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Lung Injury Induced by Paraquat, Hyperoxia and Cobalt Chloride: Effects of Ambroxol

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SUMMARY: The effects of the surfactant stimulating drug ambroxol were studied in rats given paraquat (PQ, 15 mg/kg, s.c.), intratracheal cobalt chloride ($CoCl_2$, 2.5 or 1.25 mg Co/kg) or exposed for 2 days to oxygen (> 95% O_2). Ambroxol (50 mg/kg, i.p., twice daily) was given as a pretreatment for 4 days, or up to 7 days following pneumotoxic administration. Besides body weight evolution and lethality, various pulmonary indices were measured 2 days (O_2) or 3 days (PQ and $CoCl_2$) and 7 days after pneumotoxic treatment: wet and dry lung weight, total cell count, distribution of inflammatory cells and lactate dehydrogenase in bronchoalveolar lavage fluid, and histological damage assessed by a semiquantitative injury score. Neither form of ambroxol treatment prevented the toxicity of any of the pneumotoxic agents. The use of a combination of different indices of lung damage showed that different pneumotoxic agents may cause quite distinctive patterns of injury.

INTRODUCTION

Ambroxol [*trans*-4-(2-amino-3,5-dibromobenzyl-amino) cyclohexanol hydrochloride] was originally developed as a mucolytic agent and has been used with apparent therapeutic success in patients with chronic bronchitis.[1] However, besides its mucokinetic effects, ambroxol has been shown to be a potent stimulant of the synthesis and secretion of alveolar surfactant.[1] The latter has been demonstrated in various experimental systems using different endpoints to assess surfactant quantity and quality. Thus, substantial increases in phospholipids, mainly phosphatidylcholine, have been shown to occur in lung tissue and in bronchoalveolar lavage (BAL) fluid, following the administration of ambroxol to adult rats[2,3] or rabbits.[4] Studies on isolated type 2 cells from adult rat lung have also shown that ambroxol stimulates the incorporation of precursors in surfactant phospholipids.[5]

Whilst the administration of ambroxol appears to have beneficial effects in experimental models of the infant respiratory distress syndrome,[1] its effect on other forms of lung injury which involve an impairment of surfactant function, such as the adult respiratory distress syndrome (ARDS), have been less well characterized.[6] The present experiments were therefore undertaken to verify if ambroxol would mitigate experimentally-induced lung damage in animals. Three toxicologically relevant chemical models of lung damage were investigated: the paraquat-model, the hyperoxia-model, and the cobalt-model.

Paraquat (PQ) is a herbicide with a well known lung-specific toxicity in animals as well as in man.[7] PQ specifically injures alveolar epithelial cells as a result of its specific accumulation followed by redox cycling and the generation of oxidant stress in these cells.[8] Exposure to pure oxygen is a well established cause of pulmonary injury in mammals, including man.[9] The initial lesion is considered to involve capillary endothelial cells and leads to pulmonary oedema.[8] Although both paraquat and hyperoxia probably exert their toxicity through oxidant injury, the pathogenesis of the lung damage which they eventually produce differs because of differences in cell selectivity. It could therefore be anticipated that any protective effect of ambroxol might also differ between these two agents. Exposure to cobalt-containing dust by inhalation is associated with lung disease,[10,11] and our laboratory has recently been investigating the mechanism for the toxic action of this metal.[12] The intratracheal administration of cobalt chloride ($CoCl_2$) leads to acute chemical pneumonitis. We wanted to verify whether ambroxol might afford some protection against this form of chemical lung injury.

In addition to clinical follow-up and observation of lethality, the investigation consisted of a comprehensive assessment of various indices of lung toxicity, including analysis of bronchoalveolar lavage (BAL)

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fluid and histopathological examination of lung tissue.[13]

MATERIALS AND METHODS

Experimental animals

Male SPF Wistar-rats of around 200 g were purchased from the K.U. Leuven animal centre and kept in a conventional animal house for the period of experimentation, except for the hyperoxia experiments which were carried out in the lung toxicology laboratory. Animals received food and drink ad libitum.

Compounds and modes of administration

1. Paraquat (PQ) dichloride (99.9% pure, ICI Plant Protection Division, Bracknell, Berkshire, UK) was a gift from Dr L. L. Smith (Central Toxicology Laboratory, ICI). A solution of 15 mg paraquat ion/ml was made in sterile NaCl 0.9% and a single dose of 15 mg paraquat ion/kg body weight was administered subcutaneously (s.c.) in the nape of the neck. Controls similarly received 1 ml/kg NaCl 0.9% s.c.
2. Hyperoxia. Animals were exposed for 2 days to an atmosphere of (water-saturated) 95–100% O_2 (N25, Air Liquide, Liège, Belgium) in plexiglas exposure chambers, with regular monitoring of oxygen (OA150, Servomex, Crowborough, Sussex, UK) and CO_2 (Capnograph mark II, Godart, Bilthoven, The Netherlands) concentrations. The highest CO_2 fraction recorded was 0.9%. The exposure was continuous except for short periods to make the ambroxol injections. Controls were exposed similarly to air from a compressed air cylinder.
3. Cobalt dichloride ($CoCl_2.6H_20$, Sigma, Deisenhofen, Germany), dissolved in sterile NaCl 0.9%, was administered intratracheally (i.t.) under direct laryngoscopic vision to rats anaesthetized with 1 ml/kg Hypnorm (fluanisone 10 mg, fentanyl 0.2 mg/ml, Janssen, Beerse, Belgium) i.p. Doses of cobalt administered were 2.5 or 1.25 mg cobalt/kg and the volume administered was 1 ml/kg. Controls similarly received 1 ml/kg NaCl 0.9% i.t.
4. Ambroxol (supplied by Boehringer Ingelheim, Brussels, Belgium) was dissolved (25 mg/ml) in distilled water; the solution was made fresh before each series of experiments and stored in a refrigerator or at room temperature. Ambroxol was administered intraperitoneally at a dose of 50 mg/kg twice daily. Controls received similar amounts of distilled water.

Experimental protocol

Two protective treatments schedules were used: a pretreatment and a post-treatment schedule. In the pretreatment schedule the animals received ambroxol ($n = 16$) or water ($n = 16$) twice daily for 4 days and a last injection 1 h before pneumotoxic ($n = 10 \times 2$) or vehicle ($n = 6 \times 2$) treatment. In the post-treatment schedule the animals received ambroxol ($n = 16$) or water ($n = 16$) 1 h before pneumotoxic ($n = 10 \times 2$) or vehicle ($n = 6 \times 2$) treatment, and continued to receive twice-daily injections of ambroxol or water until they died or were killed. In each group, half the animals were killed 2 (hyperoxia) or 3 (PQ and $CoCl_2$) days after the beginning of pneumotoxic treatment and the other half, or the surviving animals, 7 days after the beginning of pneumotoxic treatment. The numbers of animals indicated in the results do not exactly correspond to the values of n indicated in this protocol, because some groups contained more animals than scheduled (up to 19 instead of 16), because animals found dead are not included, and because the first experiment with $CoCl_2$ (2.5 mg/kg) was terminated at 3 days.

Endpoints measured

All animals were weighed daily: moribund and ill-looking animals were chosen in preference to others on the days selected for termination. The lungs of animals found dead were examined only grossly.

The animals were anaesthetized with an ip overdose of 120 mg/kg pentobarbital (Nembutal, Abbott, Sanofi, Brussels, Belgium); their abdominal vessels were cut, and after exsanguination the diaphragm and the thorax were opened. The left bronchus and pulmonary vessels were clamped and the left lung removed. The left lung was weighed (wet lung weight) and then placed in an oven for drying at 108°C for 48 h. Dry weight was that obtained after the tissue had been allowed to equilibrate with room air for 1 h following its removal from the oven.

The right lung was lavaged in situ via the trachea with two aliquots of 2 ml sterile NaCl 0.9% at room temperature. The instilled fluid was allowed to remain in the lung for 10 s. After its recovery the fluid was kept on ice and then centrifuged at 400 ***g*** for 10 min at 4°C. The supernatant was assayed for lactate dehydrogenase (LDH) activity using an automated method based on the NADH-reduction of pyruvate (Automatic Analyzer 737 Hitachi, Tokyo, Japan). The pellet was resuspended in 0.2 ml phosphate-buffered saline (pH 7.4) containing 1% bovine serum albumin and 0.1% sodium azide. A 40-µl aliquot of this was added to 20 ml Isoton (Coulter, Luton, UK) and cells were counted in a model DN Coulter Counter (after lysis of red blood cells using Zap-O-Globin, Coulter). Total BAL cell counts reported in the results are number of cells lavaged from the right lung. A 50-µl aliquot of the resuspended pellet (diluted, if necessary, to obtain approximately 100 000 cells) was

centrifuged in a cytocentrifuge. Slides were stained with May-Grünwald-Giemsa and the distribution of inflammatory cells (i.e. excluding erythrocytes and epithelial cells) was determined by counting 300 cells at a magnification of 1000 (under oil immersion).

Following the lavage, phosphate-buffered formaldehyde (10%) was instilled through the cannula until full expansion of the right lung lobes was achieved. Transverse sections of the middle and the basal lobe were embedded in paraffin and 5 µm sections were stained with haematoxylin and eosin or with Masson's trichrome stain. All histological sections were examined by one observer (E.K.V.) without knowledge of pneumotoxic treatment, ambroxol treatment or time of death of the animal. A semi-quantitative score was given to the severity of the injury and/or inflammation (0–5) and to the extent of these lesions (0–5, divided by two if only focal lesions). The final histology score was the product of these two scores and ranged from zero for perfectly normal looking lungs up to a maximum of 25 for the severest damage.

Statistical analysis

One-way (same treatment) or two-way (pneumotoxic treatment, ambroxol treatment) analysis of variance was used to compare the data from different groups, using the SAS statistical package.

RESULTS

Effects of ambroxol in control animals

Table 1 gives the pooled values for the pulmonary parameters found in the 35 rats which received neither ambroxol, nor pneumotoxic treatment. Analysis of variance of these data revealed no statistical differences with regard to the day of killing, the type of vehicle treatment, or the different series of experiments (except for isolated, probably meaningless differences for dry lung weight and LDH). The i.p. administration of ambroxol at a dosage of 50 mg/kg twice daily led to a small, but significant slowing of the growth of rats, e.g. weight gain over 4 days 23 ± 8 g (11 ± 3%) in H_2O-treated rats (n = 50) vs. 16 ± 7 g (7 ± 3%) in ambroxol-treated rats (n = 52), $P < 0.001$. As with many compounds the mechanisms for this slowing of body growth are unclear. Toxicity studies indicate that the ip LD_{50} for the rat is 380 mg/kg and that optimum tolerability is up to 500 mg/kg orally for a period of 4 weeks (although only up to 16 mg/kg by the intravenous route).[1] The effect found here was unlikely to be due to the handling and injecting procedures of the animals, since concurrent controls all received ip injections of distilled water in similar amounts and frequency. After the cessation of ambroxol administration, the body weight gain accelerated to attain control levels by the end of the observation period.

Table 1 Pulmonary indices in 35 control rats[a].

	Mean ± SD	Range
Final body weight (g)	262 ± 25	220–317
Left lung		
Wet weight: (mg)	390 ± 38	341–487
(mg/100 g)	149 ± 12	122–183
Dry weight: (mg)	79 ± 7	67–97
(mg/100 g)	30 ± 2	26–36
W/D ratio	4.91 ± 0.12	4.62–5.23
Right lung BAL (2 × 2 ml)		
Total cells (n × 10^3)	445 ± 133	210–802
Macrophages: (%)	97.5 ± 3.9	79–100
(n × 10^3)	435 ± 132	205–791
Neutrophils: (%)	2.2 ± 3.7	0–21
(n × 10^3)	9 ± 17	0–102
Lymphocytes: (%)	0.3 ± 0.7	0–3
(n × 10^3)	1.3 ± 3.9	0–21
Eosinophils: (%)	0.05 ± 0.18	0–1
(n × 10^3)	0.2 ± 0.8	0–5
LDH (U/ml)	46 ± 13	24–76

[a] Data from 35 male rats who only received i.p. injections of H_2O and sometimes an s.c. (n = 12) or i.t. (n = 11) injection of NaCl 0.9% at least 3 days before being killed.

There was no evidence that the administration of ambroxol adversely affected the pulmonary indices measured, since all indices were similar in ambroxol-treated and H_2O-treated animals, except for the number of BAL-macrophages which was slightly lowered by ambroxol treatment. There were no differences between indices obtained in rats which had previously received ambroxol for 4 days and those from rats which were under ambroxol treatment when killed, except for the number of cells in BAL which was lower in ambroxol-pretreated rats. However, the same difference was found between rats which had received H_2O instead of ambroxol.

We conclude that apart from isolated differences in some parameters, presumably attributable to chance, no meaningful differences occurred between the various sets of control data. We have therefore pooled the data from the vehicle-treated animals for the presentation of results (although this was not done for the statistical analyses).

Lethality and body weight

The three pneumotoxic treatments led to a substantial decrease in body weight, which was not prevented by previous or subsequent ambroxol administration (not shown).

There was a 20% lethality following the administration of paraquat to rats which did not receive ambroxol (2/10 and 2/10) and no lethality in those which did receive ambroxol (0/11 and 0/11). This apparent, and previously reported,[14] reduction in paraquat mortality by ambroxol was not confirmed by a

later experiment which caused 2/19 deaths in ambroxol-treated rats (post-treatment schedule) and 2/19 deaths in H_2O-treated rats.

Exposure to hyperoxia (>95% O_2) was limited to 48 h, because we expected that most rats would not survive a 60-h exposure.[15] No animals died during the O_2 exposure, although most rats were in poor condition by the end of the exposure. One rat from the ambroxol-pretreatment group died during the night following its removal from the hyperoxic exposure.

Ambroxol pretreatment failed to protect animals against the lethal effects of an i.t. dose of 2.5 mg/kg $CoCl_2$: there were 5/10 (50%) deaths in the no-ambroxol group and 9/12 (75%) deaths in the ambroxol-pretreated group. Such high mortality had not been anticipated, since the dose of $CoCl_2$ had been chosen on the basis of previous data obtained in hamsters in which the it LD_{50} for $CoCl_2$ had been estimated to be around 8 mg/kg (unpublished results). Rats, however, appeared to be more susceptible to i.t. $CoCl_2$ than hamsters. The high mortality in that first series precluded us from assessing the effects of ambroxol pretreatment 7 days after lung injury. In the following series, when ambroxol was given after the cobalt administration, the $CoCl_2$ dose was halved to 1.25 mg/kg. With this dose 2/10 deaths occurred in the no-ambroxol group (between 48 and 72 h), and 0/9 deaths in the ambroxol group.

Acute lung toxicity

Figure 1 shows the early effects of the pneumotoxic treatments, with or without ambroxol administration, on the indices of lung toxicity. The ranges of values on the *y*-axes have been deliberately chosen to be similar for all three pneumotoxic agents so as to allow comparisons between these agents.

Paraquat (Fig. 1A) led to significant increases in wet and dry lung weights, as well as in W/D ratio. The histologically detectable damage was minimal to moderate, but there was a clear increase in BAL-LDH. Total number of BAL cells was not increased, but there was a clear increase in the proportion of granulocytes. Neither form of ambroxol treatment had a significant effect on the parameters measured.

Hyperoxia (Fig. 1B) led to increases in wet and dry lung weights. Increases in the W/D ratio were more pronounced than following paraquat and there was considerable pleural fluid in the O_2-exposed animals: 1.9 ± 1.9 ml in the no-ambroxol group, 5.5 ± 1.8 ml in the ambroxol-pretreated group, 5.3 ± 3.5 ml in the ambroxol-post-treatment group ($P < 0.05$). Minimal damage was apparent by light microscopy, but there was no increase in BAL-LDH. Total numbers of BAL cells were not increased, but there was a small and significant increase in the proportion of neutrophils. Ambroxol treatment did not modify any of the measured parameters, except the amount of pleural fluid which was considerably increased with both treatment schedules.

Intratracheal cobalt chloride (Fig. 1C) led to dose-related increases in wet and dry lung weights, and in W/D ratios. The histological damage was readily identifiable and consisted mainly of peribronchiolar inflammation, and evidence of interstitial and alveolar oedema in some animals. There were large increases in BAL-LDH. The total number of BAL cells was only increased in the 1.25 mg/kg dose group, but in both dose groups the proportion of granulocytes was greatly increased. Ambroxol did not significantly affect the measured parameters, except for an increase in wet lung weight and W/D ratio with the ambroxol pretreatment and a decrease in BAL cells with the ambroxol post-treatment.

Residual effects

Figure 2 presents the findings 7 days after the pneumotoxic treatments. Again the *y*-axes have been deliberately chosen to allow comparisons with the early effects and between pneumotoxic agents.

Paraquat (Fig. 2A) resulted in small, but significant increases in wet and dry lung weights and in W/D ratio. In some animals, moderate histological damage was detectable, including some focal fibrosis. The BAL data had nearly returned to control. There was no effect from either ambroxol treatment. Hyperoxia (Fig 2B) also resulted in small, but significant increases in wet and dry lung weights and in W/D ratio. Again some animals had evidence of focal fibrosis. BAL data had also virtually returned to control, although there were still slightly more neutrophils among the BAL cells of hyperoxia-exposed rats. Ambroxol did not alter any of the parameters. Intratracheal cobalt chloride (Fig. 2C) also led to increased wet and dry lung weights, and W/D ratios. Moderate histological damage was still evident, including some peribronchiolar fibrosis. The BAL data were returning towards normal, but there were still increased numbers of neutrophils; the ambroxol-treated animals seemingly had more residual damage, but this was not significant.

DISCUSSION

Overall, our results showed that ambroxol failed to protect against experimental pneumotoxic injury, both when ambroxol was given as a pretreatment, and when ambroxol treatment was started with the administration of the pneumotoxic agent.

We did not measure indices of surfactant production and secretion, but the literature[1-6] is quite convincing with regard to the ability of ambroxol to

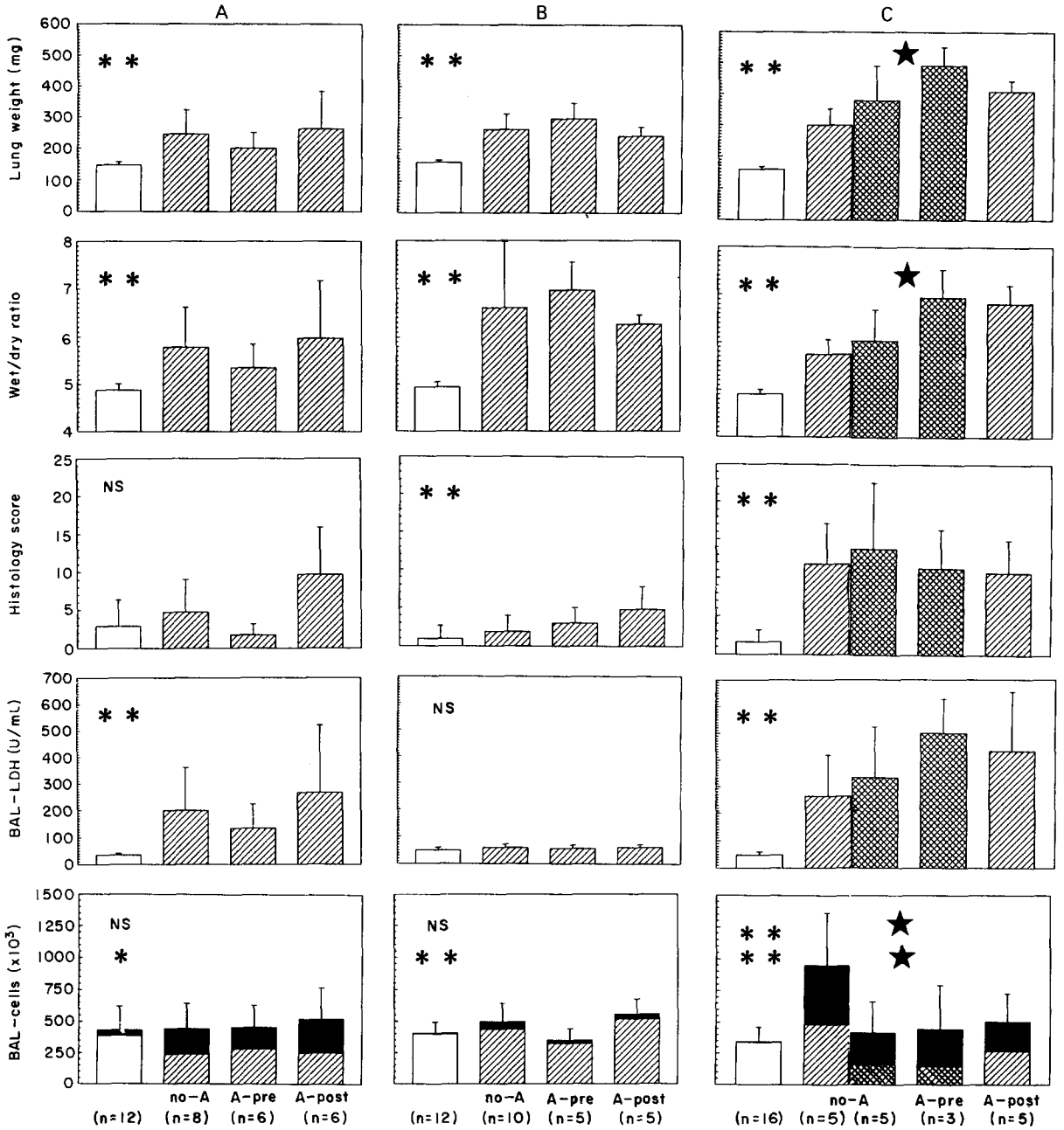

Fig. 1 Effects in rats of a 4-day pretreatment with ambroxol (A-pre) or ambroxol treatment initiated at the time of pneumotoxic administration (A-post) 3 days after the administration of paraquat (A), after 2 days of hyperoxic exposure (B) or 3 days after the i.t. administration of cobalt chloride (C), 1.25 mg/kg (▨) or 2.5 mg/kg (▩), Data from appropriate control rats not receiving pneumotoxic treatment (□) and rats receiving pneumotoxic treatment, but no ambroxol (no-A) have been pooled for clarity. Lung weights are wet weight of the left lung per 100 g body weight; BAL cells are divided into macrophages and granulocytes (■, mainly polymorphonuclear neutrophils, but also some eosinophils). Data are mean †1 SD (in the bottom graphs only the SD of the total number of cells is indicated). $*P<0.05$; $**P<0.01$ for effect of pneumotoxic treatment compared to vehicle control (irrespective of ambroxol effect); in the bottom graphs significance is indicated for the total number of cells first, then for percentage of granulocytes. ★Effect of ambroxol treatment is significantly different from no-ambroxol treatment.

induce substantial changes with the doses and animals used here. An indirect indication that ambroxol modified surfactant in our experiments is afforded by the data concerning the pleural effusion after hyperoxia. Indeed, the only clear-cut change in toxicity found in ambroxol-treated animals was a marked increase in fluid found in the pleural spaces of hyperoxia-exposed rats. Pleural exudates are a consistent feature of oxygen toxicity in the rat[9,16] and reflect increased capillary permeability without increased alveolar epithelial permeability.[16] We attribute the increased accumulation of fluid in the pleural cavity in ambroxol-treated rats to changes in alveolar surface forces caused by altered surfactant production. Such changes are likely to alter both alveolar permeability and lymphatic drainage,[17] and hence the amount of

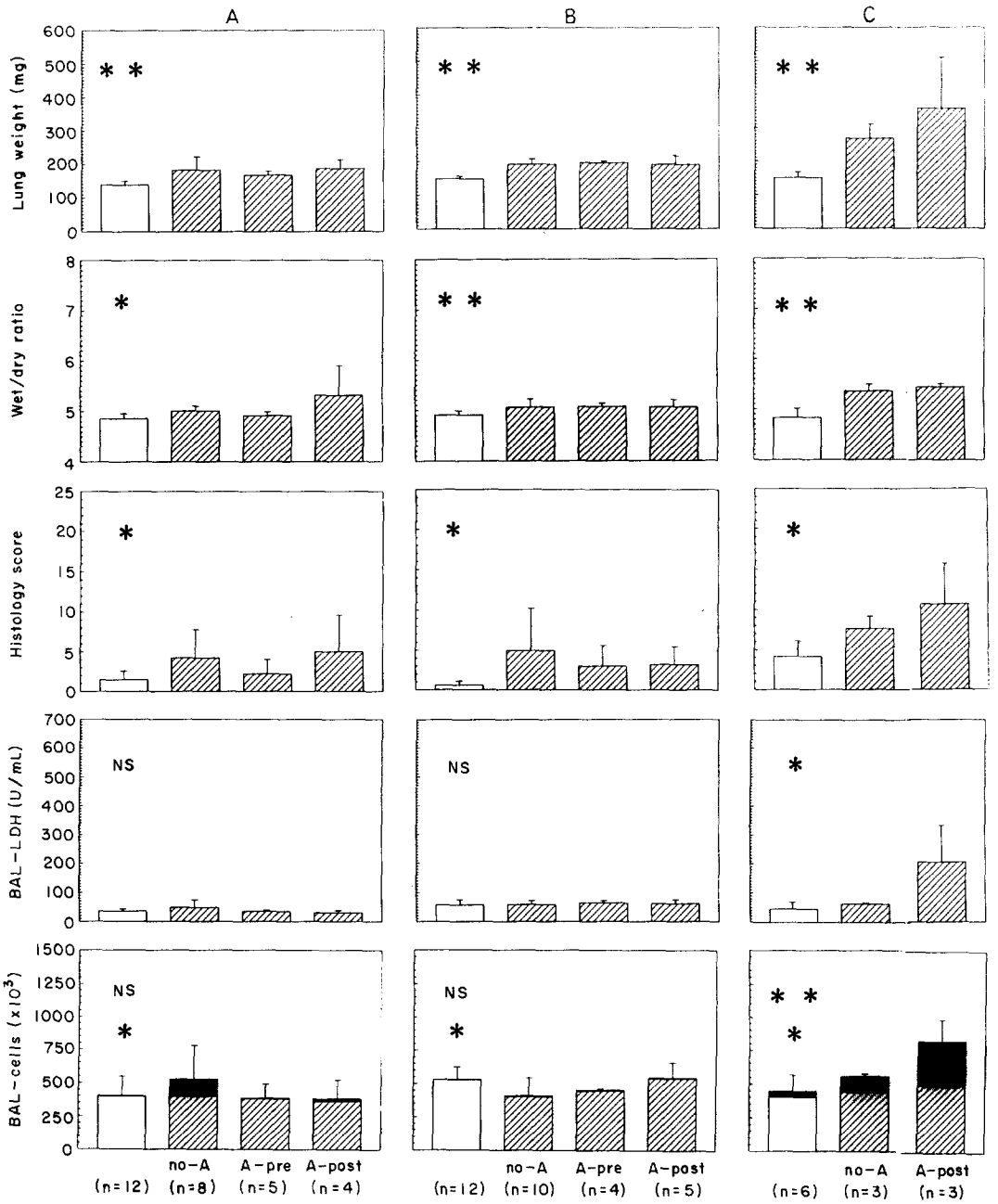

Fig. 2 Effects in rats of a 4-day pretreatment with ambroxol (A-pre) or ambroxol treatment initiated at the time of pneumotoxic administration (A-post) 7 days after the administration of paraquat (A), oxygen (B) or cobalt chloride (C). See legend of Figure 1 for further explanation of symbols.

fluid in the pleural spaces. While such changes are entirely compatible with the known pharmacological action of ambroxol, it is doubtful that they are beneficial in terms of protection against hyperoxic injury.[16] Certainly, in the present study they did not appear to be of any beneficial consequence.

Ambroxol apparently prevented paraquat-induced deaths,[14] but this could not be confirmed. Ambroxol also appeared to prevent cobalt-induced deaths in the 1.25 mg/kg dose group. However, the more affected rats were terminated preferentially, and it is possible that this 'bias' was not equal between the no-ambroxol group and the ambroxol-pretreatment group. The early indices of lung damage did not differ between these two groups, except that total BAL cells were fewer in the ambroxol-treated animals. However, this may in fact be evidence for more severe damage in the latter animals, because the harvesting of cells by the gentle lavage procedure used here is probably less efficient in oedematous lungs, particularly when alveolar flooding has occurred. At any rate, the average weight loss and the residual damage as assessed 7 days after $CoCl_2$ appeared to be more pronounced in the ambroxol-treated rats.

Whilst the primary objective of this study, i.e. testing the effects of ambroxol on toxic lung injury,

proved disappointing, this investigation has provided additional evidence that lung injury can be assessed by a variety of means and that a combination of indices allows a better and relatively easier characterization of toxicity than that based on a histological assessment alone.[13]

The cellular targets for paraquat and hyperoxia are different. Paraquat accumulates in alveolar epithelial cells and damages these cells through a process of increased cellular oxidation.[8] The dose of paraquat given here corresponded to an LD_{20}.[18] This led to overt changes in lung weight, with evidence of pulmonary oedema (W/D ratio), injury of cells lining the air spaces (LDH in BAL) and an influx of polymorphonuclear neutrophils (in BAL). All this occurred without statistically significant evidence of cell damage by histology. This low histological sensitivity is probably partly accounted for by the fact that the lungs had been lavaged before being instilled with fixative so that the air spaces appeared relatively empty and free from inflammatory cells, and also by the fact that the histological examination was performed without any knowledge of the time of death and treatment given to the animal. That the histological assessment and scoring system were nevertheless valid is attested by the overall consistency of the histological results in our study. Some control rat lungs were found to exhibit very discrete inflammatory changes in the parenchyma (with sometimes neutrophils in the BAL fluid). These were possibly due to some infection, since although the rats were specific pathogen-free on receipt, they were then kept in a conventional animal house. However, no frank pneumonia was found in any of the control animals.

The initial target for hyperoxia is the capillary endothelium of the lungs.[8] Endothelial injury causes pulmonary oedema (and pleural effusions), with slight, but nevertheless statistically detectable histological damage and alveolar inflammation, but no signs of cell death in the BAL fluid. It is probable that some of these parameters would have been altered had the exposure been more prolonged and led to alveolar epithelial damage and more inflammation.[15]

The effects of intratracheal cobalt chloride were more spectacular than those of either paraquat or oxygen. The mechanism of cell injury by this metal salt are not known, but our laboratory has been involved in both in vivo and in vitro studies of the toxicity of cobalt compounds, which are known to cause fibrosing alveolitis in some occupationally exposed subjects.[10,11] The present experiments confirm that $CoCl_2$ has the potential to acutely damage the lungs and indicate that rats may be somewhat more sensitive tham hamsters, the species used in our previous experiments.[12]

With regard to the residual effects of lung injury, our data show that whereas BAL findings had mostly returned to normal by 7 days, lung weights were still slightly, but detectably elevated. This sensitivity of lung weights is probably due, in part, to the very small variability of organ weights.[13] The increases in dry lung weight presumably reflect interstitial thickening by cellular and non-cellular material; foci of fibrosis were histologically detectable in some animals. At this later stage it would probably have been useful to measure lung hydroxyproline content to assess the degree of collagen deposition.

In conclusion, we have shown by using a comprehensive set of parameters of lung toxicity that three different pneumotoxic agents caused different patterns of lung injury, and that this injury could not be substantially modified by the administration of ambroxol.

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