV. She was killed 72 hours later by electrocution and gross and histopathological examinations were done.

RESULTS

Clinical Signs

Lamb #88 collapsed at the end of the IV infusion of 3MI, started panting, fell down, went into lateral recumbency, had several generalized seizures, and then died.

⁹T-61^R Euthanasia Solution, Hoechst, Sommerville, NJ

Ewe #86 lived for 24 hours following 3MI administration, had pronounced respiratory distress as demonstrated by irregular breathing pattern 12 hours after 3MI administration. Her respiratory rate was 200/min before 3MI administration and 60/min 24 hours later.

Ewe #92 lived for 72 hours following 3MI administration. She also showed respiratory distress similar to that of ewe #86. Her breathing pattern became irregular 24 hours following administration, and signs were still visible 72 hours following administration of 3MI. Her respiratory rate varied from 200/min before the administration to 180/min 5 hours later, and then maintained at 40 to 64/min.

Gross and Histopathological Findings

Lamb #88 - died during the 3MI infusion. The only gross pathological change found was moderately pale lungs with a lighter color on the surface than usual. Microscopically, 30% of the bronchi and bronchioles were closed, and also 30% of them were affected with peribronchitis and peribronchiolitis. Multifocal edema with cellular debris, red blood cells, and mononuclear cells were present in the alveoli. Almost all alveoli were smaller than normal because they were surrounded by expanded interstitium which was approximately twice as wide in the edematous areas. Very few pneumocytes were present in the alveoli when compared with other experimental lambs (Figures 13 & 14, page 37).

Ewe #86 - killed 24 hours after the infusion of 3MI. Grossly, edema and emphysema were visible on the lung surface and the lungs did not collapse completely. Petechial hemorrhages were present in the tracheal mucosa. The trachea and bronchi were partially filled with a

bloody froth. The heart was dilated. Microscopically, all bronchi and bronchioles were open, with no cellular or fluid content, but 80% of them were affected with peribronchitis and peribronchiolitis. The pneumocytes were normal in size, but less numerous than in lambs. The alveoli were smaller than usual because they were depressed by swollen interstitium. Multifocal areas of one or more alveoli contained slightly eosinophilic fluid. Walls of about 25% of the alveoli were ruptured and red blood cells were present in the lumen. Approximately 50% of the interstitial spaces were infiltrated with numerous red blood cells, which caused the interstitium width to increase up to 10 times. The vessels appeared normal. No other organs of this ewe were examined histologically (Figure 15, page 37).

Ewe #92 - killed 72 hours after the infusion of 3Ml. Grossly, the lungs contained bloody froth in the trachea and bronchi. The left and right cranial lobes were adhered to the chest wall by fibrinous adhesions. The liver was degenerated, with some localized calcifications present in the bile duct wall just proximal to the gall bladder (Figure 16, page 37).

Microscopically, the bronchi and bronchioles were all open, with no abnormal content; but all were affected with peribronchitis and peribronchiolitis. The alveoli were smaller than normal, because they were depressed by expanded interstitium. Multifocal areas of alveolar hemorrhage were also present in the lungs. Approximately 50% of the alveoli contained red blood cells and numerous pneumocytes with mononuclear cells. Some of the alveolar walls were ruptured. Edema was present in some alveoli where slightly eosinophilic fluid was

found. In some areas, hemorrhage caused the interstitium to become wider. Approximately 25% of the interstitial spaces were infiltrated with mononuclear cells. The vasculature was normal. Mild alveolar emphysema was present under the pleural surface.

Mild sarcocystosis was found in the heart section, up to 5 cysts were present in one microscopic field. Mild necrosis was present in both the kidney and the liver. Glomeruli were hypercellular, glomerular capillaries were not distinct. The membranes of tubular cells were very indistinct and cytoplasm of these cells was stained lighter than normal with a granular appearance. Cytoplasmic vacuolization was also present. Nuclei were stained lighter than normal, with nucleoli easily visible. Small dark granules were present in karyoplasm. All changes were diffuse.

Liver cells were stained lighter than normal, and were surrounded by a very indistinguishable cell membranes. The cytoplasm was granular and vacuoles were present in some cells. Karyoplasm was stained lighter than normal, and contained small granules. Nucleoli were easily visible. All the changes were diffuse.

PAPER 2

THE PATHOLOGICAL EFFECT OF THREE-METHYLINDOLE

ON LUNG SURFACTANT COMPOSITION IN LAMBS

INTRODUCTION AND LITERATURE REVIEW

Lung surfactant is a phospholipid-protein complex, a mixture containing more than 50% of saturated dipalmitoylphosphatidylcholine, and approximately 25% of unsaturated phosphatidylcholine, 5 to 10% of phosphatidylglycerol, 5% of cholesterol, 8 to 10% of protein, which is mostly apolipoprotein A.¹ The overall phospholipid composition of all mammalian species is similar.¹ But Harwood et al. found that lung surfactant from sheep contained 58% of dipalmitoylphosphatidylcholine, 11% of lysophosphatidylcholine, 10% of unesterified fatty acids, 6% of triacylglycerol, 4.4% of phosphatidylglycerol, 3.5% of phosphatidylinositol, 2 to 3% of cholesterol and sphingomyelin each and 0.5% of others.² Lipid extracts from bovine lung lavage fluid from newly slaughtered cows contained 3% of neutral lipids, and 97% of phospholipids, from which phosphatidylcholine comprised 79% and phosphatidylglycerol 11%.³ Phosphatidylethanolamine, phosphatidylinositol, lysobis-phosphatidic acid and sphingomyelin were found in smaller amounts. Phosphatidylcholine was 53% disaturated, phosphatidylglycerol was 23% disaturated. The remaining species were mostly monoenoic; the protein content was 10% or 5%, being dependent on the analytical method. Extraction with chloroform-methanol removed approximately 90% of the protein, but had no effect on the surfactant properties, when evaluated by a pulsating bubble technique.³

The reason for phosphatidylcholine saturation seems to be its pliability, which is better when palmitic acid is attached to C1 and

C2 of the glycerol skeleton. This enables saturated phosphatidylcholine to be more pliable at the body temperature.^h

When lung surfactant is deficient in phosphatidylglycerol, it still exerts normal surface active properties. That confirms indirectly the overall importance of phosphatidylcholine in surfactant composition.⁴

Quite recently, several studies have been published about the phospholipid analysis from lung washings by high performance liquid chromatography (HPLC).⁵⁻⁸ This method is quite expedient and is reliable for routine analysis.

HPLC procedures use normal phase (stationary phase - polar, eluents - nonpolar) or reversed-phase chromatography (stationary phase - nonpolar, eluents - polar) for phospholipids,⁹ and basically two solvent systems: acetonitrile - water or hexane - isopropanol - (water).⁵⁻⁸ These two solvent systems allow the detection of phospholipids in the ultraviolet light range, around the wavelength of 200 nm, because at this wavelength they do not interfere.ⁱ Though, the acetonitrile-water system is more suitable for sensitive detection of phospholipids.ⁱ

^hDr. W. Klopfenstein, Kansas State University: personal communication, May 1986.

ⁱDr. Larry Seitz, US Grain Marketing Research Center, Manhattan, KS: personal communication, September 1986.

By HPLC applied to amniotic fluid or lung washings, different authors were able to detect and separate almost all phospholipids present.⁵⁻⁸ They usually used a precolumn (guard column) to achieve better results.⁵⁻⁸

MATERIALS AND METHODS

Following a gross necropsy examination, after severing the left lung and clamping the left bronchus, the right lung was lavaged with approximately 2 liters of 0.9% physiological saline at 40°C, by pouring it via a funnel inserted into the trachea. The right lung was gently massaged by hand for 30 sec and the recovered fluid was used for phospholipid analysis, by using the method of Engen et al.¹⁰

The lavage fluid, usually more than 1 pint, was transferred into two 250 ml containers and centrifuged at 3,000 g for 15 min to separate the cellular elements. The supernatant was then centrifuged in eight 40 ml tubes at 31,000 g for 1 hour at 4°C. The pellets were suspended in distilled deionized water, transferred into 1 tube and centrifuged again at 31,000 g for 30 min at 4°C. The pellet was then suspended in 5 ml of 0.74% potassium chloride in distilled deionized water, and transferred into a separation funnel. Five milliliters of methanol and 10 ml of chloroform, both of HPLC quality, were added and the funnel was shaken gently for 1 min. After standing at 4°C overnight, the lower chloroform portion was collected into a vial and evaporated under nitrogen at room temperature. The lipids remaining in the vial were reconstituted with 0.5 ml of methanol and chloroform each, both chemicals being of HPLC quality. Then the vial was filled

with nitrogen and stored at -30°C in a freezer till chromatography was provided. Before chromatography, samples were evaporated and diluted in hexane-isopropanol 1:1.

The standards were purchased from Supelco^J: lecithin, phosphatidic acid, phosphatidylethanolamine, lysolecithin, sphingomyelin, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol; and from Sigma^K: palmitoyl-L- α -lysophosphatidylcholine, phosphatidylcholine, dipalmitoyl-phosphatidylglycerol, dipalmitoyl-phosphatidylserine, dipalmitoyl-phosphatidylethanolamine, dipalmitoyl-phosphatidylcholine.

The HPLC system:

Chromatography was provided on the Hewlett-Packard chromatograph, model 1084B, equipped with a photodiode array detector, and also with an autosampler, an autoinjector, an integrator, and an oven. The detector was model 1040A consisting of a mainframe, a master controller (HP 85 computer), and a dual disc drive (HP 82901).

Chromatograms were formed by this instrument by monitoring the difference between absorbances at sample and reference wavelengths. For all analyses the reference length was centered at 550 nm with a 50 nm bandwidth. The sample wavelength was 200 nm with a 4 nm bandwidth. The separation was provided on a Dupont ZORBAX SIL column⁸ (25 cm x 4.6 mm I.D., 5 to 6 μm particle size), maintained at 35°C , at the flow rate of 1.5 ml/min.

^JSupelco, Inc., Bellefonte, PA

^KSigma Chemical Co., St. Louis, MO

The mobile phase was acetonitrile/water 80/20 (solvent A) and 100% acetonitrile (solvent B), both of HPLC quality. We maintained the gradient of 88% B from 0 to 5 min. to 25% B at 15 min to 25% B at 35 min and 88% B again at 40 min. Both solvents were degassed with heat and vacuum before the separation, and kept at room temperature during the separation.

The chromatograph drew a curve with peaks for individual phospholipids, integrated the areas under the peaks, and expressed them as integrator counts. We divided the size of every given area in counts with the amount of phospholipids harvested from each animal, because we diluted the harvested phospholipids with the same amount of solvent, and used the same amount of that mixture injected into the column. The details are given in a copy of a chromatogram from lamb #83 in the Appendix, on page 63. That lamb was killed 24 hours after 3MI administration.

The results of the phospholipid analysis were evaluated statistically by a one-way analysis of variance.

RESULTS

The composition of phospholipids present in lung washings is given in Table 4, the mean values for individual phospholipids in each group are given in Table 5.

When evaluated statistically, the only significant difference found was in the amount of sphingomyelin in groups 1 and 4, which might be an accidental finding. No other significant changes have been found at the level of $p = 0.05$.

DISCUSSION

The HPLC procedure found zero values for lysophosphatidylcholine (LPC) in lung washings from lambs #123 (control group), #6 (24 hour group), #4 (72 hour group), #93 (propylene glycol group), and zero values for sphingomyelin in lung washings from lambs #4 (72 hours group) and #93 (propylene glycol group). The amount of phospholipids harvested from the lungs of those animals was small: 2.84 mg from #93, 4.91 mg from #4, 6.64 mg from #123, 7.43 mg from #6, and this may account for the negative findings. The phospholipid quantities from other animals were larger (for example: 8.41 mg from #40 - control group, also contained all 5 phospholipids).

One lamb (#123) in group 1 (control) had 14,681 counts of sphingomyelin found in the chromatograph, while others in that group had 3,638 to 8,048 points. Also, one lamb (#93) from group 4 (propylene glycol) had a zero value. The other 3 lambs in that group had 3,143 to 4,402 counts of sphingomyelin, similar values to group 1. The quantity of phospholipids from lamb #93 was the lowest (2.84 mg), which may have been below the detectable levels for sphingomyelin in this lamb. This may account for the statistical difference of sphingomyelin between groups 1 and 4.

Lung washings from lambs in our study did not contain blood and this was helpful for HPLC analysis. If hemolyzed blood phospholipids are present, it makes it nearly impossible to separate phospholipids from blood cells and serum from phospholipids coming from lung washings.

The phospholipid composition of lung surfactant from lambs administered 3MI was not significantly different from the control or propylene glycol group. This finding correlates with the negative pathological and histopathological findings in the experimental lambs.

The phospholipid standards helped us identify each HPLC peak, but we were not able to positively identify lysophosphatidylcholine and this may be due to the fact that our standard was saturated, while the lung washings contained unsaturated lysophosphatidylcholine. To produce detectable pathological lesions or changes in the chemical composition of lung phospholipids may require either higher dosages of 3MI, longer administration, or the use of adult sheep which may be more sensitive to 3MI toxicity than lambs.

We chose the HPLC analysis instead of thin layer chromatography (TLC) because TLC is quite tedious and relatively unreliable if the phospholipids are being detected by densitometry. A reliable and accurate analysis can be made by TLC if a quantitative determination of phosphorus is made.¹¹ To improve the resolution of individual phospholipids, two-dimensional TLC is used in addition.

Both methods, TLC and HPLC, require standards for the detection of phospholipids present in the analyzed samples. The saturation of fatty acids on the phospholipid molecule is crucial for HPLC, because the UV detection used with HPLC is dependent on detecting the amount of unsaturated bonds in the molecule. Phospholipids with more unsaturated bonds in their fatty acids will demonstrate greater

presence when compared with phospholipids containing saturated fatty acids in their molecule.¹

SUMMARY

The HPLC procedure was able to separate phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol, sphingomyelin, and maybe lysophosphatidylcholine which was hard to identify with its standard, because the retention time of these two compounds were not identical. That might have been due to difference in saturation of the lysophosphatidylcholine molecule.

The HPLC did not detect significant differences in lung surfactant phospholipid composition in any of the groups except there was a difference in sphingomyelin in groups 1 and 4, but this was evaluated as an inaccurate finding. One animal in group 4 had a value for sphingomyelin of 2.84 mg. The amount of lung surfactant phospholipids from other animals varied between 4.91 and 31.03 mg. The sphingomyelin content (2.84 mg) of that animal might have been below the detectable level by that method.

The HPLC procedure was quite expedient, less tedious than thin layer chromatography, and was reliable enough to analyze the lung washing samples from lambs treated with 3-methylindole.

¹Dr. Larry Seitz, US Grain Marketing Research Center, Manhattan, KS: personal communication, September 1986.

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APPENDIX: Paper 2

Table 4: Composition of phospholipids* from lung surfactant (High performance liquid chromatography results**)

| Group | Lamb # | PG | PE | PC | SPH | LPC |
|---|--------|--------|---------|---------|--------|--------|
| 1 (control) | 40 | 34,619 | 33,281 | 171,070 | 8,048 | 3,623 |
| | 46 | 27,653 | 41,972 | 172,889 | 6,413 | 10,207 |
| | 76 | 43,776 | 34,409 | 188,211 | 3,638 | 6,484 |
| | 123 | 35,494 | 131,626 | 218,554 | 14,681 | 0 |
| 2 (3MI, killed after 24 hours) | 6 | 44,684 | 58,485 | 219,785 | 3,061 | 0 |
| | 47 | 29,950 | 25,867 | 152,148 | 4,473 | 4,207 |
| | 49 | 31,914 | 26,812 | 152,520 | 4,781 | 4,026 |
| | 83 | 37,953 | 25,488 | 153,467 | 2,416 | 4,772 |
| | 84 | 37,925 | 25,254 | 141,063 | 4,600 | 3,971 |
| 3 (3MI, killed after 72 hours) | 4 | 39,367 | 55,544 | 182,281 | 0 | 0 |
| | 44 | 25,416 | 25,126 | 155,377 | 10,013 | 4,720 |
| | 48 | 37,180 | 32,613 | 165,013 | 5,371 | 3,059 |
| | 85 | 44,893 | 31,098 | 188,550 | 6,530 | 3,408 |
| 4 (killed 24 hours after prop. glycol administration) | 80 | 24,898 | 22,543 | 123,917 | 3,538 | 4,402 |
| | 91 | 23,938 | 18,321 | 137,148 | 4,402 | 6,229 |
| | 93 | 31,444 | 46,599 | 213,873 | 0 | 0 |
| | 124 | 36,263 | 23,932 | 146,297 | 3,148 | 5,651 |

*PG = phosphatidylglycerol, PE = phosphatidylethanolamine, PC = phosphatidylcholine, SPH = sphingomyelin, LPC = lysophosphatidylcholine

**The values given are integrator counts calculated by the chromatograph computer, integrated from the areas under individual peaks, divided by the amount of isolated lung surfactant (in mg) to unify the results for comparison.

APPENDIX: Paper 2

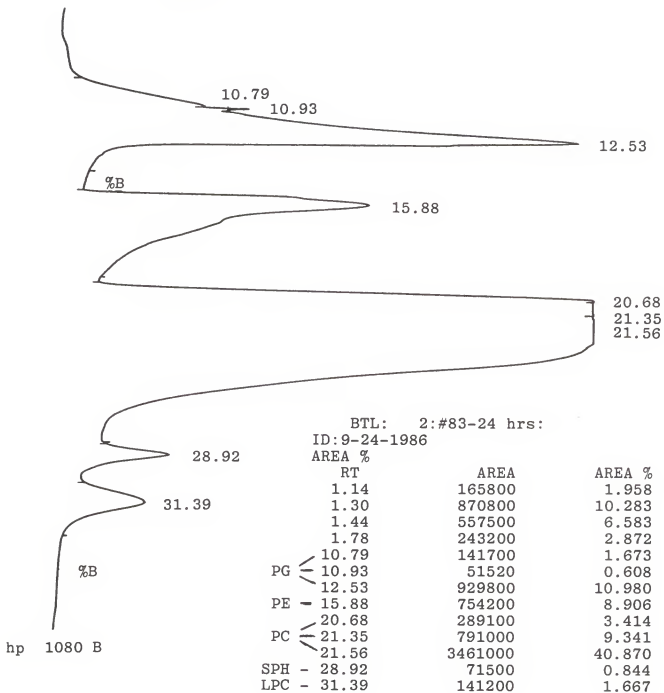
Table 5: Mean* values of individual phospholipids in lung surfactant of 4 experimental groups of lambs.

| Group | PG | PE | PC | SPH | LPC |
|---|--------|--------|---------|---------|-------|
| 1 (control) | 35,386 | 60,322 | 187,681 | 8,195** | 6,771 |
| 2 (3MI, killed after 24 hours) | 36,485 | 32,381 | 163,797 | 3,866 | 4,244 |
| 3 (3MI, killed after 72 hours) | 36,714 | 36,095 | 172,805 | 7,305 | 3,729 |
| 4 (killed 24 hours after prop. glycol administration) | 29,136 | 27,849 | 155,309 | 2,772** | 4,070 |

*The values given are integrator counts calculated by the chromatograph computer, as in Table 4, and the means were calculated for each group.

**Values were significantly different at $p \leq 0.05$.

Figure 25: Chromatogram of lung surfactant phospholipids from lamb #83



THE PATHOLOGICAL EFFECT OF THREE-METHYLINDOLE ON LUNGS
AND LUNG SURFACTANT COMPOSITION IN LAMBS

by

JOE A. KUBICEK

MVDr., University of Veterinary Medicine, Brno, 1967

AN ABSTRACT OF A MASTER'S DISSERTATION

submitted in partial fulfillment of the
requirements for the degree

MASTER OF SCIENCE

College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1987

ABSTRACT

Seventeen lambs of Suffolk and cross breedings, 5 to 6 months old, females and castrated males, were divided randomly into 4 groups. Four control lambs in group 1 were clinically examined 3 times within 24 hours, and killed without treatment. Five lambs in group 2 received 0.03 g of 3-methylindole (3MI) per kg of body weight, diluted in propylene glycol, intravenously and intraperitoneally, and were killed 24 hours later. Four lambs in group 3 received 0.03 g of 3MI per kg of body weight as group 2, and were killed 72 hours later. Four lambs in group 4 received propylene glycol intravenously and were killed 24 hours later. All lambs in groups 2, 3 and 4 were also examined clinically at 12 hour intervals as the lambs in group 1.

All lambs were necropsied, examined grossly, and their left lung, heart, kidney and liver were examined histopathologically. The lung sections were also frozen in a mixture of freon 22 with dry ice, and stained for lung surfactant phospholipids.

No significant clinical changes were found. Pathologically, all 17 lambs suffered from chronic interstitial pneumonia, possibly of viral origin; no typical lesions as described for 3MI were found in lambs. Two adult ewes and one lamb, who collapsed during the 3MI infusion, exhibited pathological changes of acute pulmonary edema and emphysema, which are typical of 3MI. These findings are included in the appendix. The dosage of 3MI given to lambs was found to be below detectable toxicity levels. Lambs appear to be less sensitive to 3MI than adult sheep, and sheep in general are reported to be more tolerant to 3MI than other ruminants.

The right lung from all lambs was lavaged with saline, and the fluid was examined by high performance liquid chromatography (HPLC) for lung surfactant composition. The procedure was able to separate phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol, sphingomyelin, and possibly lysophosphatidylcholine. The HPLC procedure did not detect significant differences in lung surfactant composition in any of the groups except the difference in sphingomyelin in groups 1 and 4, which was evaluated as an inaccurate finding.