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# PATHOPHYSIOLOGIC MECHANISMS OF THREE PULMONARY EDEMAGENIC COMPOUNDS: THE ROLE OF TOXIC OXYGEN SPECIES

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Inhalation of phosgene (CG), perfluoroisobutylene (PFIB), or bis(trifluoromethyl)-disulfide (TFD) results in the production of pulmonary edema, but the mechanism involved has not been determined. To study this mechanism, the effects of eight drugs thought to affect the development of this type of edema were studied in rats exposed to these gases. The compounds included N-acetylcysteine (thiol), ibuprofen (cyclooxygenase inhibitor), pentigetide (inflammation modulator), lodoxamide (xanthine oxidase inhibitor). and hydroxyurea (neutrophil depleter) and three lazaroids (two 21-aminosteroids and one 2-methylaminochroman). The protection from lung toxicity that was found, if any, varied according to the drug used, gas involved and timing of drug administration. Although the results are consistent with a mechanism of toxic gas lung injury involving toxic oxygen radicals, other mechanisms are not excluded.

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## PREFACE

The work reported herein was conducted under USAMRICD Protocol #1-03-89-000-A-525, entitled "Pathophysiologic Mechanisms of Three Pulmonary Edemagenic Compounds: The Role of Toxic Oxygen Species." The data is recorded in USAMRICD notebooks numbers 054-89 and 035-90. The work was initiated in August 1988 and completed in April 1991.

## ACKNOWLEDGEMENTS

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# INTRODUCTION

The purpose of this study was to gain insight into the mechanism by which certain toxic gases produce chemical injury to the lungs when inhaled. The mechanisms responsible for producing lung edema after exposure to phosgene (CG), bis(trifluoromethyl)disulfide (TFD), or perfluoroisobutylene (PFIB) remain unknown. Some of the pulmonary responses to acute lung injury include complement activation, aggregation of granulocytes and platelets, stimulation of arachidonic acid metabolism and generation of toxic oxygen species [Said, 1985]. Selected lung peptides also may play a role in either pathologic or protective mechanisms involved in acute lung injury [Dey and Said, 1985]. The current scientific and medical literature indicates that these responses are often overlapping, and the generation of toxic oxygen species may be a common molecular action in each case.

Toxic oxygen species include superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide ($H_2O_2$), hydroxyl radical ($OH^{\cdot}$), singlet oxygen ($^1O_2$) and peroxide radical ($ROO^{\cdot}$, where R = lipid). There is mounting evidence suggesting that the reduction of molecular oxygen to any of these moieties results in damage to biologic membranes, and can be generated by pulmonary epithelia and endothelia, macrophages and neutrophils. The tissue damage is characterized by lipid peroxidation, nucleic acid damage, carbohydrate depolymerization, and protein denaturation [Taylor, 1985]. These reactive oxygen metabolites are generated by specific enzymes (e.g., xanthine oxidase), by autoxidation and by energy transfer reactions, all normal processes in aerobic metabolism. Similarly, there are normal antioxidant defenses that prevent cellular toxicity and are characterized as enzymatic (e.g., superoxide dismutase, catalase, glutathione peroxidase) or non-enzymatic (e.g., vitamins E and C, glutathione, cysteine and other thiols). When oxidant production overcomes the endogenous defense system, tissue damage ensues. A logical approach to determining if lung damage is related to toxic oxygen species is to manipulate the enzymatic/non-enzymatic control over the balance of these oxygen radical forces. Indeed, the pathogenesis of pulmonary edema has been attenuated by enzymes that promote the removal of toxic oxygen moieties [Milligan, 1988] and by leukopenia inducers [Shasby, 1982].

Although toxic oxygen species generation is suggested as a likely mechanism of pulmonary injury in the aforementioned studies, it is not known if this is a mechanism for the injury observed after inhalation exposure to CG, TFD or PFIB. In this study, rats were administered intervention compounds based largely on the above biochemical schema.

## MATERIALS AND METHODS

*Experimental Paradigm*. Male Sprague-Dawley rats (140-325 g) were assigned to one of four test groups: Group A, 6 rats treated with the selected drug and exposed to the selected toxic gas; Group B, 6 rats treated with the drug vehicle and exposed to the gas; Group C, 2 rats treated with the chosen drug and exposed to air; and Group D, 2 rats treated with drug vehicle and exposed to air. For statistical analysis, the results from groups C and D (for each drug respectively) were pooled. The drug or vehicle was administered either 15 to 30 minutes before or 15 to 30 minutes after exposure to the gas, with the exception of hydroxyurea. Hydroxyurea was given twice daily for three days prior to exposure, and blood leukocyte counts were determined to insure that leukopenia had been attained (Orthodiagnostics ELT 8/DS Lazer Hematology Analyzer, Westwood, MA). Ten-minute exposures to the gases were conducted. In the initial experiments, the animals were observed for 24 hours after exposure. It was decided later to shorten the post-exposure period to 5 hours, usually using a higher concentration of the toxic gas to obtain pulmonary edema within the shortened observation period. In determining whether or not an adequate exposure to the toxic gas had been obtained, we arbitrarily categorized each experiment as comprising an "adequate exposure" when the lung weight to body weight ratio of the vehicle-treated, gas-exposed rats (group B) was found to be at least twice that of the vehicle-treated air-exposed rats (group D).

*Drugs administered*. Drugs were N-acetylcysteine 200 mg/kg (Bristol-Myers Co., Evansville, IN) (vehicle, 0.9 % saline), pentigetide 2 mg/kg (Immunetech Pharmaceuticals, San Diego, CA) (vehicle, phosphate buffered saline), ibuprofen 50 mg/kg (Upjohn Co., Kalmazoo, MI) (vehicle, Placebo for Ibuprofen Sterile Solution, 50 mg/ml, Study Lot #40,524, Upjohn Corp.), lodoxamide tromethamine 10 mg/kg (Upjohn Co., Kalmazoo, MI) (vehicle, 0.9% saline), hydroxyurea 1500 mg/kg (Sigma Chemical Co., St. Louis, MO) (vehicle, 0.9% saline) and three experimental Upjohn lazaroid compounds, U78517F (vehicle, distilled water) U75412E (vehicle, 0.05N saline), and U74006F (vehicle, 0.9%saline). The latter three compounds were all given at 10 mg/kg. All drugs were given as a single dose, except hydroxyurea, which was given daily for three days. The drug or its vehicle was administered intraperitoneally (ip), with the exception of hydroxyurea. Hydroxyurea was given ip to the phosgene-exposed rats, and by gavage to the rats exposed to TFD and PFIB.

*Inhalation exposure apparatus*. Each rat was exposed to the toxic gas or air for 10 minutes through his nose and mouth. The generation and maintenance of appropriate concentrations of the required gases have been described previously (Keeler *et al*., 1990). A head-stock type of restraint (Process Instruments Corp., Brooklyn, NY) was used to hold the rats in fixed position in the gas manifold.

*Toxic Compounds Studied*. Gases were administered in concentrations that would produce pulmonary edema within the observation period and were continuously

monitored during exposure by an infra-red spectrometer (Miran 1A, Foxboro Co., Sharon, MA). Concentrations administered were CG (170 mg/m$^3$), TFD (200 mg/m$^3$), and PFIB (130-150 mg/m$^3$) [24-hr observation periods]; CG (200-230 mg/m$^3$), TFD (200-260 mg/m$^3$), PFIB (150-200 mg/m$^3$) [5-hr observation periods].

*Necropsy and Disposition of Tissue Samples*. Rats that died prior to the 5- or 24-hour endpoints were necropsied at time of death. Survivors were sacrificed by guillotine at the 5- or 24-hour endpoint after exposure and necropsied. The lungs were removed and trimmed free of adjacent tissues, then blotted and weighed. The right caudal lobe was then excised, weighed, and dried to a constant weight at 100° C. The right cranial lobe was immersion fixed in Zenker's fixative. The left lung was inflated with and then immersion fixed in 10% formalin. The heart, thymus and a mesenteric lymph node, as well as portions of the trachea, spleen, and left kidney, were also preserved in formalin.

*Histopathology*. Representative sections of the lungs were reviewed by pathologists and graded on a scale of 1 to 4. No blinding procedures were utilized in the scoring. Criteria were as follows: 0 = normal; 1 = minimal lesion (widened lymphatic and perivascular spaces, slight increase in perivascular and/or alveolar macrophages and/or eosinophils, occasional amounts or wisps of eosinophilic edema fluid in alveoli); 2 = mild lesion (multifocally, <20% of alveoli contain traces of eosinophilic edema fluid and increased number of alveolar macrophages perivascular spaces are widened and interstitial inflammatory cells are slightly increased); 3 = moderate lesion (20-50% of alveoli contain eosinophilic edema fluid , scattered alveoli have strands of fibrin within alveoli or perivascularly, septae are thickened and inflammatory cells within alveoli and interstitium are increased); 4 = severe lesion (diffuse alveolar edema, <50% of alveoli are involved, widespread fibrin deposition, marked thickening of alveolar septa, increase alveolar and interstitial inflammatory cells).

*Statistical Analysis*. Two primary indices of the severity of lung edema were analyzed by 2-tailed one way analysis of variance, (1) the lung weight to body weight ratio (x 100), and (2) the right caudal lobe wet weight to dry weight ratio. If a significant difference between the means was found, at a probability level of $p < 0.05$, a comparison between groups was performed using Newman-Keuls Analysis. Two additional parameters were tested in some of the experiments: (1) the proportions of the treated and the untreated rats that died within the 24-hour post-exposure period were compared using Fisher's Exact Test; and (2) the lung injury scores generated by histopathology examination were compared between the treated and untreated toxic gas-exposed rats and analyzed using the Mann-Whitney U Test. No formal analysis of the data for normality of distribution was made.

## RESULTS

Results are shown in the bar graphs of Figures 1 through 45, and statistically significant results for the lung weight ratios are described in the narrative summary which follows. In the intergroup comparisons, the presence of a significant difference at the $p < 0.05$ level is indicated as follows:

* indicates a significant difference between group B, the gas-exposed *vehicle*-treated group, and group A, the gas-exposed *drug*-treated group.

† indicates a significant difference between group B, the gas-exposed *vehicle*-treated group, and group C, the air-exposed *drug*-treated group.

‡ indicates a significant difference between group B, the gas-exposed *vehicle*-treated group, and group D, the air-exposed *vehicle*-treated group.

No significant differences were found between groups C and D in these experiments, and no symbols are shown for those comparisons. To avoid complexity, comparisons of groups A and C and of groups A and D are not presented. Therefore only comparisons between group B and the A, C, and D groups are noted here. An absence of any symbol over group B indicates that comparison of group B with groups A and C was made and found to be not significant.

*Results by drug and gas studied.*

(Note, those experiments in which a long (24-hour) observation period was utilized are distinguished by asterisks in Tables 1 and 2)

(1) N-ACETYLCYSTEINE Pre-treatment:

a. *vs.* CG exposure, Figures 1a & 1b. Although an initial experiment showed significant reduction in the WW/DW ratio, no significant reduction in the LW/BW ratio was found. When the experiment was repeated with a higher phosgene concentration, no significant reductions were noted.
b. *vs.* TFD exposure, Figure 2.
c. *vs.* PFIB exposure, Figures 3a and 3b. An initial experiment failed to secure an adequate exposure. When repeated at a higher dose of PFIB, a significant reduction in the WW/DW ratio was found. The LW/BW ratio trended similarly but did not reach statistical significance.

(2) PENTIGETIDE Pre-treatment:

a. *vs.* CG exposure, Figures 4a & 4b. On an initial experiment, significant reductions in LW/BW and WW/DW ratios were found. On repetition at a higher phosgene concentration and a shorter observation period, this was not confirmed.
b. *vs.* TFD exposure, Figure 5. A significant *increase* in LW/BW ratio was noted, but the WW/DW ratio was not significantly increased.

c. *vs.* PFIB exposure, Figures 6a & 6b. On an initial experiment, an adequate exposure was not obtained. When the experiment was repeated at the same PFIB concentration, adequate exposure was obtained but no significant differences in treatment and vehicle groups was found.

(3) IBUPROFEN Pre-treatment:

a. *vs.* CG exposure, Figure 7.
b. *vs.* TFD exposure, Figure 8.
c. *vs.* PFIB exposure, Figures 9a & 9b. An initial exposure to PFIB did not result in an adequate exposure. When the experiment was repeated at a higher PFIB concentration, the LW/BW ratio indicated significant reduction in edema by the ibuprofen pre-treatment. The WW/DW ratio trended in the same direction but did not reach statistical significance.

(4) LODOXAMIDE Pre-treatment:

a. *vs.* CG exposure, Figure 10. A significant *increase* in WW/DW ratio was found, but the increase in LW/BW ratio was not significant.
b. *vs.* TFD exposure, Figure 11.
c. *vs.* PFIB exposure, Figures 12a & 12b. In the initial experiment, an adequate exposure was not obtained. When repeated at a higher PFIB concentration, no significant differences between drug and vehicle-treated groups were noted.

(5) HYDROXYUREA Pre-treatment:

a. *vs.* CG exposure, Figure 13.
b. *vs.* TFD exposure, Figures 14a & 14b. In two experiments, at two concentrations of TFD, significant reductions in lung edema accumulations apparently resulted from pretreatment with hydroxyurea.
c. *vs.* PFIB exposure, Figures 15a, 15b, 15c, & 15d. On two initial experiments (Figures 15a and 15b), an adequate exposure was not obtained. On a third experiment at a greater PFIB concentration, a significant decrease in the WW/DW ratio was found, but the decrease in LW/BW ratio was not significant. On a fourth repetition at a slightly lower PFIB concentration, no significant differences between the gas-exposed groups were noted, but a similar trend was noted.

(6) U78517F Pre-treatment:

a. *vs.* CG exposure, Figure 16.
b. *vs.* TFD exposure, Figure 17. A significant reduction was found in the LW/BW ratio, but the WW/DW ratio was not significantly reduced.
c. *vs.* PFIB exposure, Figure 18. Significant reductions were found in both LW/BW and WW/DW ratios.

(7) U75412E Pre-treatment:

a. *vs.* CG exposure, Figure 19.
b. *vs.* TFD exposure, Figure 20a, 20b, & 20c. On an initial experiment an adequate exposure was not obtained. When the experiment was repeated at the same concentration, a significant *increase* in the LW/BW ratio was found in the drug-treated, gas-exposed group, but this was not accompanied by a significant difference in the WW/DW ratio. When the experiment was repeated again, at a higher concentration, a significant decrease in the LW/BW ratio was found in the drug-treated, gas-exposed group. The WW/DW ratio trended in the same direction but did not reach statistical significance.
c. *vs.* PFIB exposure, Figure 21.

(8) U74006F Pre-treatment:

a. *vs.* CG exposure, Figures 22a & 22b. On an initial experiment a significant reduction on LW/BW ratio was found, but the reduction in WW/DW ratio was not significant. On repetition of the experiment at the same CG concentration, no significant differences in the ratios for treated and untreated rats were found.
b. *vs.* TFD exposure, Figures 23a & 23b. On an initial experiment, an adequate exposure was not obtained. On repetition of the experiment at a higher concentration, the WW/DW ratio indicated significant reduction in the edema in the drug-treated group, when compared with the vehicle-treated group. The LW/BW ratio trended in the same direction but did not reach statistical significance.
c. *vs.* PFIB exposure, Figure 24.

(9) ACETYLCYSTEINE Post-treatment:

a. *vs.* CG exposure, Figure 25.
b. *vs.* TFD exposure, Figure 26.
c. *vs.* PFIB exposure, Figure 27a, 27b, 27c, & 27d. On an initial experiment, an adequate exposure was not obtained. When the experiment was repeated at higher PFIB concentration, a reduction in the LW/BW index was found. The change in the WW/DW index was minimal. When the experiment was repeated at an even higher concentration of PFIB, no significant differences were found between drug and vehicle-treated groups.

10) PENTIGETIDE Post-treatment:

a. *vs.* CG exposure, Figures 28a & 28b. No significant differences between the gas-exposed groups were found, in two experiments at differing gas concentrations.
b. *vs.* TFD exposure, Figure 29.
c. *vs.* PFIB exposure, Figures 30a & 30b. An initial experiment failed to achieve an adequate exposure. On repetition at the same PFIB concentration, no significant differences were noted between gas-exposed groups.

11) IBUPROFEN Post-treatment:

a. *vs.* CG exposure, Figures 31a & 31b. On an initial experiment, significant reductions in LW/BW and WW/DW ratios were found. On repetition at a higher phosgene concentration with a shorter observation period, no significant reductions were found.
b. *vs.* TFD exposure, Figure 32.
c. *vs.* PFIB exposure, Figures 33a & 33b. On an initial experiment, an adequate exposure was not obtained. When the experiment was repeated at a higher concentration, no significant differences were found between the gas-exposed groups.

12) LODOXAMIDE Post-treatment:

a. *vs.* CG exposure, Figure 34.
b. *vs.* TFD exposure, Figure 35.
c. *vs.* PFIB exposure, Figure 36.

13) U78517F Post-treatment:

a. *vs.* CG exposure, Figure 37. Significant reductions were found in both LW/BW and WW/DW ratios.
b. *vs.* TFD exposure, Figure 38. An adequate exposure was not obtained.
c. *vs.* PFIB exposure, Figure 39.

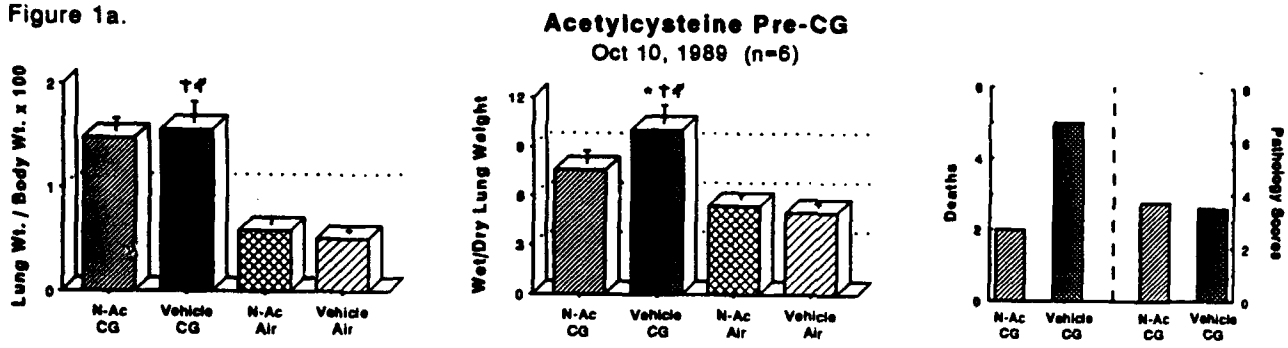
14) U75412E Post-treatment:

a. *vs.* CG exposure, Figures 40a & 40b. No significant differences between the gas-exposed groups were found at two CG concentrations, in two experiments. WW/DW ratios were not obtained in the second experiment.
b. *vs.* TFD exposure, Figures 41a, 41b, & 41c. In two initial experiments, adequate exposures were not obtained. When the experiments were repeated at a higher TFD concentration, no significant differences between the gas-exposed groups were noted.
c. *vs.* PFIB exposure, Figure 42.

15) U74006F Post-treatment:

a. *vs.* phosgene exposure, Figure 43.
b. *vs.* TFD exposure, Figures 44a & 44b. On an initial experiment a significant increase in lung weights was not produced by the gas exposures. When the experiment was repeated at a higher gas concentration, no significant differences in the ratios for treated and untreated rats were found.
c. *vs.* PFIB exposure, Figure 45.

Figure 1a.

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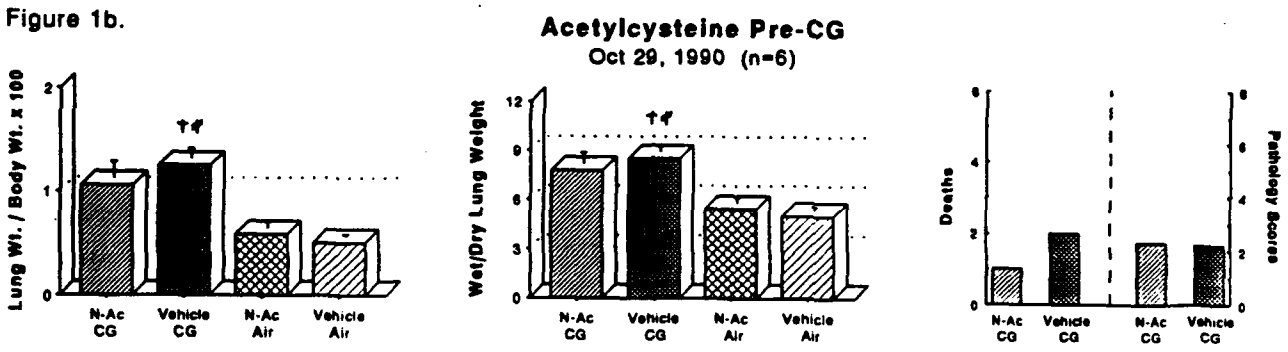


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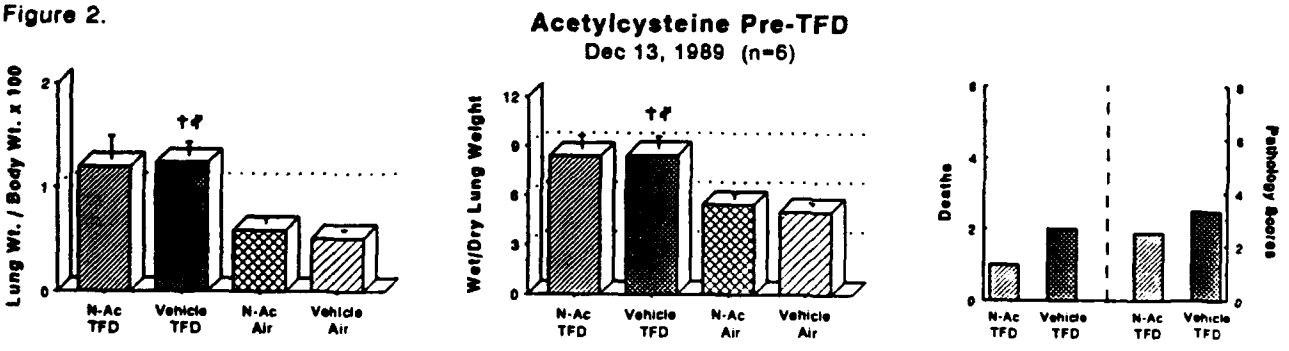


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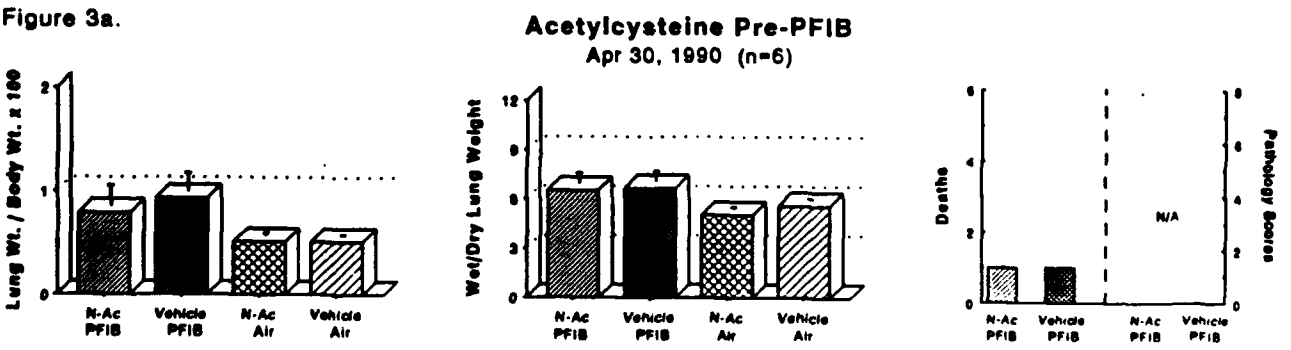

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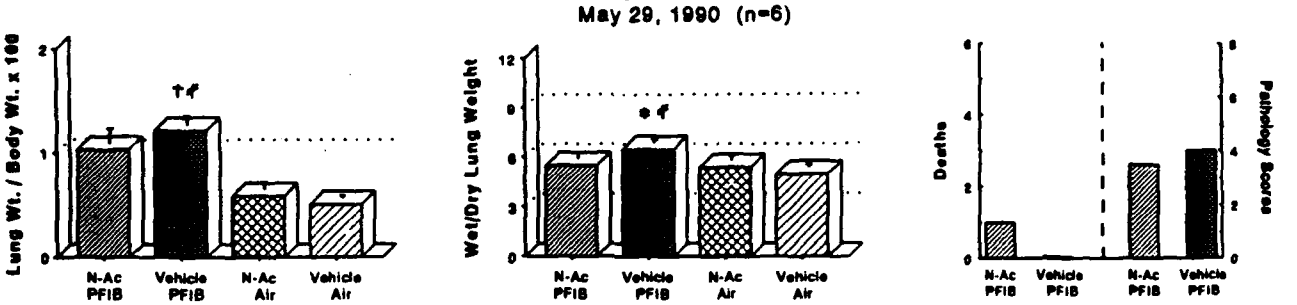


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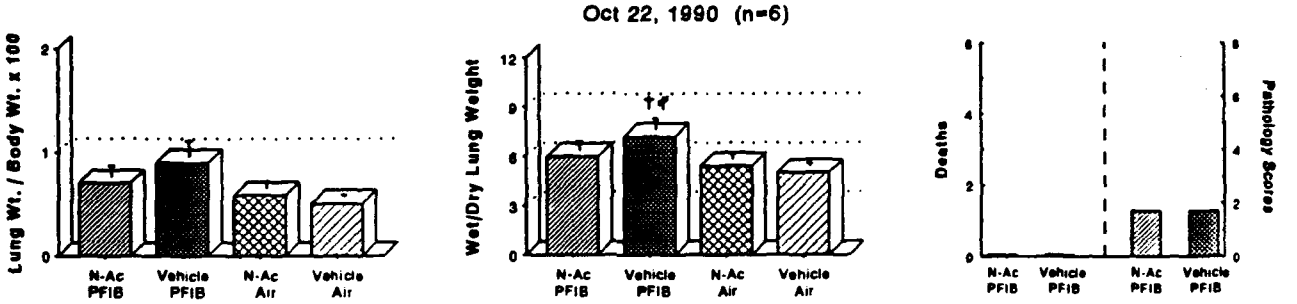


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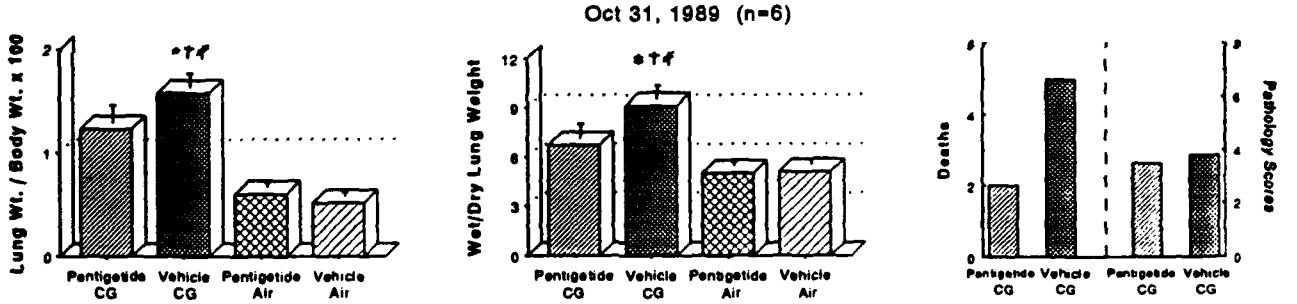


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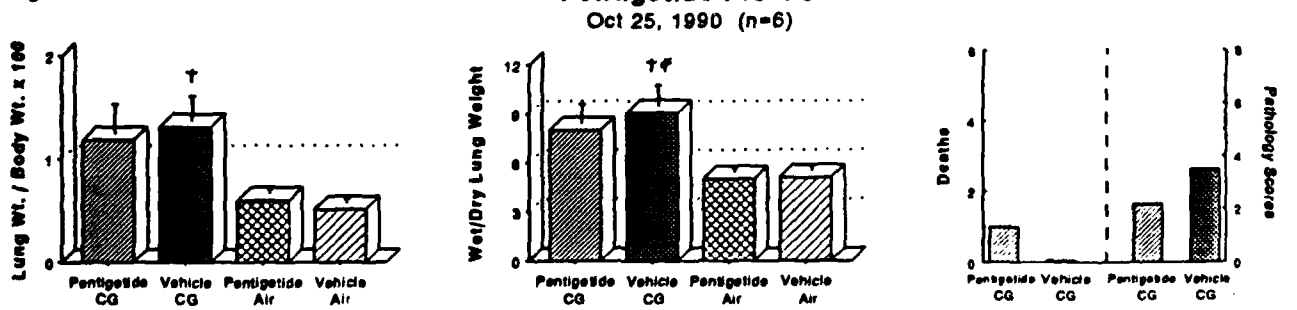

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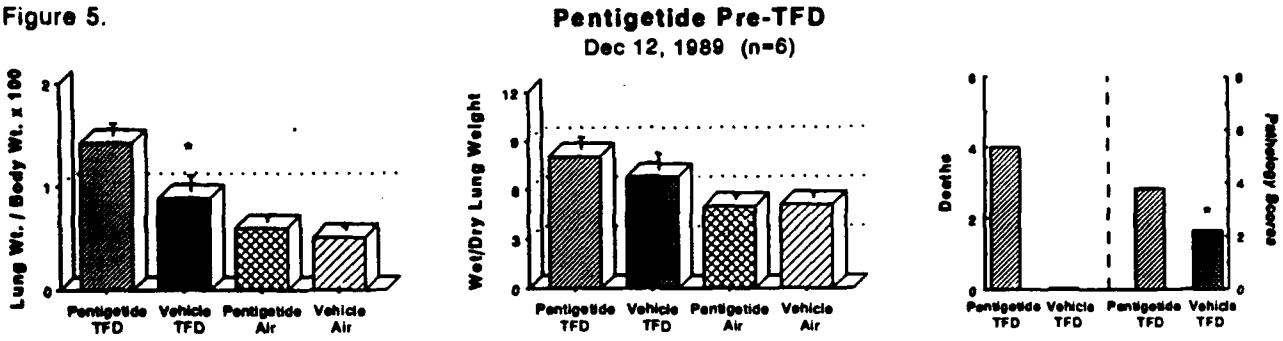


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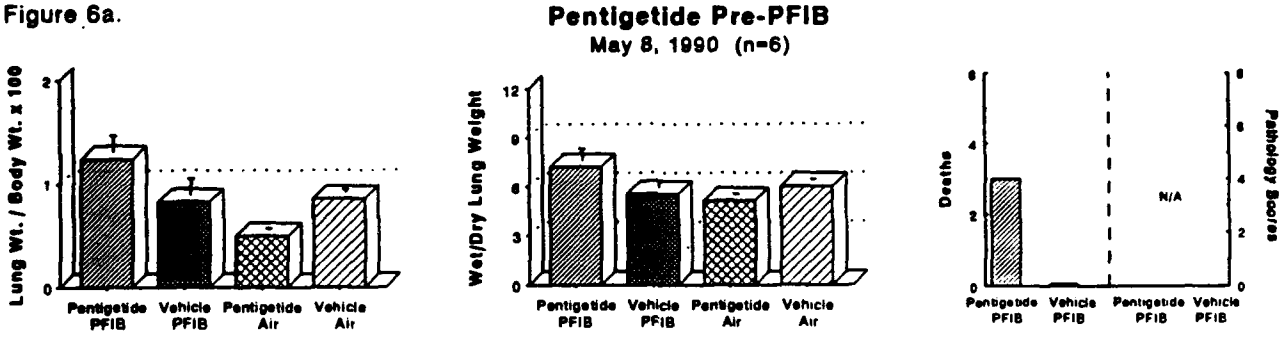


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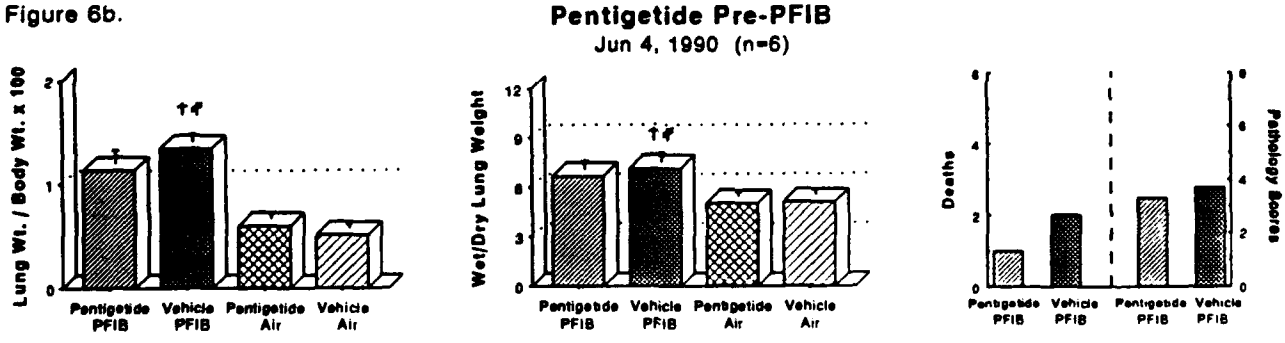


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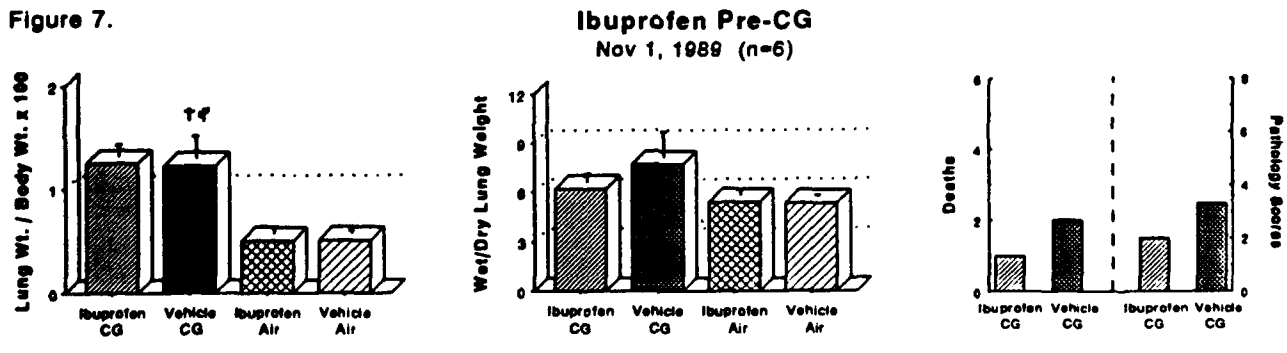

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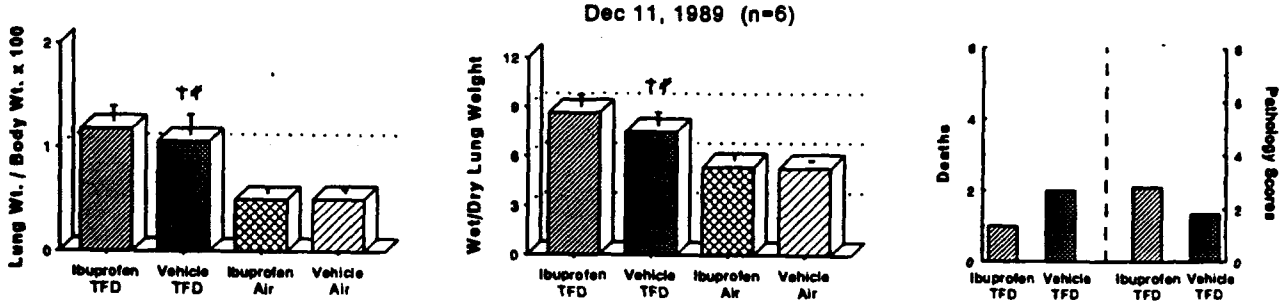


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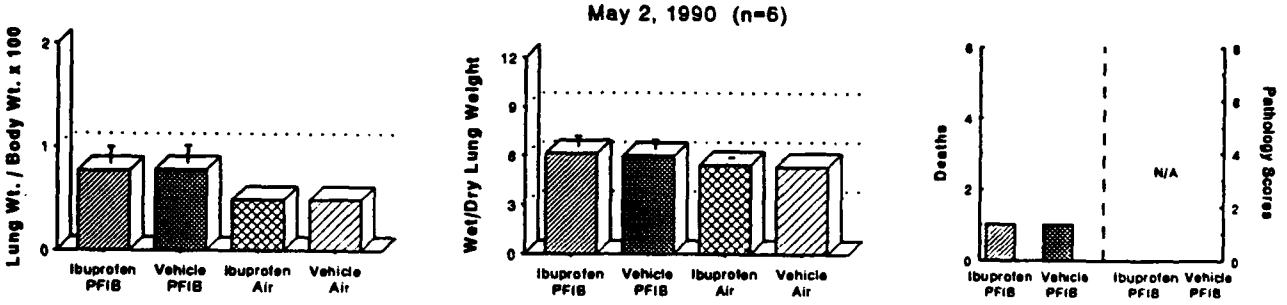


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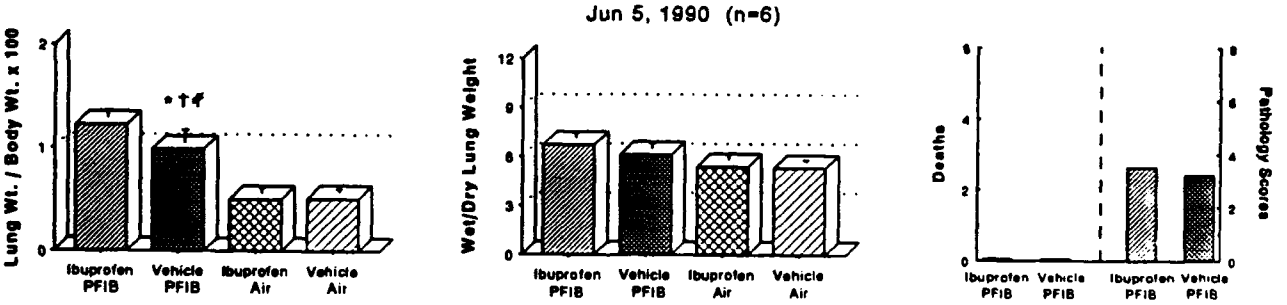


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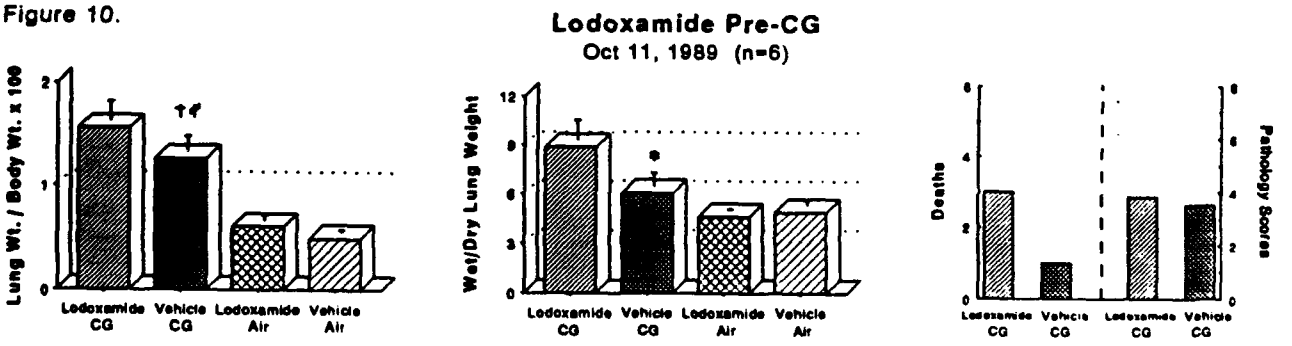

Figure 11.

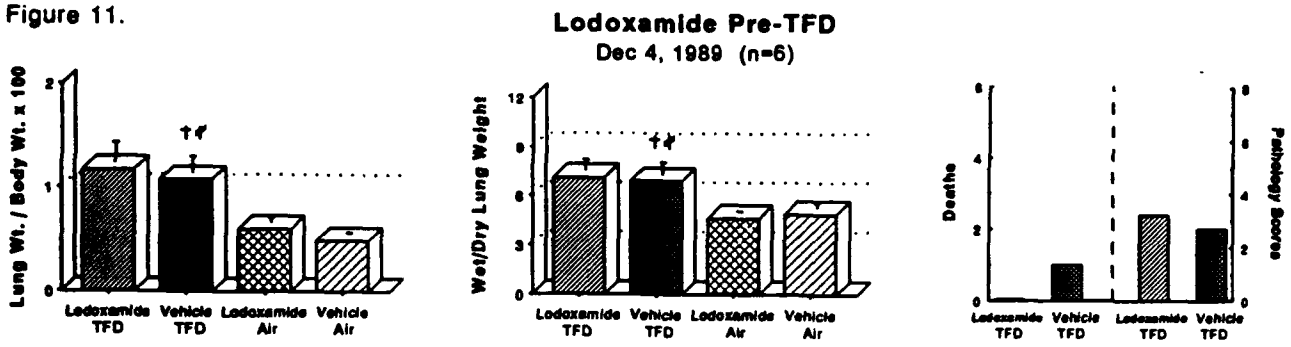


Figure 12a.

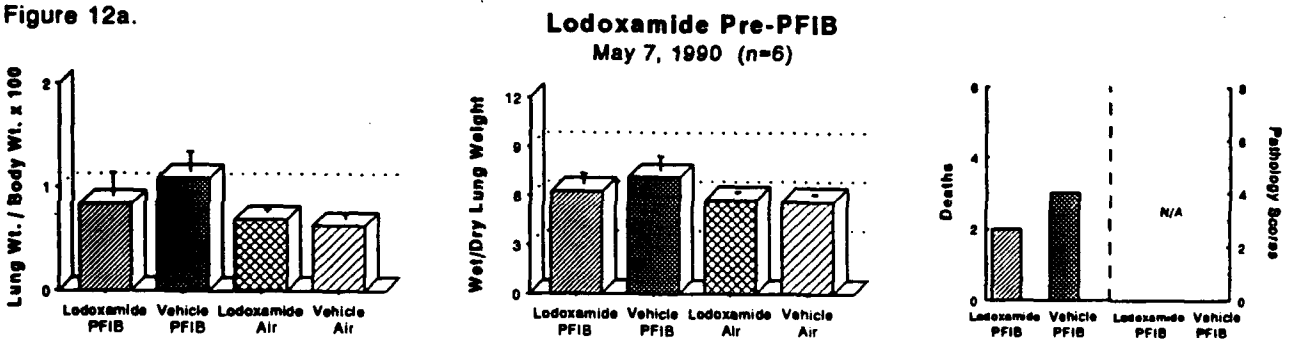


Figure 12b.

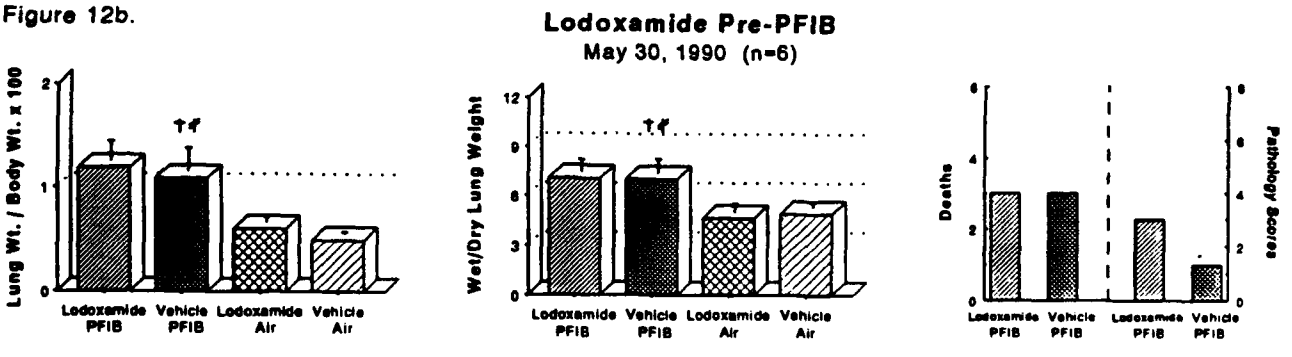


Figure 13.

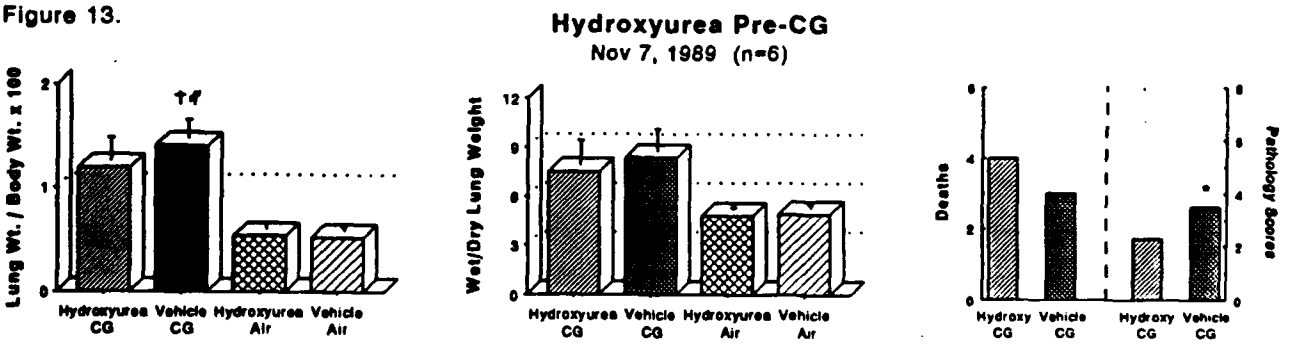

Figure 14a.

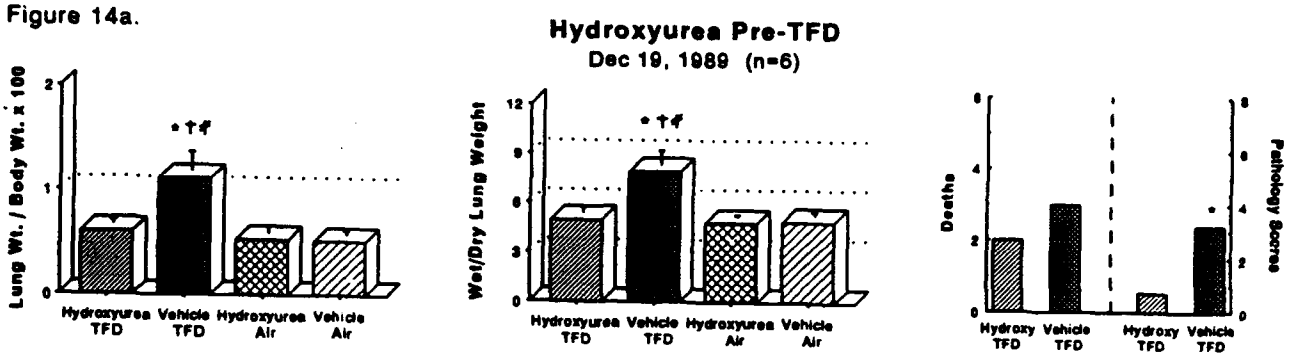


Figure 14b.

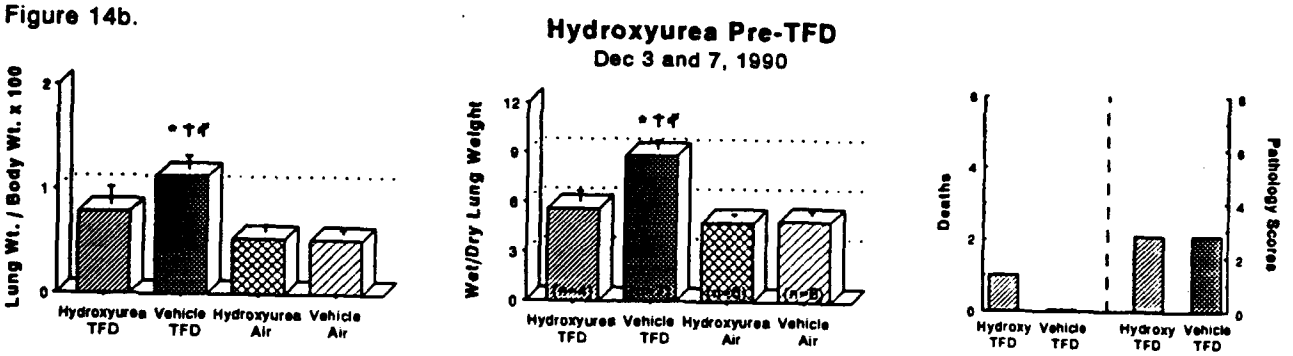


Figure 15a.

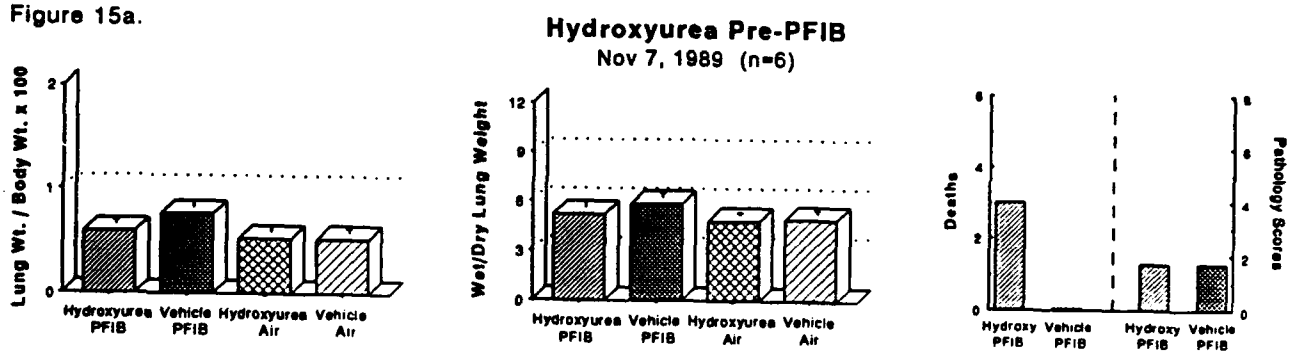


Figure 15b.

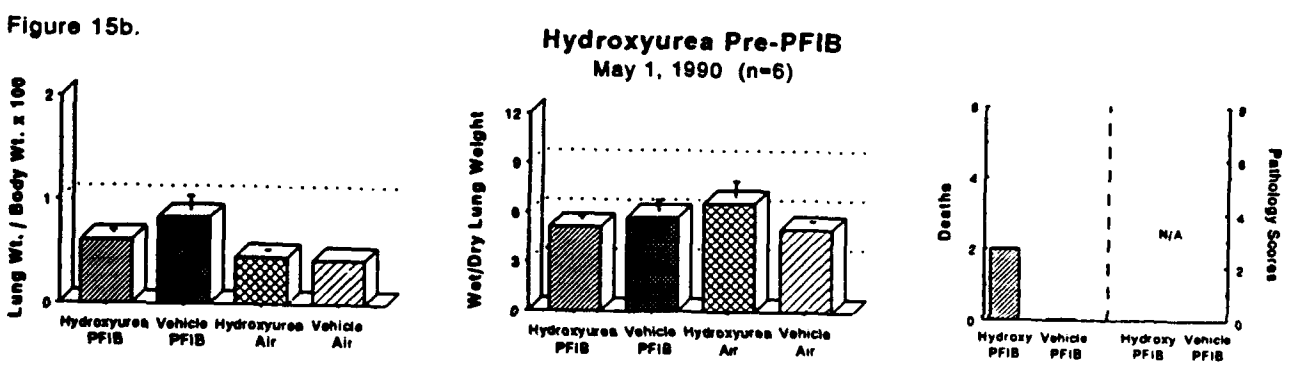

Figure 15c.

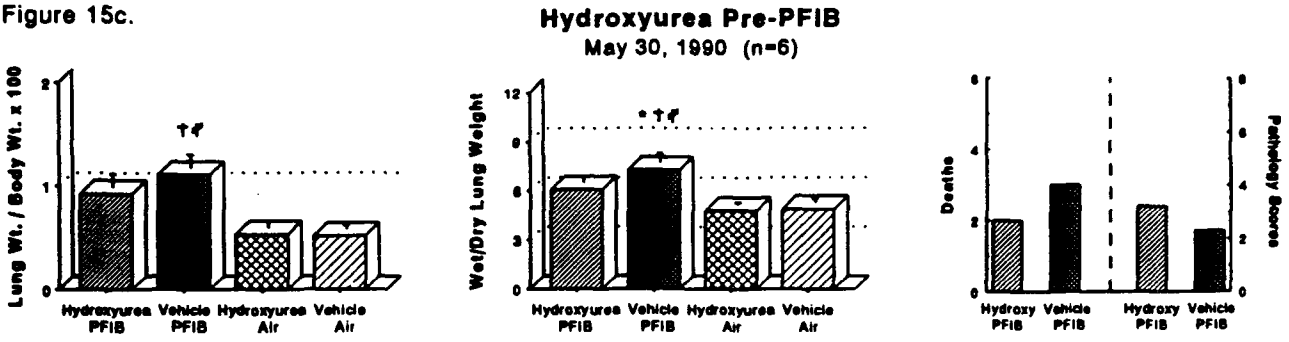


Figure 15d.

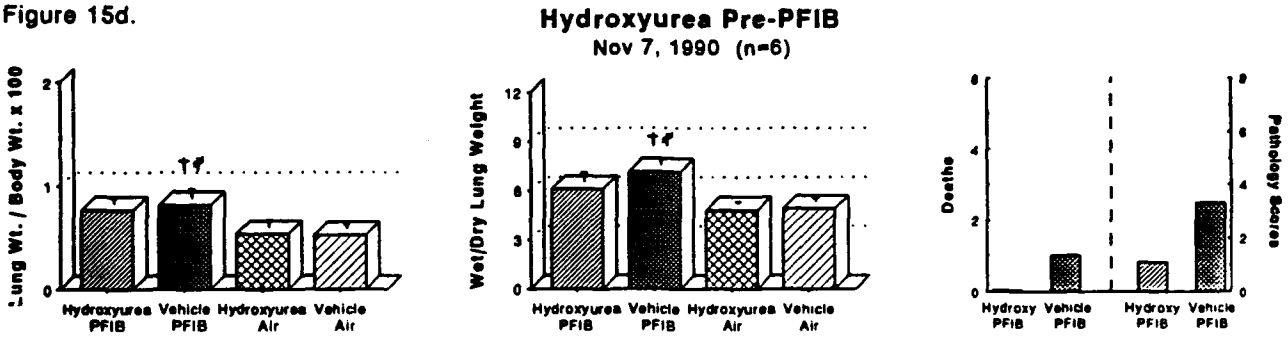


Figure 16.

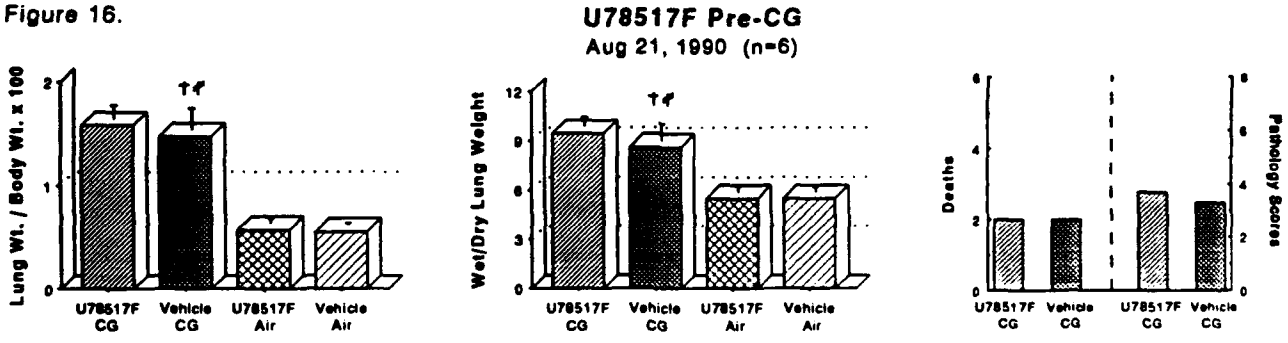


Figure 17.

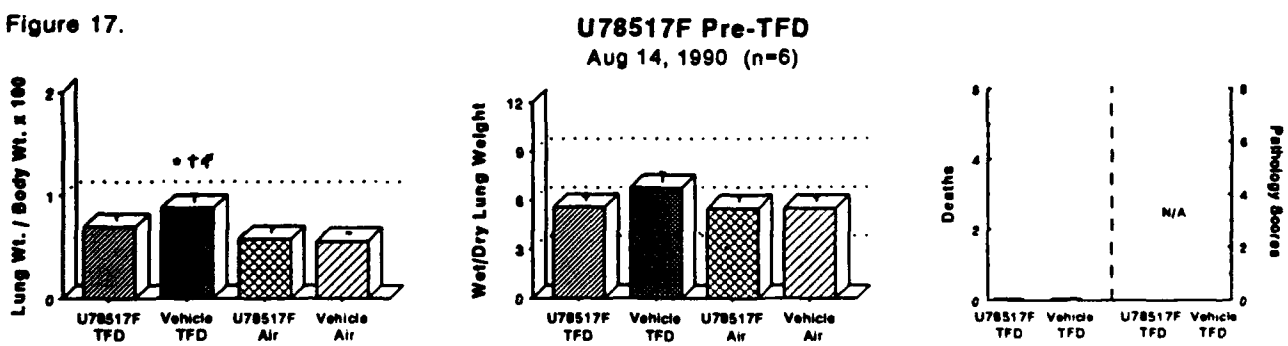

Figure 18.

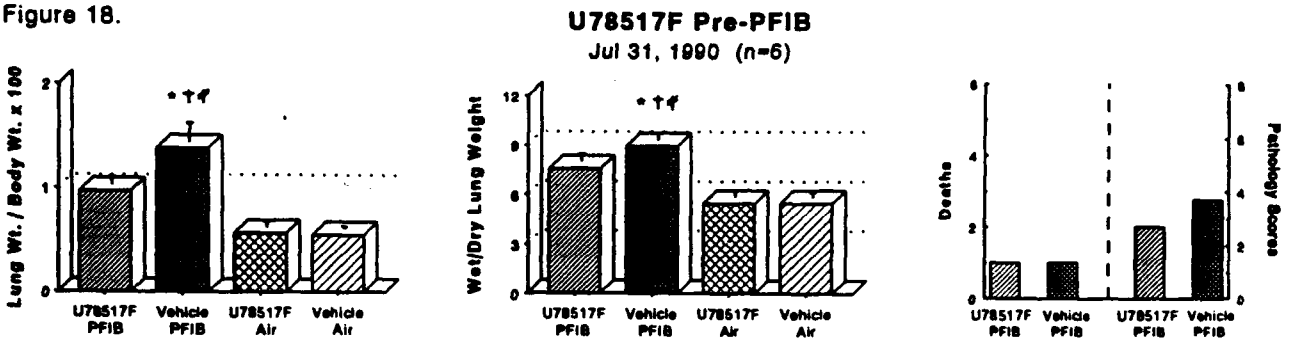


Figure 19.

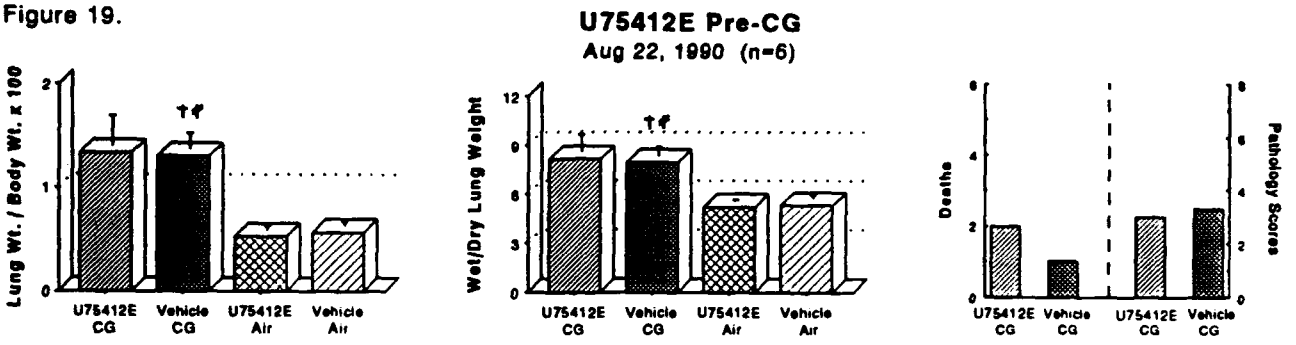


Figure 20a.

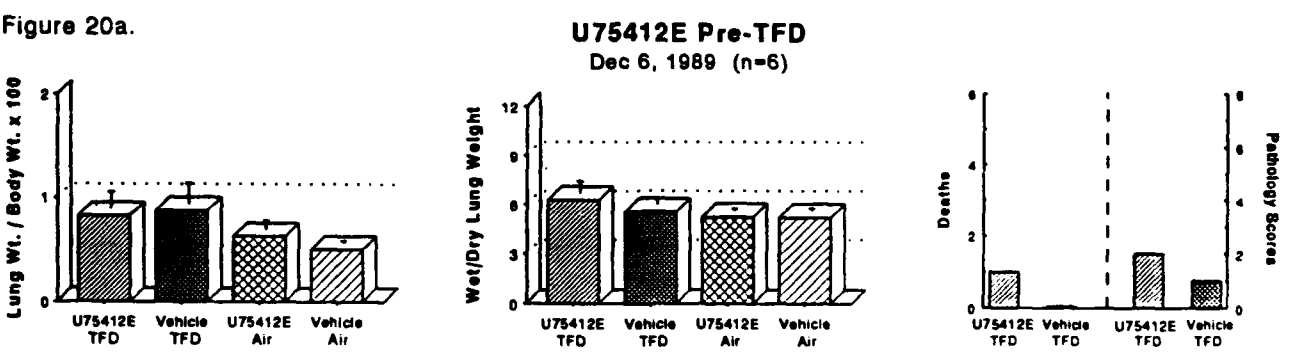


Figure 20b.

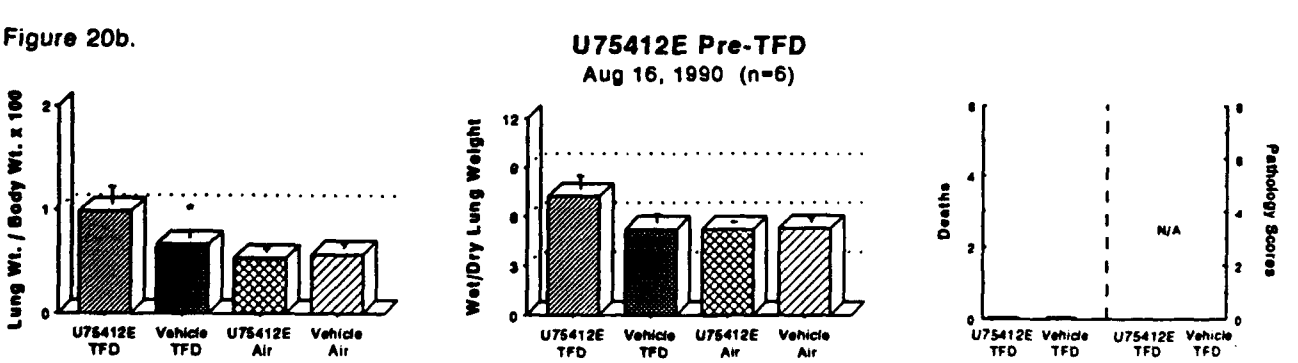

Figure 20c.

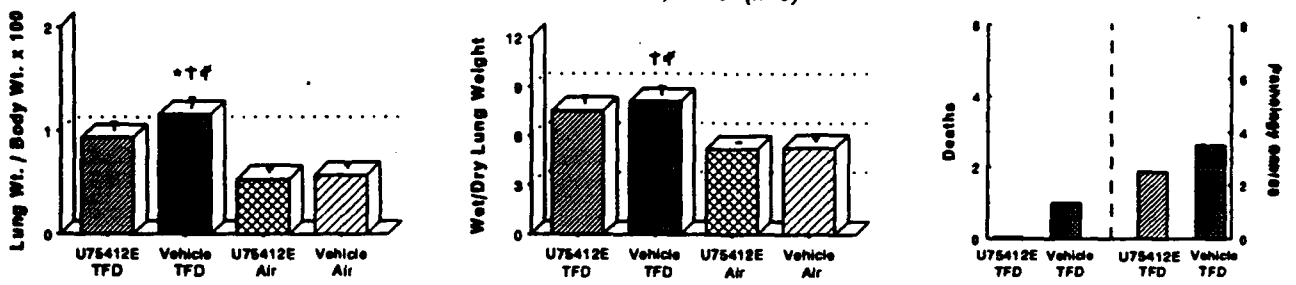


Figure 21.

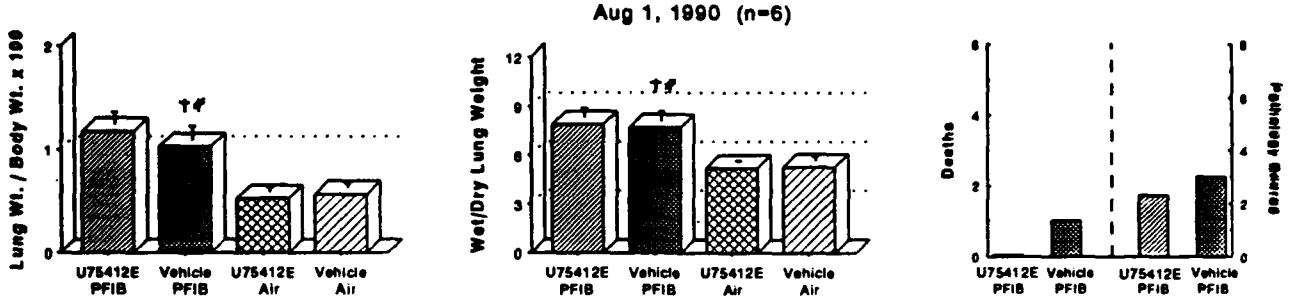


Figure 22a.

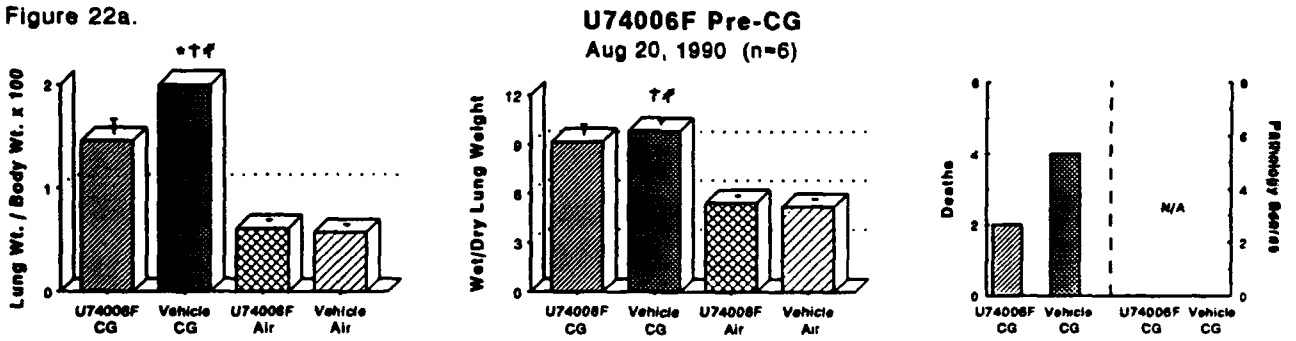


Figure 22b.

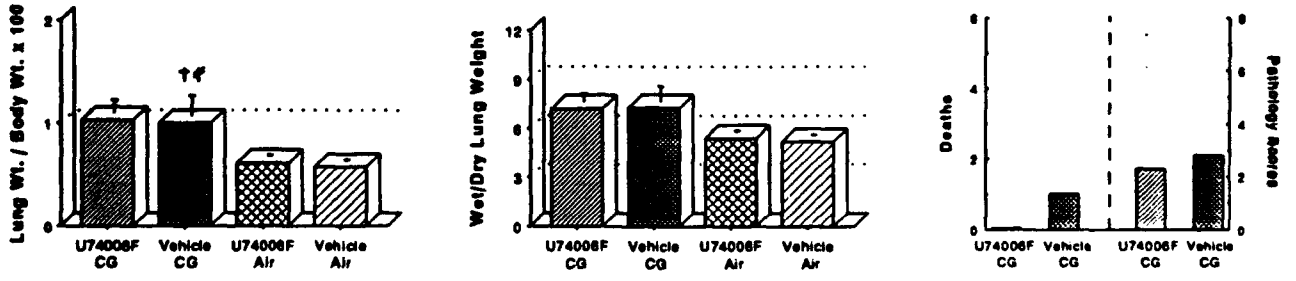

Figure 23a.

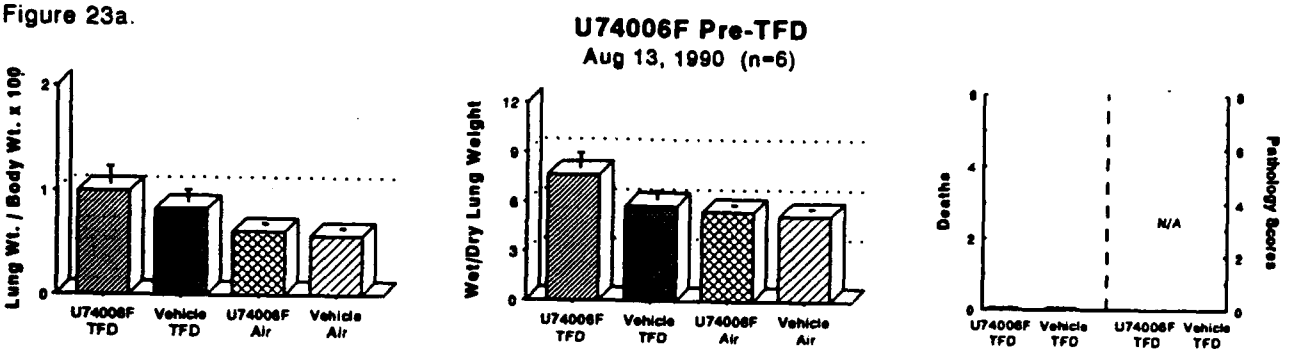


Figure 23b.

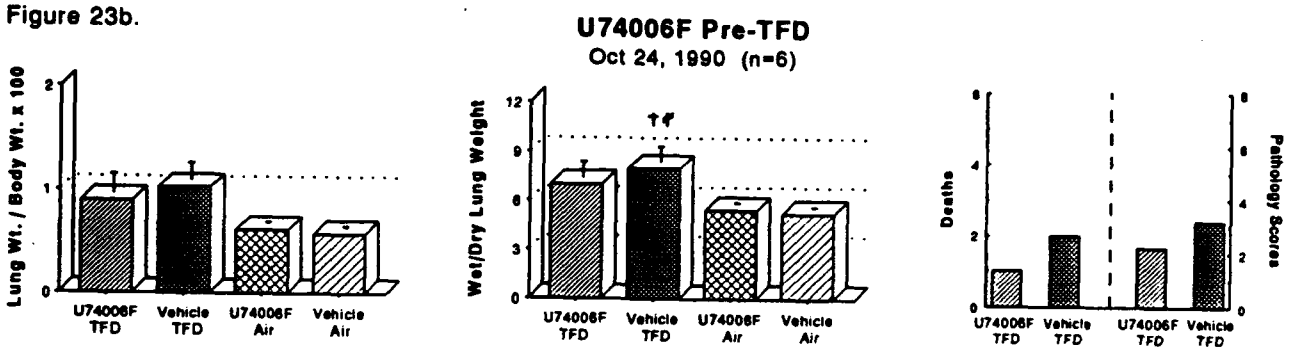


Figure 24.

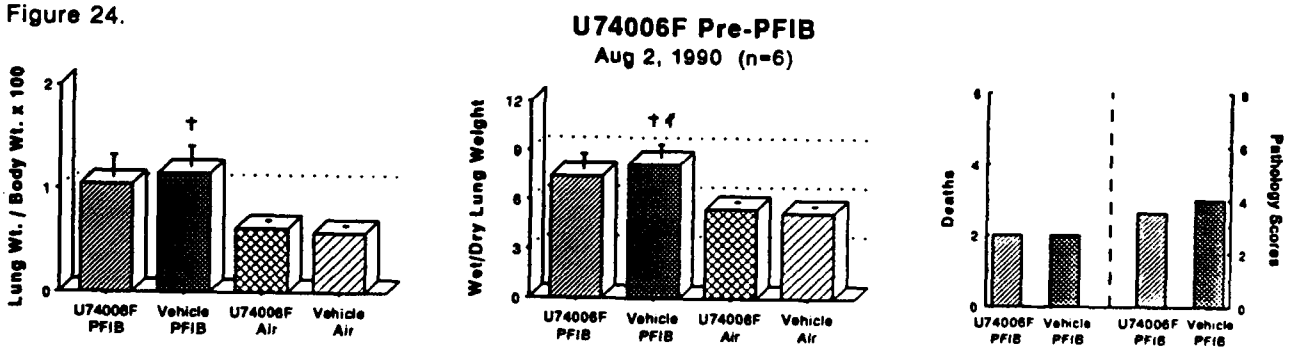

Figure 25.

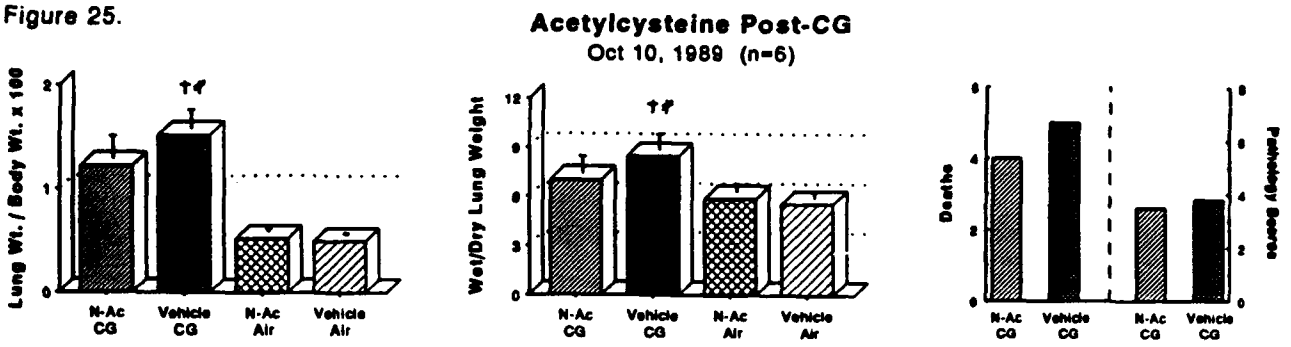


Figure 26.

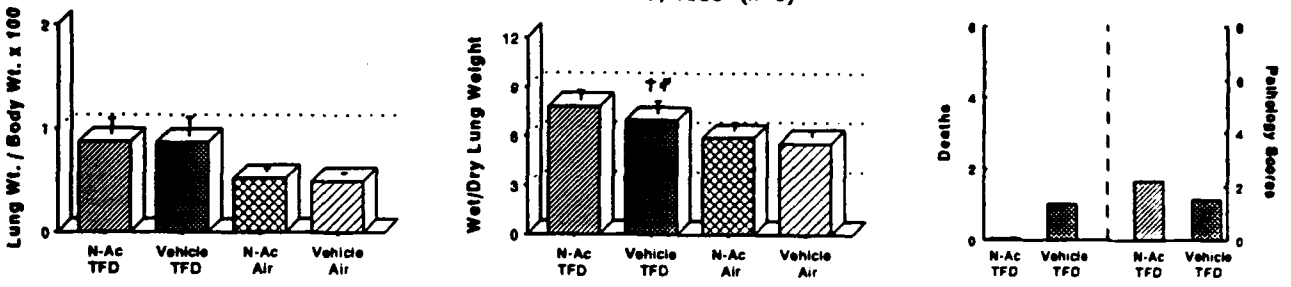


Figure 27a.

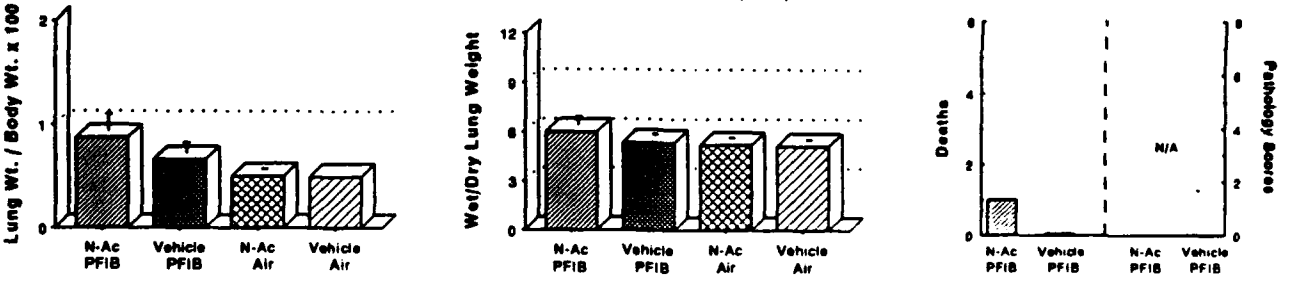


Figure 27b.

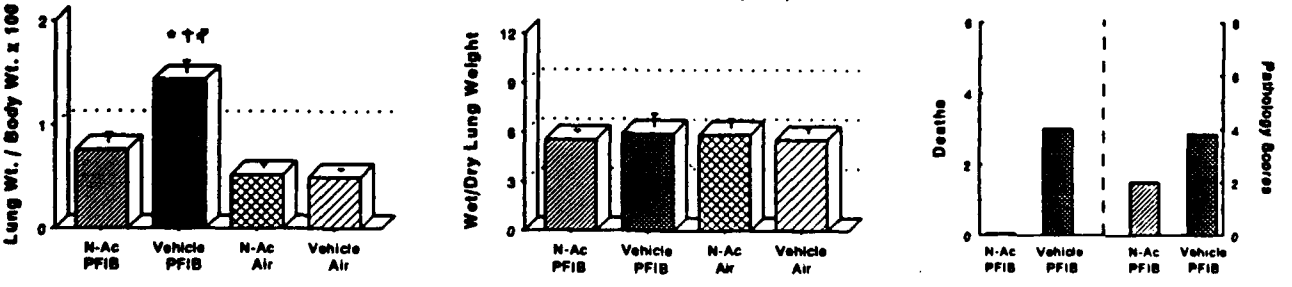

Figure 27c.

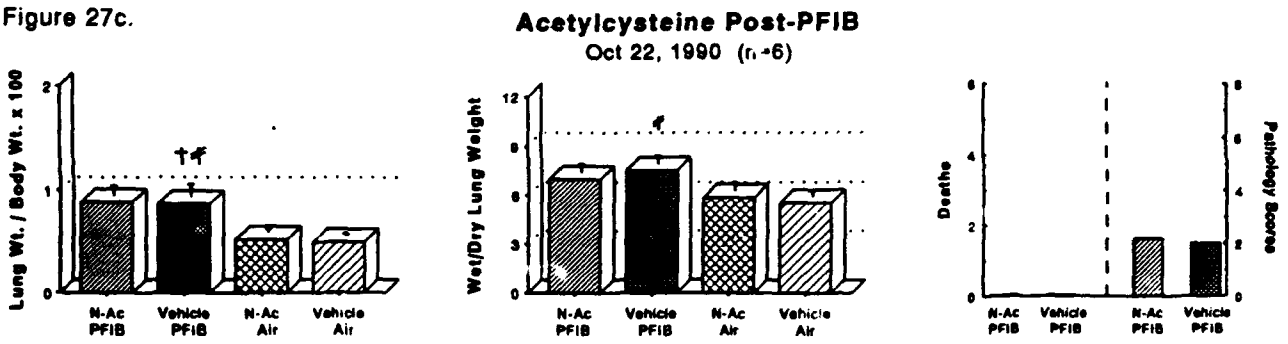


Figure 27d.

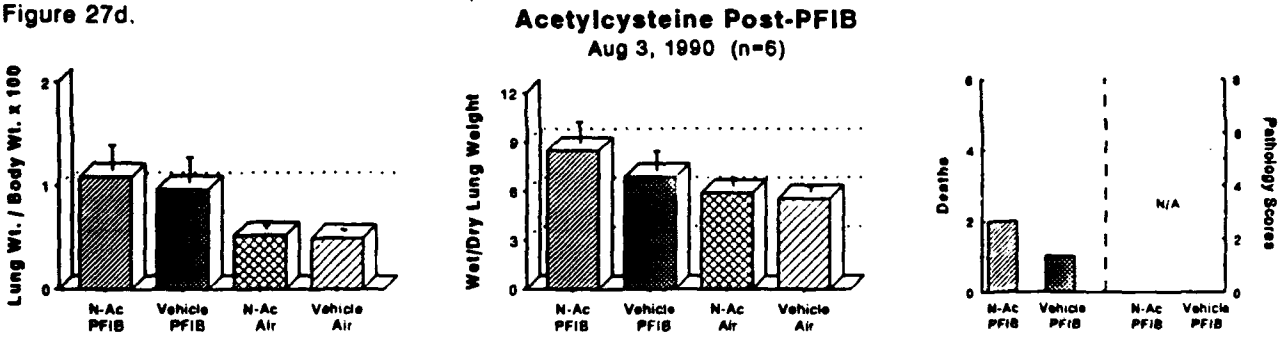


Figure 28a.

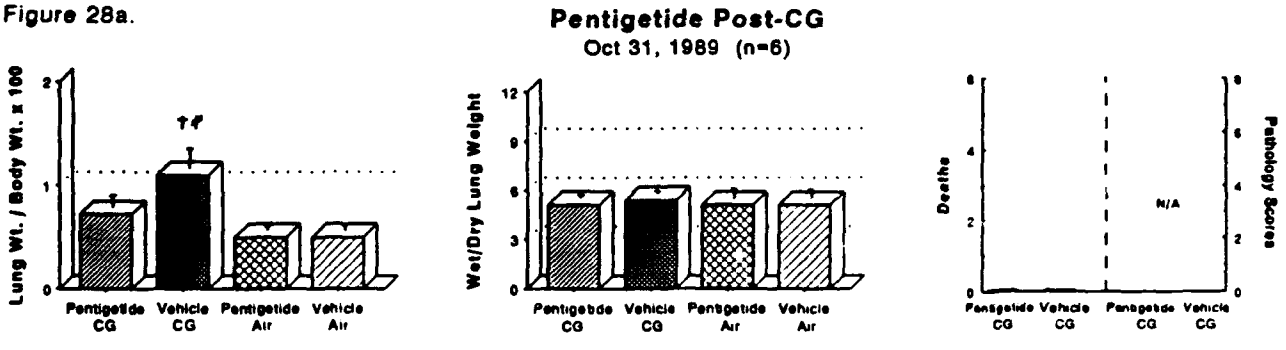


Figure 28b.

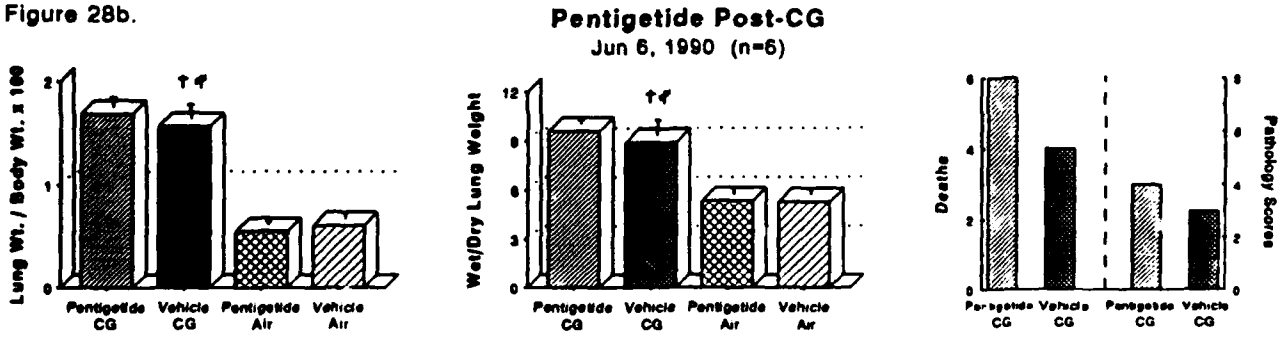

Figure 29.

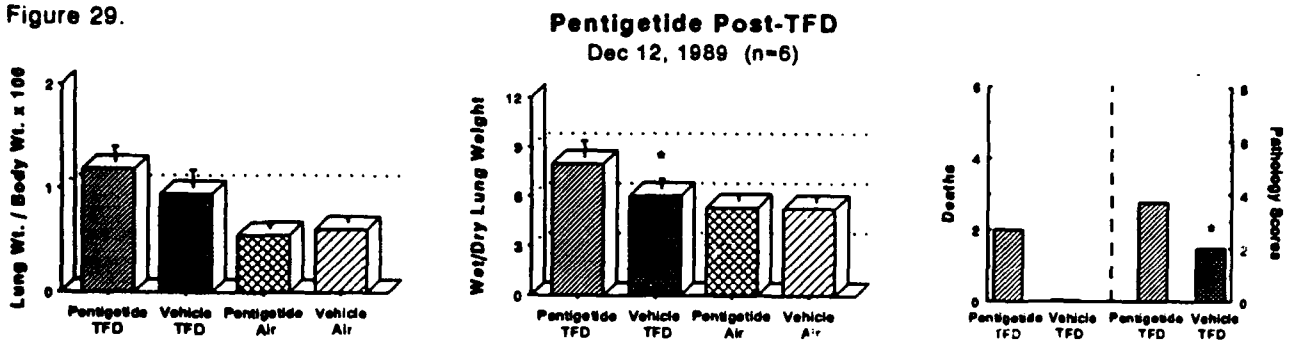


Figure 30a.

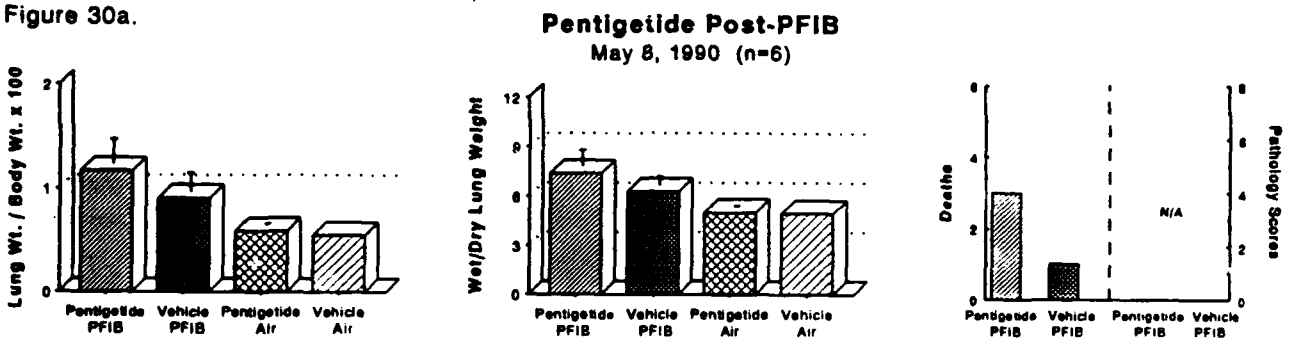


Figure 30b.

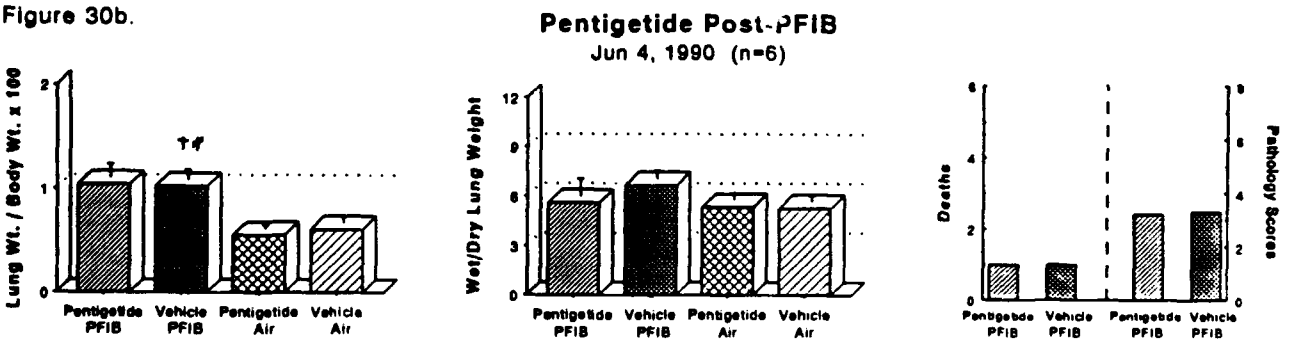


Figure 31a.

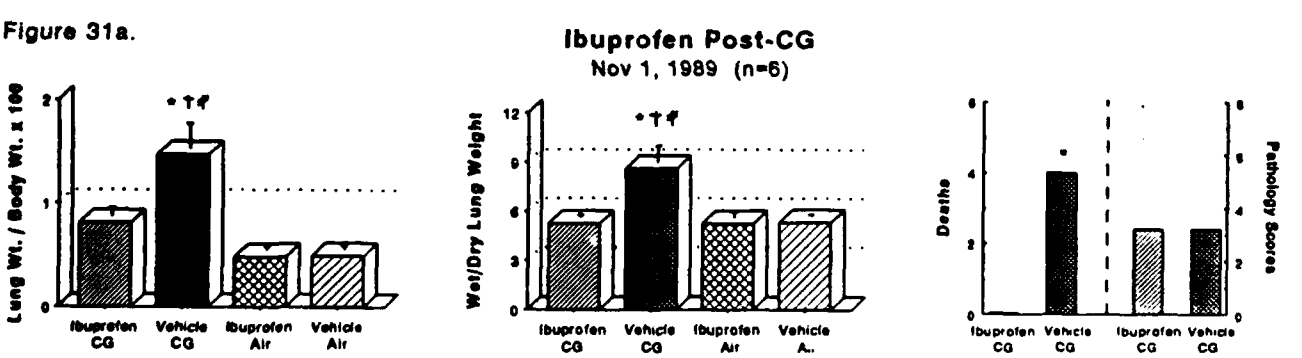

Figure 31b.

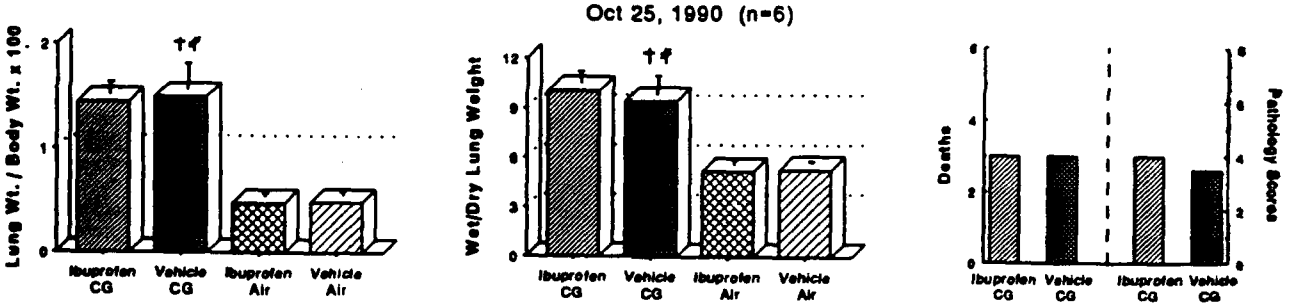


Figure 32.

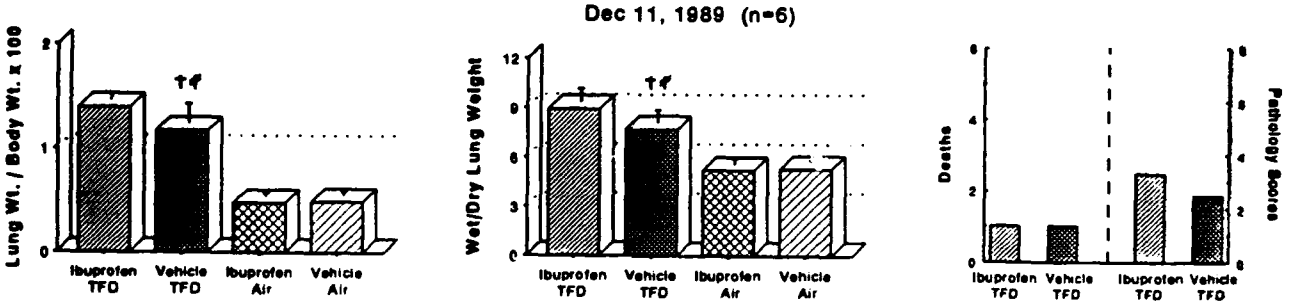


Figure 33a.

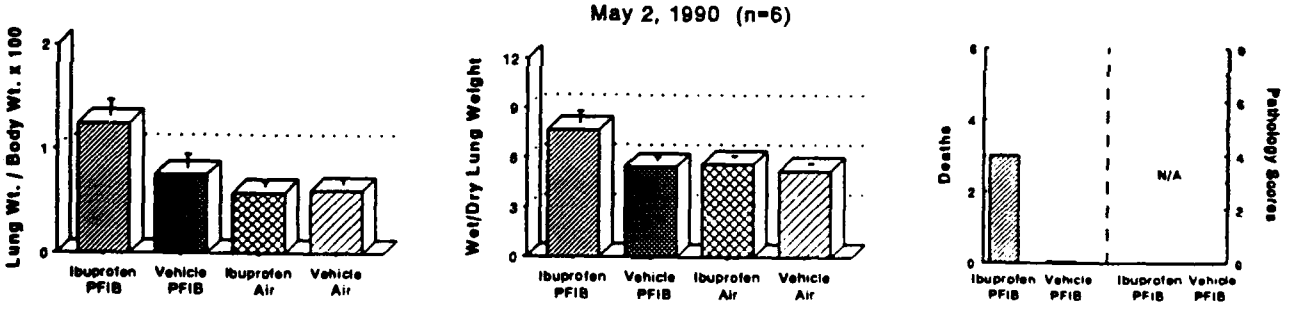


Figure 33b.

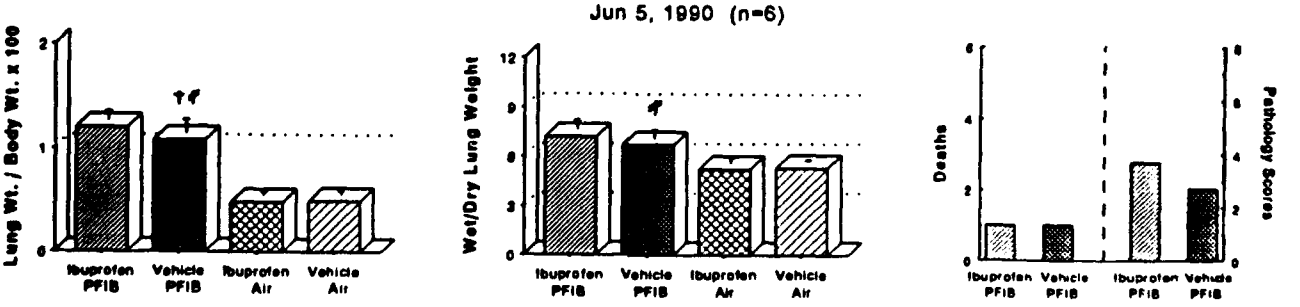

Figure 34.

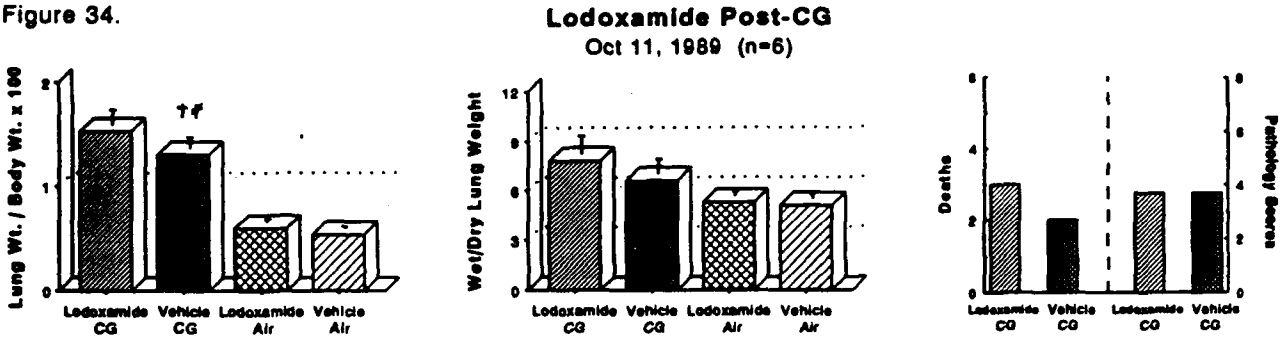


Figure 35.

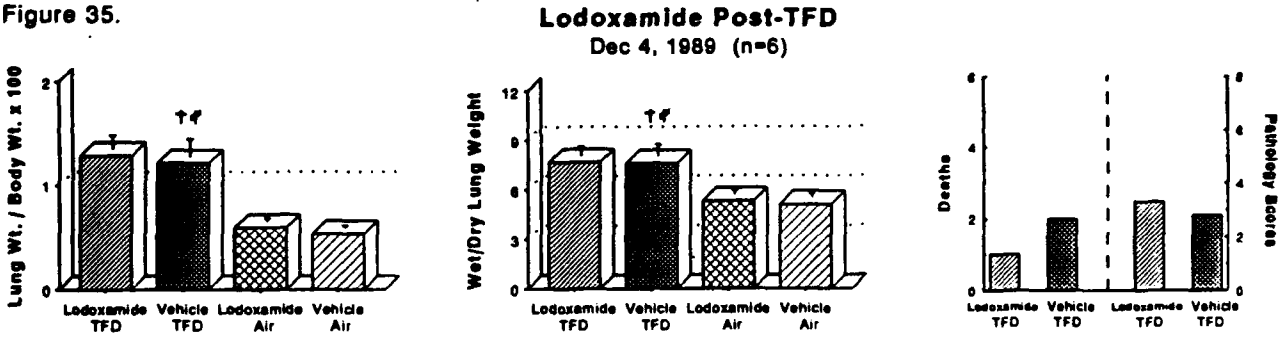


Figure 36.

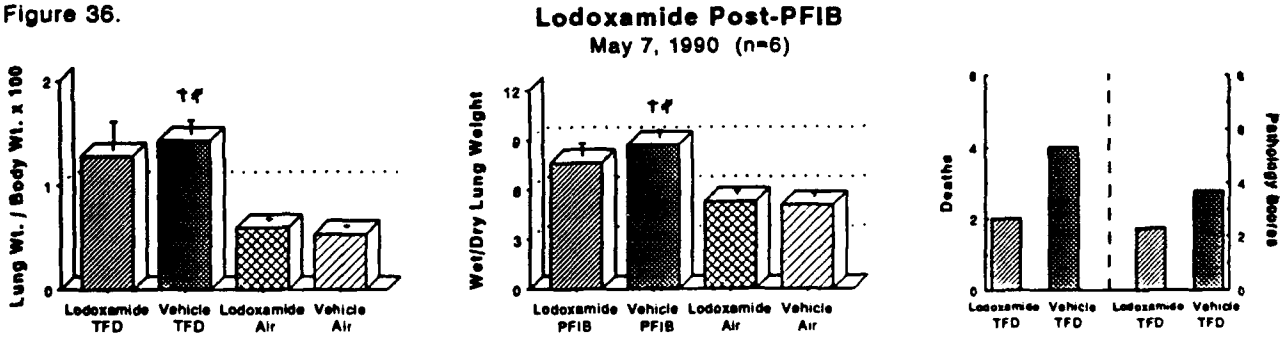


Figure 37.

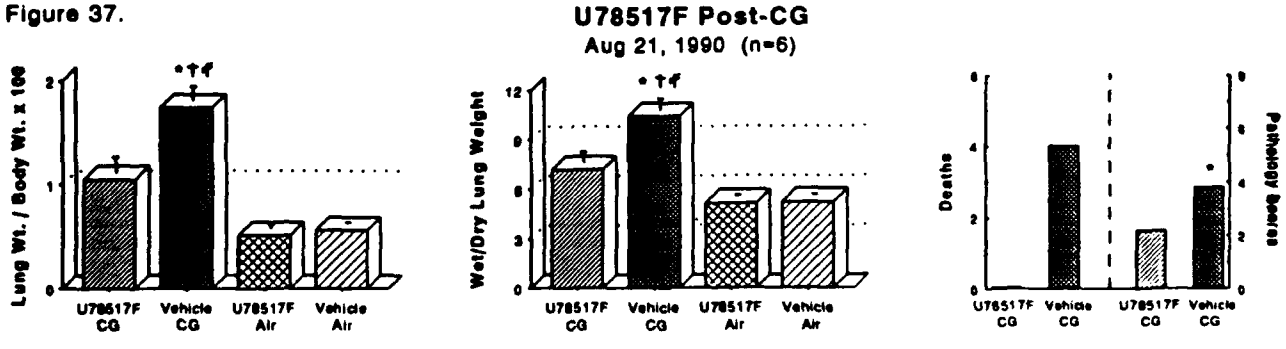

Figure 38.

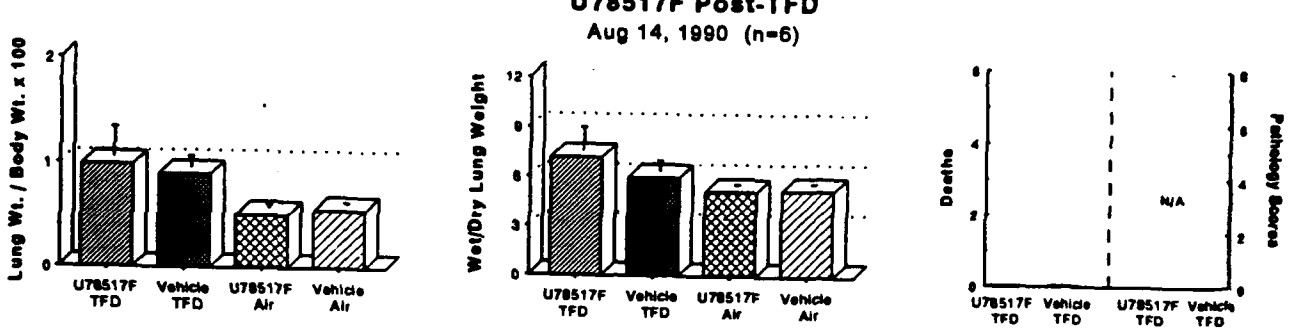


Figure 39.

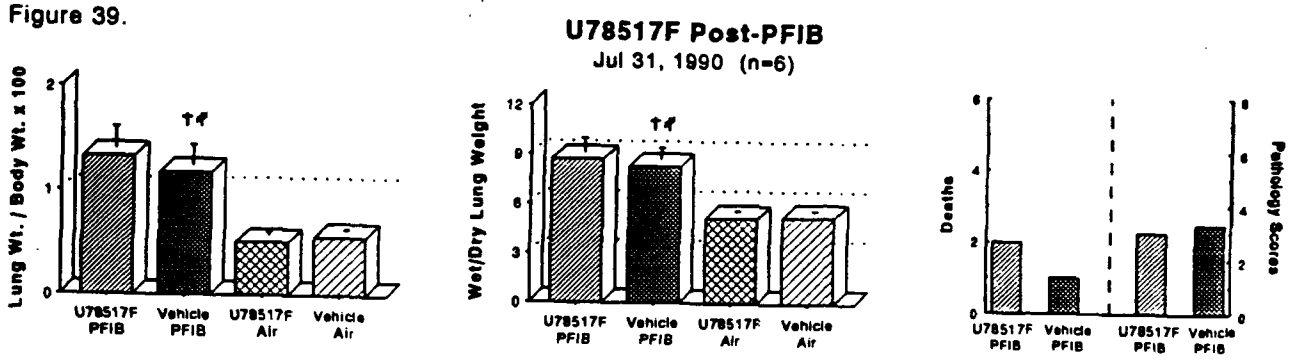


Figure 40a.

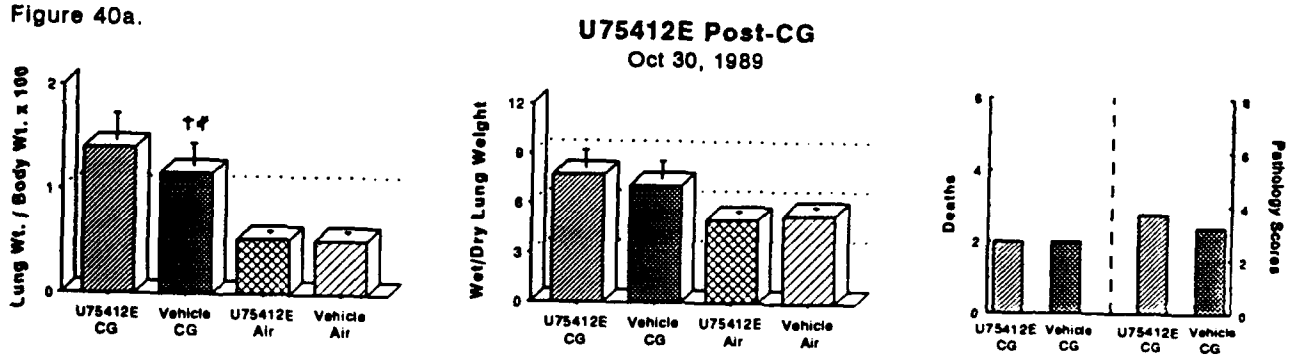


Figure 40b.

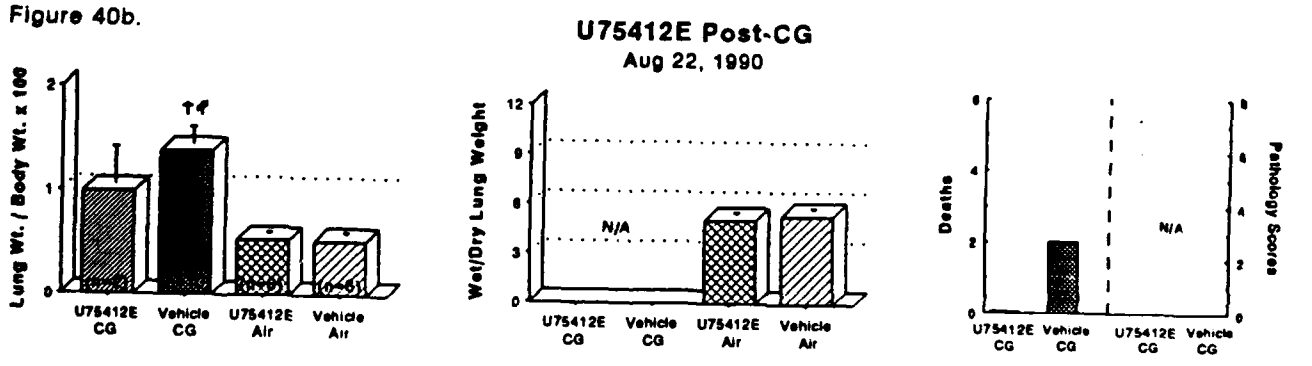

Figure 41a.

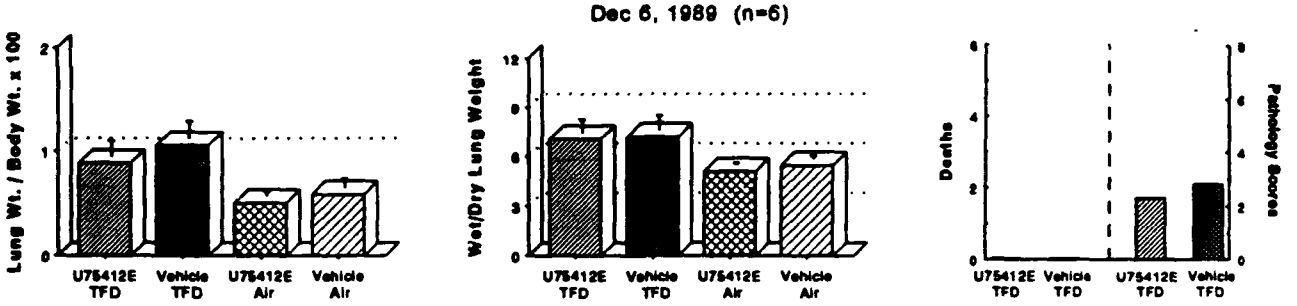


Figure 41b.

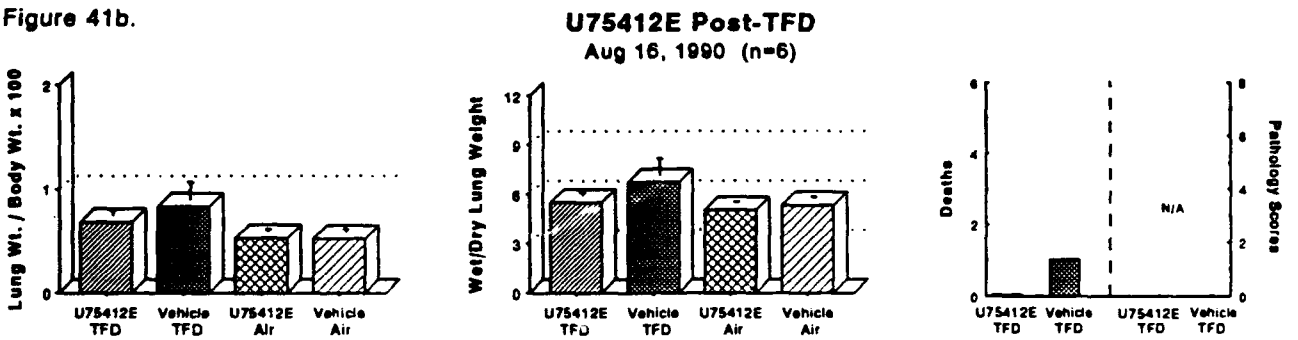


Figure 41c.

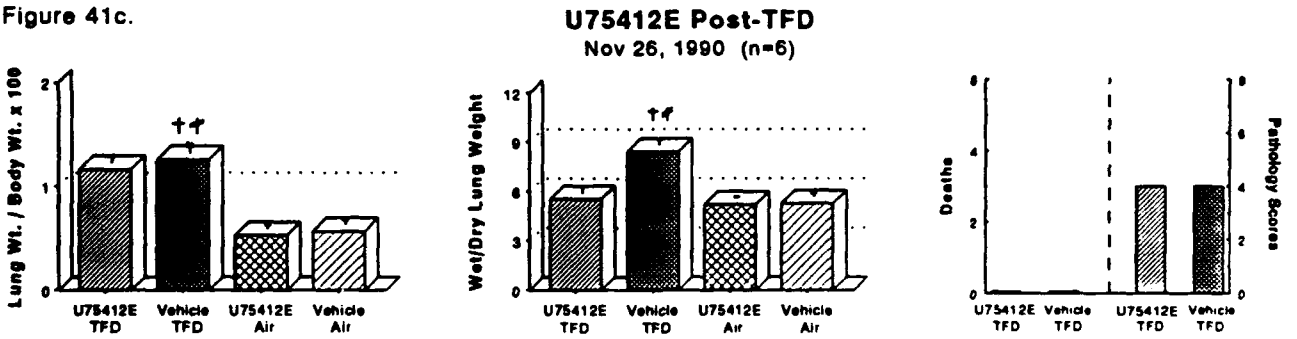


Figure 42.

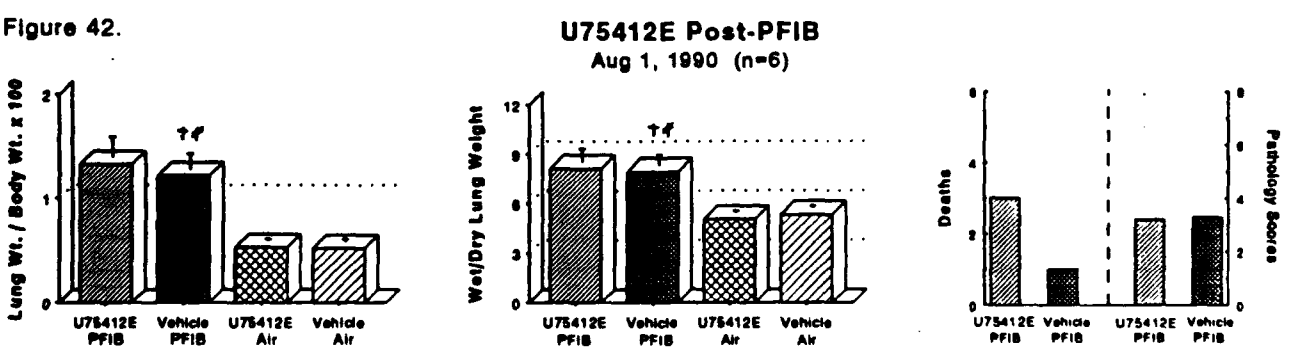

Figure 1a.

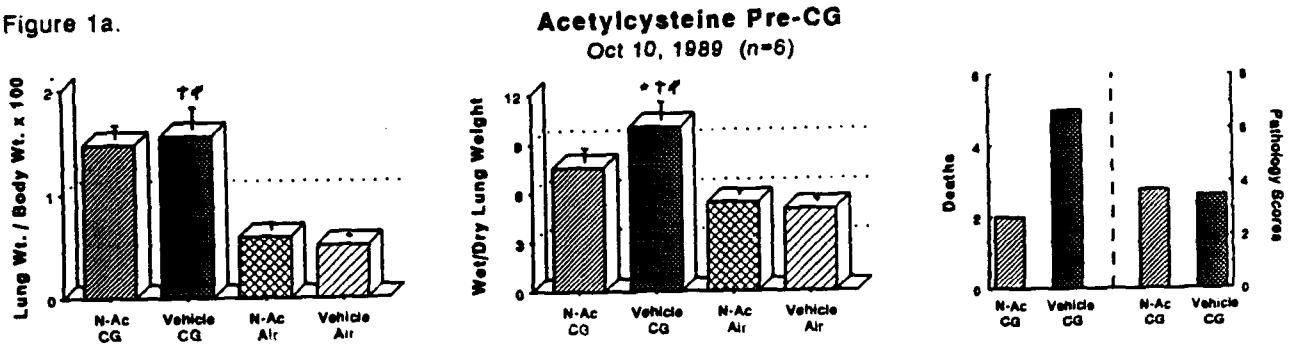


Figure 1b.

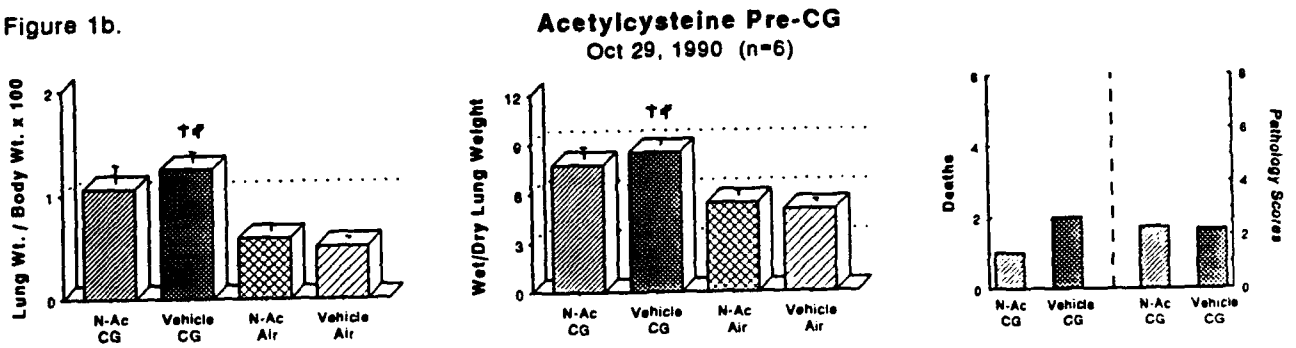


Figure 2.

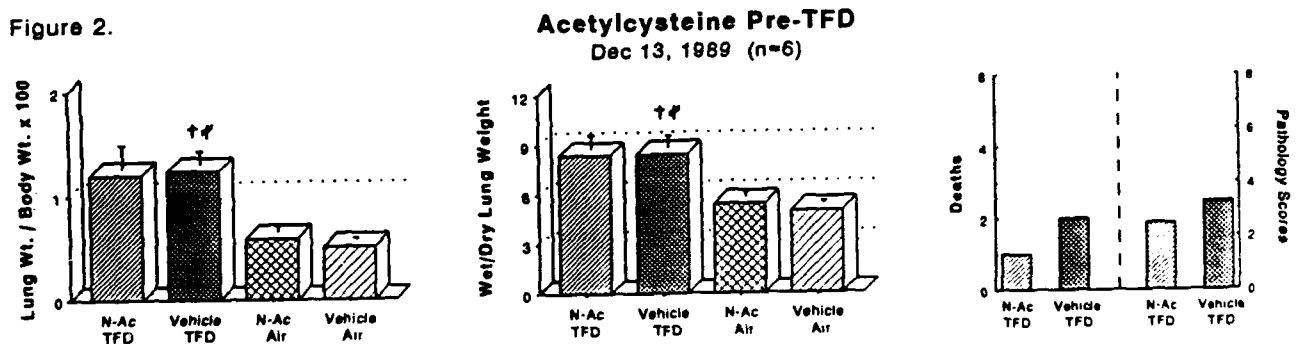


Figure 3a.

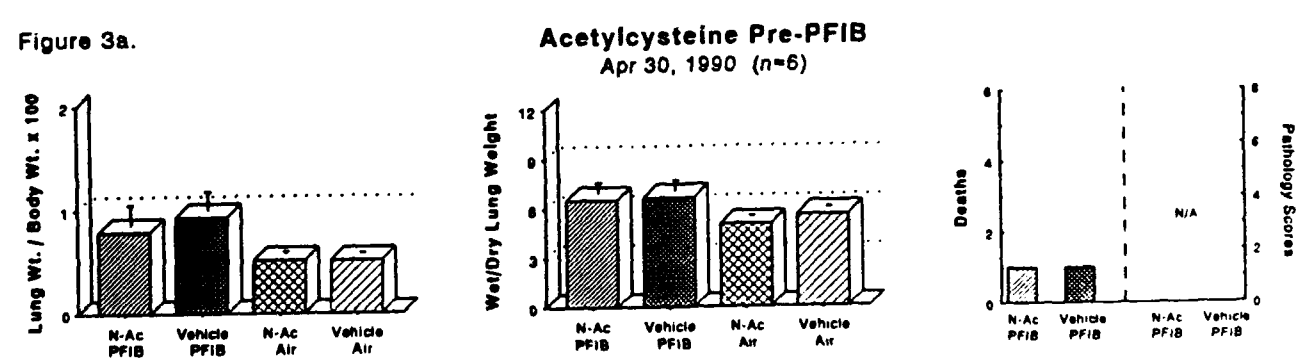

Table 1. LUNG WEIGHT INDICES.

| | PRE-TREATMENT | | | | | | POST-TREATMENT | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LW/BW | | | WW/DW | | | LW/BW | | | WW/DW | | |
| DRUG | CG | TFD | PFIB | CG | TFD | PFIB | CG | TFD | PFIB | CG | TFD | PFIB |
| N-acetylcysteine | (170)* | (200)* | (130)* | + (170)* | (200)* | (130)* | (170)* | (200)* | (130)* | (170)* | (200)* | (130)* |
| | (200) | | (140)* | (200) | | (140)* | | | + (140)* | | | (140)* |
| | | | (150) | | | (150) | | | (150)* | | | (150)* |
| | | | | | | | | | (200) | | | (200) |
| pentigetide | + (170)* | – (200)* | (150)* | + (170)* | (200)* | (150)* | (170)* | (200)* | (150)* | (170)* | - (200)* | (150)* |
| | (200) | | (150) | (200) | | (150)* | (220)* | | (150)* | (220)* | | (150)* |
| ibuprofen | (170)* | (200)* | (140)* | (170)* | (200)* | (140)* | + (170)* | (200)* | (140)* | + (170)* | (200)* | (140)* |
| | | | – (150)* | | | (150)* | (200) | | (150)* | (200) | | (150)* |
| lodoxamide | (170)* | (200)* | (150)* | (170)* | (200)* | (150)* | (170)* | (200)* | (150)* | (170)* | (200)* | (150)* |
| | | | (160)* | | | (160)* | | | | | | |
| hydroxyurea | (170)* | + (200)* | (130)* | (170)* | + (200)* | + (130)* | | | | | | |
| | | + (230) | (150)* | | + (230) | (150)* | | | | | | |
| | | | (160)* | | | (160)* | | | | | | |
| | | | (150) | | | (150) | | | | | | |
| U78517F | (200) | + (200) | + (150) | (200) | (200) | + (150) | + (200) | (200) | (150) | + (200) | (200) | (150) |
| U75412E | (200) | (200) | (150) | (200) | (200) | (150) | (170)* | (200)* | (150) | (170)* | (200)* | (150) |
| | | – (200) | | | (200) | | (200) | (200) | | | (200) | |
| | | + (230) | | | (230) | | | (230) | | | (230) | |
| U74006F | + (200)* | (200)* | (150)* | (200)* | (200)* | (150) | (200)* | (200) | (150) | (200)* | (200) | (150) |
| | | (200) | (260) | | (200) | (200) | | (260) | | | (260) | |

– indicates the lung weight index of the drug-treated gas-exposed group was significantly greater than that of the vehicle-treated group.

+ indicates the lung weight index of the drug-treated gas-exposed group was significantly smaller than that of the vehicle-treated group.

* indicates 24 observation periods. Absence of asterisks indicates 5-hour observation periods.

Gas concentrations are indicated in parentheses ($mg/m^3$).

*Tabular Summary of Mortality Comparisons*. The numbers of rats that died, in each drug- or vehicle-treated group of six, are compared in Table 2, which shows the drug studied, gas to which the rats were exposed, and the length of the observation period of each experiment. In only two instances were ibuprofen and U78517F (both used as post-treatment of CG exposure) followed by significantly fewer deaths in the drug-treated group than in the vehicle-treated group at $p < .05$ using Fisher's exact test.

Table 2. MORTALITY COMPARISONS.
(Drug vs vehicle-treated, gas-exposed groups).

| | PRE-TREATMENT | | | POST-TREATMENT | | |
|---|---|---|---|---|---|---|
| DRUG | CG | TFD | PFIB | CG | TFD | PFIB |
| N-acetylcysteine | 2 vs 5 (170)*<br>1 vs 2 (200) | 1 vs 2 (200)* | 1 vs 1 (130)*<br>1 vs 0 (140)*<br>0 vs 0 (150) | 4 vs 5 (170)* | 0 vs 1 (200)* | 1 vs 0 (130)*<br>0 vs 1 (140)*<br>0 vs 0 (150)*<br>0 vs 0 (200) |
| pentigetide | 2 vs 5 (170)*<br>1 vs 0 (200) | 4 vs 0 (200)* | 3 vs 0 (150)*<br>1 vs 2 (150) | 0 vs 0 (170)*<br>6 vs 4 (220)* | 2 vs 1 (200)* | 3 vs 1 (150)*<br>1 vs 1 (150)* |
| ibuprofen | 1 vs 2 (170)* | 1 vs 2 (200)* | 1 vs 1 (140)*<br>0 vs 0 (150)* | 0 vs 4 (170)*<br>3 vs 3 (200) | 1 vs 1 (200)* | 3 vs 0 (140)*<br>1 vs 1 (150)* |
| lodoxamide | 3 vs 1 (170)* | 1 vs 1 (200)* | 2 vs 3 (150)*<br>3 vs 3 (160)* | 3 vs 2 (170)* | 1 vs 2 (200)* | 2 vs 4 (150)* |
| hydroxyurea | 4 vs 3 (170)* | 2 vs 3 (200)*<br>1 vs 0 (230) | 3 vs 0 (130)*<br>2 vs 0 (150)*<br>2 vs 3 (160)*<br>0 vs 1 (150) | | | |
| U78517F | 2 vs 2 (200) | 0 vs 0 (200) | 1 vs 1 (150) | 0 vs 4 (200) | 0 vs 0 (200) | 2 vs 1 (150) |
| U75412E | 2 vs 1 (200) | 1 vs 0 (200)<br>0 vs 0 (200)<br>0 vs 1 (230) | 0 vs 1 (150) | 2 vs 2 (170)*<br>0 vs 1 (200) | 0 vs 0 (200)*<br>0 vs 1 (200)<br>0 vs 0 (230) | 3 vs 1 (150) |
| U74006F | 2 vs 4 (200)*<br>0 vs 1 (200) | 0 vs 0 (200)*<br>1 vs 2 (260) | 2 vs 2 (150)* | 2 vs 2 (200)* | 2 vs 1 (200)<br>3 vs 2 (260) | 1 vs 2 (150) |

* indicates 24 observation periods. Absence of asterisks indicates 5-hour observation periods.
Comparisons shown indicate deaths during observation period for drug-treated compared to vehicle-treated groups.
Gas concentrations are indicated in parentheses ($mg/m^3$).

*Tabular Summary of Histopathology Studies*. The microscopic studies of the lung sections were scored for severity of lung damage, as described in materials and methods section, and the scores are shown in Table 3. Where the scores are absent no pathology studies were performed.

Table 3. PATHOLOGY SCORES.
(Drug vs vehicle-treated, gas-exposed groups).

| | PRE-TREATMENT | | | POST-TREATMENT | | |
|---|---|---|---|---|---|---|
| DRUG | CG | TFD | PFIB | CG | TFD | PFIB |
| N-acetylcysteine | 3.7:3.5 (170)*<br>2.3:2.2 (200) | 2.5:3.3 (200)* | 1.7:1.7(130)*<br>3.5:4.0 (140)*<br>1.7:1.7 (150) | 3.5:3.8 (170)* | 2.2:1.5 (200)* | 2.0:3.8 (140)*<br>2.5:2.0 (150)*<br>2.1:2.0 (200) |
| pentigetide | 3.5:3.8 (170)*<br>2.2:3.5 (200) | 3.8:2.2 (200)* | 3.3:3.7(150)*<br>3.3:3.7 (150) | 4.0:3.5 (220)* | 3.7:2.0 (200)* | 3.2:3.3 (150)*<br>3.2:3.3 (150)* |
| ibuprofen | 2.0:3.3 (170)* | 2.8:1.8 (200)* | 3.5:3.2 (150)* | 3.2:3.2 (170)*<br>4.0:3.5 (200) | 3.3:2.5 (200)* | 3.7:2.7 (150)* |
| lodoxamide | 3.8:3.5 (170)* | 3.2:2.7 (200)* | 3.0:1.3 (160)* | 3.7:3.7 (170)* | 3.3:2.8 (200)* | 2.3:3.7 (150)* |
| hydroxyurea | +2.3:3.5 (170)* | +0.7:3.2 (200)*<br>2.8:2.8 (230) | 2.3:3.5 (150)<br>3.2:2.3 (160)*<br>1.1:3.3 (150)* | | | |
| U78517F | 3.7:3.3 (200) | | 2.7:3.7 (150) | +2.2:3.8 (200) | | 3.0:3.3 (150) |
| U75412E | 3.0:3.3 (200) | 2.2:1.0 (200)<br>2.5:3.5 (230) | 2.3:3.0 (150) | 3.7:3.2 (170)* | 2.3:2.8 (200)*<br>3.8:3.3 (200)<br>4.0:4.0 (230) | 3.2:3.3 (150) |
| U74006F | 2.3:2.8 (200) | 2.2:3.3 (260) | 3.5:4.0 (150)* | 2.3:3.8 (200)* | 2.8:3.7 (260) | 3.5:2.7 (150) |

Each pair of figures represents the average pathology score in the drug treated group followed by colon, followed by the pathology score in vehicle treated group.

* indicates 24-hour observation periods. Absence of an asterisk indicates five hour observation periods.

\+ significant at the $p < 0.05$ level by Mann-Whitney test.

Gas concentrations are indicated in parentheses ($mg/m^3$).

## DISCUSSION

The purpose of these studies was to evaluate the possibility that generation of toxic oxygen species is a molecular mechanism by which inhalation of PFIB, TFD and CG causes pulmonary edema. The drugs chosen for study in these experiments are believed to interfere, directly or indirectly, with generation of toxic oxygen species or aid in the disposition of toxic radicals. N-acetylcysteine is thought to contribute to intracellular replenishment of glutathione, which subsequently acts as a sacrificial oxidant to conjugate active oxygen species (Moldeus *et al*., 1986). Pentigetide is thought to moderate immune inflammatory reactions (Hamburger, 1975) with their associated inflammatory oxygen radical production. It has also been reported to inhibit histamine release (Plummer *et al*., 1989) from calcium ionophore triggered mast cells. Ibuprofen, in addition to its cyclooxygenase inhibitory effects in preventing prostaglandin production (Vane, 1971), is thought to chelate iron, thus interfering with iron-mediated hydroxyl radical generation (Kennedy *et al*., 1990). Ibuprofen is also thought to act through hydroxyl radical scavenging (Aruoma and Halliwell, 1988). Further, ibuprofen has been shown to attenuate the production of hypochlorous acid from the reaction of halides with the hydrogen peroxide produced in respiratory bursts of neutrophils (Carey *et al*., 1990). Lodoxamide is an inhibitor of rat lung xanthine oxidase (White, 1981). Xanthine oxidase catalyzes the production of uric acid from xanthine, generating superoxide radicals and hydrogen peroxide. These in turn combine to form highly active hydroxyl radicals through the metal catalyzed Haber-Weiss reaction. Hydroxyurea produces leukopenia. When activated, leukocytes produce toxic oxygen radicals as part of their defense against foreign organisms. Hydroxyurea is thought to interfere with an enzyme, ribonucleoside diphosphate, which is utilized in the production of deoxyribonucleic acid (DNA) (Goodman *et al*., 1980). DNA in turn is essential in the replication of leukocytes. Interference with the enzyme leads to leukopenia, reducing the potential for toxic oxygen radical production.

The manner in which lazaroid drugs protect against oxidative stress is not clearly known. One suggested method (Braughler *et al*., 1987) is by chelating iron, thus reducing the amount of oxygen radicals produced by iron catalyzed Fenton reactions. Other suggested mechanisms include scavenging lipid hydroperoxides and superoxide radicals (Hall *et al*., 1988).

These studies were conducted over a two-year period, during which time improvements were made in techniques and procedures. Shortening the post-exposure observation periods allowed completion of experiments in a day. Analysis of the results indicates this did not result in failure to observe significant edema at necropsy. This may be concluded from the fact that the proportion of short observation period experiments in which significant lung weight increases resulted from the gas exposures was greater than the proportion (57%) observed in the experiments using the longer observation periods. Likewise, the ability of the procedures to permit detection of differences in the drug-treated as opposed to the vehicle-treated gas-exposed groups did not appear to be less in the short as opposed to the long observation period procedures. The proportion

of experiments in which significant differences were noted between drug and vehicle-treated, gas-exposed groups was equal (26%) in the short and long observation period groups. The possibility that results may be different using the two observation periods cannot be excluded, but seems unlikely.

Difficulty in maintaining appropriate gas concentrations at the nose and mouth of the rats may have contributed to the variation in lung weight ratios found. Additional variation in these ratios may gave resulted from accumulation of blood in the tracheobronchial tree after euthanasia.

In deciding that a given drug reduced the development of the lung edema from exposure to a toxic gas, we relied primarily on two indices of increased lung weight, namely lung to body weight (LW/BW) ratio and wet to dry caudal lobe weight (WW/DW) ratio (for convenience, we present the lung to body weight ratios multiplied by 100). Both indices reflect the degree of lung edema, but each is subject to different experimental and recording errors. We have used them as cross-checks on each other. In all but two instances, the indices trend in the same direction, but statistical significance was often present for one index, and absent in the corresponding paired index. An increase in the lung to body weight ratio reflects pulmonary edema as long as there is no concomitant body weight loss (Currie *et al*., 1987). With the exception of the experiments involving hydroxyurea, there were no differences in average body weight between groups A and B. Therefore, in these experiments, the differences in LW/BW ratios should reflect differences in the amount of lung edema. The lung weight to dry weight ratio is considered a good index of pulmonary edema if marked edema is present. In lesser degrees of edema, the proportional increases in wet weight (due to water) and dry weight (due to protein leakage) may be comparable (Currie *et al*., 1985).

In a large series of experiments such as these (71 experiments), when statistical analysis is made at a double tailed probability level of $p < .05$, approximately two (.025 x 71) positive results might be expected if none of the drugs had a beneficial effect in reducing the amount of lung edema produced. In these experiments, however, significantly *less* weight gain in the drug-treated group was noted in 20 experiments (a *greater* weight gain in the drug-treated group was noted in four experiments). In the absence of a preventive effect of any drug studied, this proportion would be expected to occur in less than 1 out of 1000 series of such experiments. Therefore, the results of the experiments reported here clearly reflect a reduction in lung edema due to some of the drugs studied, even though occasional inconsistencies were found.

If the incidence of prevention of lung weight gain by the drug used is compared between the pre-treatment and the post-treatment experiments overall, it is found that 14 out of 49 pre-treatment experiments (29%) indicate prevention of lung weight gain, compared to only 3 out of 32 post-treatment experiments (9%). This suggests that pre-treatment may be more effective than post-treatment, at least for some of the drugs examined here. Nevertheless, the finding that post-treatment appeared helpful in three instances suggests that a post-treatment therapy may be developed.

On the basis of presumed mode of action of these drugs it is difficult to formulate a role for any of them in *accentuating* lung weight gain after gas exposure. The finding that in four instances (i.e., 6% of the experiments) an apparently significant weight gain

was noted appears more likely due to random difficulties with restraint during exposure, or aspiration of blood during the necropsy procedure.

In the *mortality* results, the effect of the drugs in reducing the number of deaths during the post-exposure observation periods was examined. The ratio of drug-treated to vehicle-treated deaths was significant, using Fisher's exact test, in only two instances. Ibuprophen and U78517F, used as post-treatment of CG exposure, was followed by significantly fewer deaths in the drug-treated group than in the vehicle-treated group. The ratio was favorable (i.e., more rats died in the vehicle group than in the drug-treated group) in 14 out of 21 experiments examining pre-treatment results (this excludes the hydroxyurea experiments, in which dehydration due to diarrhea may have influenced results). When post-treatment mortality results are compared, the ratio was favorable in only 10 out of 21 experiments.

In the *pathology scoring of the lung sections*, the effect of the drugs in reducing the severity of lung pathology was examined. The effects were favorable (pathology scores were greater in the vehicle-treated than in the drug-treated groups) in 12 out of 17 pre-treatment experiments and in 4 out of 12 post-treatment experiments.

More often than not the mortality ratios and the pathology scores lay in the same direction as the lung weight indices, but in some experiments, either or both trended in a direction opposite to the lung weight ratios. No pattern to these inconsistencies was discerned. The number of animals studied was too small to allow any conclusions as to whether or not any of the drugs had effects on mortality or lung pathology different from their effects on pulmonary weight gain.

The current profusion of theories as to how toxic gases might produce tissue injury (Halliwell *et al*., 1988) makes it difficult to use the results of these experiments to decide between various possible mechanisms of toxic lung injury. The results are entirely consistent with toxic oxygen species as a partial or major source of the injury. In view of the reduction in lung edema which appears to have resulted from pretreatment with hydroxyurea, leukocytes appear to be involved to a significant extent in the injury produced by TFD, and possibly CG and PFIB. In view of the effectiveness of U78517F, iron chelation, lipid hydroperoxide and superoxide ion scavenging are all possible mechanisms of therapeutic effect. But, in these experiments, each drug was studied in only one dosage, one route of administration, and with two timings of administration. In the absence of a therapeutic or preventive effect, one cannot be sure that another dose, route of administration or dose timing might have shown effectiveness. Thus, absence of therapeutic effect in these experiments cannot be used to rule out the postulated mechanism of that particular drugs action as a source of prevention of the toxic effect of the gas used for producing the edema.

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1. After distribution of subject USAMRICD technical report, it was discovered that Figures 1a, 1b; 2, and 3a, were duplicated on page 25 and that the figures that should have been on page 25, 43, 44a, 44b, and 45, were omitted.

2. Enclosed is a corrected page 25/26. Please insert this into your copy of USAMRICD-TR-92-02 in place of the present page 25/26.

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Figure 43.

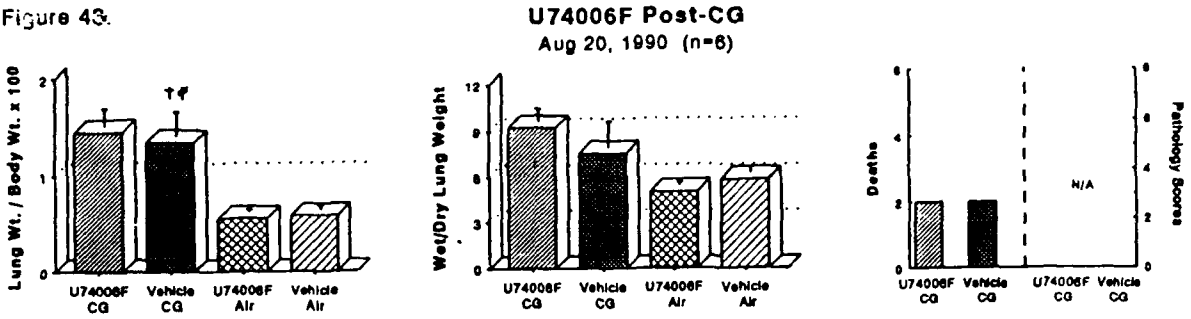


Figure 44a.

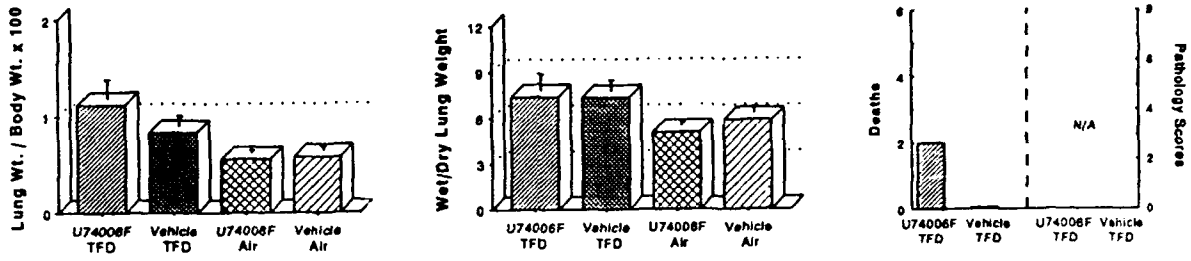


Figure 44b.

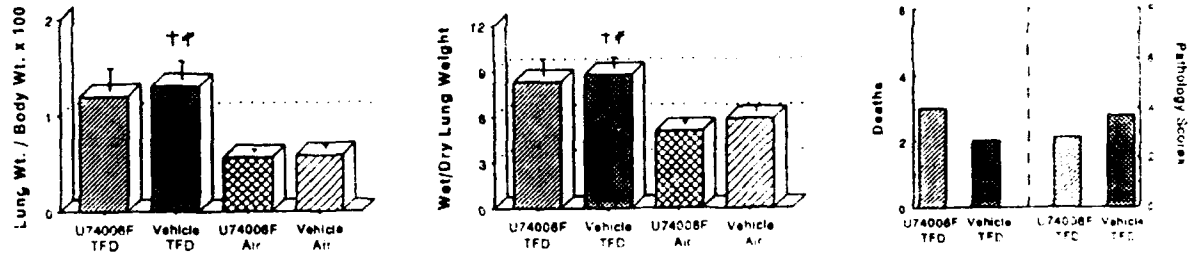


Figure 45.

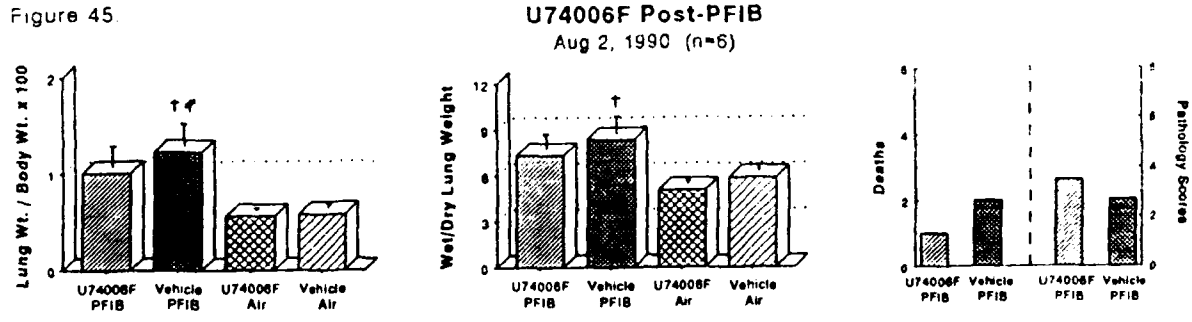

Table 1. LUNG WEIGHT INDICES.

| | PRE-TREATMENT | | | | | | POST-TREATMENT | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LW/BW | | | WW/DW | | | LW/BW | | | WW/DW | | |
| DRUG | CG | TFD | PFIB | CG | TFD | PFIB | CG | TFD | PFIB | CG | TFD | PFIB |
| N-acetylcysteine | (170)* (200) | (200)* | (130)* (140)* (150) | + (170)* (200) | (200)* | (130)* (140)* (150) | (170)* | (200)* | (130)* + (140)* (150)* (200) | (170)* | (200)* | (130)* (140)* (150)* (200) |
| pentigetide | + (170)* (200) | - (200)* | (150)* (150) | + (170)* (200) | (200)* | (150)* (150)* | (170)* (220)* | (200)* | (150)* (150)* | (170)* (220)* | - (200)* | (150)* (150)* |
| ibuprofen | (170)* | (200)* | (140)* - (150)* | (170)* | (200)* | (140)* (150)* | + (170)* (200) | (200)* | (140)* (150)* | + (170)* (200) | (200)* | (140)* (150)* |
| lodoxamide | (170)* | (200)* | (150)* (160)* | (170)* | (200)* | (150)* (160)* | (170)* | (200)* | (150)* | (170)* | (200)* | (150)* |
| hydroxyurea | (170)* | + (200)* + (230) | (130)* (150)* (160)* (150) | (170)* | + (200)* + (230) | + (130)* (150)* (160)* (150) | | | | | | |
| U78517F | (200) | + (200) | + (150) | (200) | (200) | + (150) | + (200) | (200) | (150) | + (200) | (200) | (150) |
| U75412E | (200) | (200) - (200) + (230) | (150) | (200) | (200) (200) (230) | (150) | (170)* (200) | (200)* (200) (230) | (150) | (170)* | (200)* (200) (230) | (150) |
| U74006F | + (200)* | (200)* (200) | (150)* (260) | (200)* | (200)* (200) | (150) (200) | (200)* | (200) (260) | (150) | (200)* | (200) (260) | (150) |

- indicates the lung weight index of the drug-treated gas-exposed group was significantly greater than that of the vehicle-treated group.
+ indicates the lung weight index of the drug-treated gas-exposed group was significantly smaller than that of the vehicle-treated group.
* indicates 24 observation periods. Absence of asterisks indicates 5-hour observation periods.
Gas concentrations are indicated in parentheses ($mg/m^3$).