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Investigation of the Ultrastructural Changes and Hydroxyproline Levels in Mice Lungs Induced by Bleomycin Treatment

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Summary
The present study was planned to investigate the ultrastructural changes and hydroxyproline level in the lungs of the mice induced by bleomycin treatment. Following 4 weeks of bleomycin treatment, the lungs were examined histologically (using light and electron microscopes) and the hydroxyproline level was measured at 4th, 6th, 8th, and 10th weeks of the experiment. Biochemical and structural changes that normally occur in lung fibrosis were also observed in our study. For example; lung hydroxyproline level increased progressively ($p<0.05$) and structural changes advanced to interstitial fibrosis from 4th to 10th weeks of the experiment. Alveolar septum thickening, type II pneumocytes increase and mononuclear cell infiltration (lymphocyte, monocyte and macrophage) were seen. In addition, membrane-bound rod-shaped amorphous structures in the cytoplasm of alveolar macrophages were increased significantly ($p<0.05$).
It is concluded that, bleomycin treatment may produce fibrosis due to increase in interstitial connective tissue, hydroxyproline level and the number of alveolar macrophages. However, more studies are needed to demonstrate whether there is a relationship between the increased membrane-bound rod shaped amorphous structures and the occurrence of fibrosis.

Introduction
Bleomycin is known to cause lung fibrosis in humans and experimentally in animals.
Bleomycin, a mixture of antibiotic and antineoplastic glycopeptides, produces only minor hematological and gastrointestinal toxicity but is limited in its clinical usefulness by a dose-related pulmonary toxicity. Bleomycin-induced interstitial pneumonitis and subsequent pulmonary fibrosis have been described in patients and in several animal species, including monkeys, dogs, rodents, etc. *(Hersterberg et al.,1981; Horuichi et al.,1990; Jensen et al.,1990; Strausz et al.,1990)*. The biochemical mechanism of bleomycin induced lung injury is not known. However, superoxide and hydroxyl radicals are generated during the metabolism of bleomycin. These activated oxygen species are able to initiate peroxidation of membrane lipids, leading to the degeneration of membrane structure and function *(Singer et al.,1986, Tryka., 1989)*.
The well known major side effect of bleomycin is pulmonary fibrosis which is usually characterized by an insidious onset of dyspnea 4-10 weeks after initiation of therapy *(Ganick et al.,1980)*. Several investigators demonstrated the changes caused by bleomycin in experiments at light microscopic level *(Ward et al.,1988; Hirose et al.,1993)*, but there were only a few ultrastructural reports.
The present study was planned to investigate the ultrastructural changes and hydroxyproline levels induced by bleomycin treatment in the lungs of mice.

Materials and Methods
52 male Swiss albino mice, free of any disease, weighing, 20-30 g (approx. 60 days of age) were used in this study. Animals were housed according to NIH guidelines in two separate cages and fed

with laboratory chow and water ad libitum and randomly divided into two comparable groups. Bleomycin (bleomycin sulfate) was purchased from a standard commercial source (Bleocin, from Mustafa Nevzat Ilaç Sanayi A. Ş.). Our dose selection for bleomycin (10 mg/kg) was based on the previous study of Sikic et al., 1978. These investigators suggested an optimal dose of 10-20 mg/kg s.c. twice weekly for 4 to 6 weeks for development of chronic bleomycin lung toxicity in mice. A period of 10 weeks was designed for this experiment due time taken for the onset of side effects. The experimental design of this study is shown in table 1.

Table 1: Experimental dosing.

GROUPS	Drugs (dose)*	Mice †			Time (week)§
	s.c.	Number	Initial Body Weight (g) (mean±SEM)	Final Body Weight (g) (mean±SEM)	
Control	Saline (0.3 ml/animal)	24	25.4±0.77	36.04 ± 0.80	4,6,8,10
Bleomycin	Bleomycin (10 mg/kg)	28	25.7±0.80	29.65 ± 0.85	4,6,8,10

* Drugs were administered twice a week for 4 weeks.
† Number and body weight of mice in each groups at the beginning of the experiment. Six mice from control group and 7 mice from bleomycin were randomly selected to be killed at each time interval.
§ Weeks after beginning of experiments at which mice were killed.
s.c.: Subcutaneously, i.p.: Intraperitoneally

Initial weights of the animals were recorded. Animals were selected randomly from each group and were sacrificed by craniocervical dislocation under anesthesia (7.5 mg/animal of ketamin, i.p.). The lungs were removed and dissected free of central bronchi and vessels. The left lungs were used in biochemical assays for hydroxyproline determination and the right lungs were processed for routine electron microscopic examination. Tissues were fixed in 2.5 % glutaraldehyde in PBS (phosphate buffer solution) and were postfixed in 1 % osmium tetroxide in phosphate buffer. Following dehydration, tissues were embebded in araldite CY 212. Semithin and thin sections were cut on a LKB Nova Ultramicrotome from the blocks. Semithin sections were stained with 1% toluidine blue and then examined and color pictures taken on a Olympus BHS/BHT light microscope. Thin sections were stained with aqueous uranyl acetate and Reynold's solution and examined and photographed with a Zeiss EM 9S2 electron microscope.

Hydroxyproline Determination

We used the method of Jamall et al.,1981, with small modifications. All reagents used in this study were of analytical grade. Ehrlich's reagent solution: 10 g of p-dimethylaminobenzaldehyde (Merck) in 11 ml of 60% (w/w) perchloric acid. This solution was stored in a brown pyrex flask for a maximum period of 2 weeks. Prior to each experiment, 3 ml of this solution was mixed with 8 ml of isopropanol to yield the working Ehrlich's reagent solution. Hydroxyproline solution: A

solution containing 100 mg/100 ml of l-hydroxyproline (Merck) was prepared in 80% isopropanol. Standard solutions were prepared freshly by appropriate dilutions with 50% isopropanol. Acetate-citrate buffer: The buffer was prepared by dissolving 57 g sodium acetate trihydrate (Merck), 87.5 g trisodium citrate (Merck), and 5.5 g citric acid monohydrate (Merck) in 400 ml of isopropanol (Merck) and completed to 1 litre with distilled water. The pH of the solution was 6.5 and had to be adjusted to pH 6.0 by the dropwise addition of 12 N hydrochloric acid. Chloramine T solution: A solution containing 543 mg of chloramine T (Sigma) was prepared in 10 ml acetate-citrate buffer. This solution was freshly prepared before each working. All reagents were stored in a refrigerator at 4 °C and brought to room temperature prior to use.

Assay for hydroxyproline in hydrolysates

Left lung was removed and blotted with filter paper, cut into small pieces, weighed, and placed in a screw-capped tube. After adding 2 ml of 6 N hydrochloric acid to the tube, it was hydrolyzed at 150 °C for 1 h in a Hycel Thermal Block. Aliquots of 10 microlitre lung hydrolysate, in triplicate, were added to separate tubes and evaporated to dryness under vacuum over sodium hydroxide/calcium sulfate desiccant. Sets of samples were spiked with 0, 0.3, 0.6, 0.9 and 1.2 microgram hydroxyproline standard in 1.2 ml of 50% isopropanol. A 0.2 ml aliquot of chloramine T solution was added to each tube. After a 10-min interval, 1.0 ml of working Ehrlich's reagent solution was added to all tubes. All samples were mixed and then incubated at 50 °C for 90 min. The absorbances at 558 nm were read, using water as reference and corrected for the reagent blank. Absorbance values were plotted against microgram of added hydroxyproline and the negative intercept on the x axis was found by linear regression analysis. The absolute value of this intercept was taken to represent the hydroxyproline content of the tissue. Data were expressed as milligrams hydroxyproline per gram of wet lung tissue.

Statistical analysis

Data were presented as mean±SEM. Students t test for mean body weight and Mann-Whitney U test for mean lung hydroxyproline content were used to test differences among the means. In all cases, a $p<0.05$ was considered to be significant.

Results

Body Weight

All animals used in this study survived and gained weight progressively. Although there was no significant difference between the initial weights of the animals in both groups, the increase in the mean body weight of the control groups (from 25.40 ± 0.77 to 36.04 ± 0.80) was significantly higher than that of the bleomycin (from 25.70 ± 0.80 to 29.65 ± 0.85) treated group ($p<0.05$ or $p<0.01$) (fig 1).

Lung hydroxyproline content

Lung hydroxyproline content of mice in the bleomycin treated group showed a significant increase ($p<0.05$) starting from the last day of drug administration (4th week) to the tenth week (fig 2). There was no significant change in the lung hydroxyproline content of the control group. The increase in hydroxyproline levels in bleomycin treated mice became more significant in the 6th week of the experiment (2 weeks after the cessation of bleomycin administration) (Table 2).

Structural findings

Tissue samples obtained at the 4th, 6th, 8th and 10th weeks of the experiment were examined respectively by using light and electron microscopes.

Light microscopy findings

Lungs of mice in the control group showed normal lung structure and no lesion (Fig. 3). Thickness due to the infiltration of mononuclear cells (lymphocyte, monocyte, macrophage), increase in the type II pneumocyte and in the thickness of the connective tissue in the interalveolar septum were observed in peribronchial and perivascular spaces in the bleomycin group. These findings showed a progressive increase from 4th to 10th week (Fig. 4abc). All findings were consistent with pulmonary fibrosis.

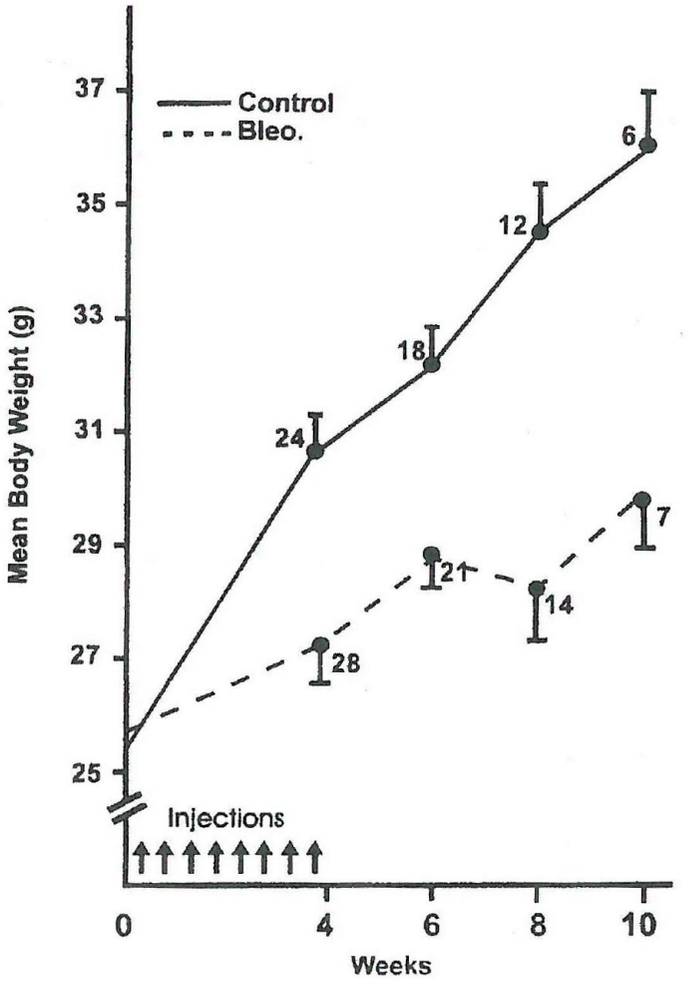

Fig. 1: Changes in body weights (mean±S.E.M.) of mice. Bleomycin was injected for 4 weeks. (_____): Control group; (- - - - -): Bleomycin treatment group.

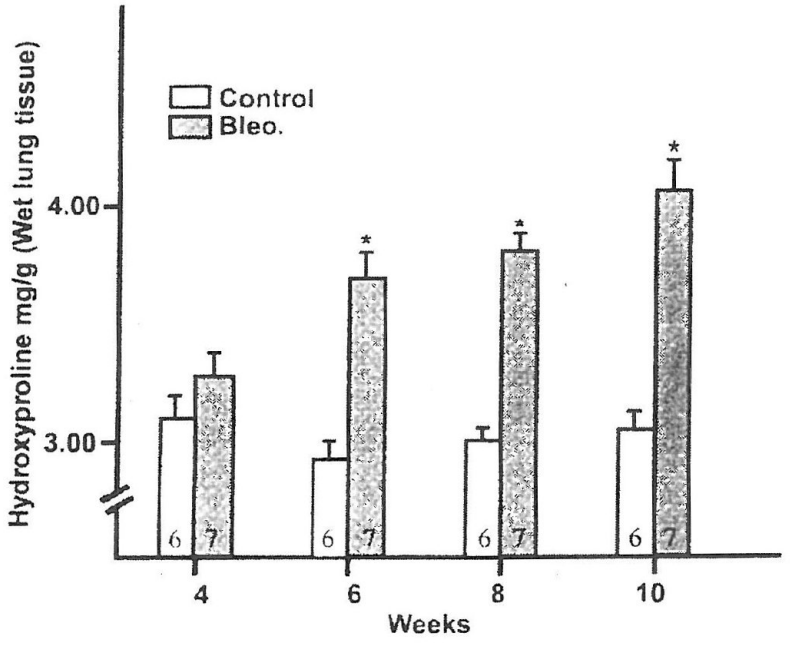

Fig. 2: Hydroxyproline content (mean±SEM) of lung. Administration was finished at 4th week and first examination was performed after a day (*:p<0.05).

Table 2: Lung hydroxproline contents (mean±SEM) of two groups.

		Hydroxyproline (mg/g wet lung tissue)			
GROUPS	n	4 th week	6 th week	8 th week	10 th week
Control	6	3.15±0.09	2.9±0.07	3.02±0.09	3.05±0.05
Bleomycin	7	3.42±0.07	3.74±0.11	3.79±0.08	4.11±0.13
p(Control-Bleomycin)		>0.05	<0.05	<0.05	<0.05

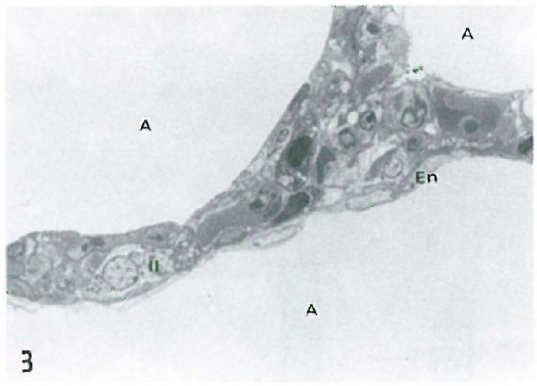

Fig. 3: Light micrograph of a mouse interalveolar septum of the control group. Note the thinness of the septum. A: Alveolar air space; En: Endothelial cell, II: Type II pneumocyte; methylene blue-Azur II, x1200).

Electron microscopy findings

Our electron microscopical examinations supported the light microscopical findings. The same pathological changes were observed in the bleomycin treated group while the control group showed the normal ultrastructure of the lung. All cell types in infiltration sites were clearly distinguished, among the accumulations of thick collagen fibre bundles (Fig.5). In addition, rod-shaped, amorphous structures were determined in the cytoplasms of alveolar macrophages (Fig 6a, 6b). These structures were observed to be membrane-bound under higher magnifications (Fig 6c). Almost all of the alveolar macrophages were found to be full of these structures.

Discussion

This experiment was carried out to investigate the ultrastructural changes and hydroxyproline level induced by bleomycin treatment in the lungs of mice. Mean body weights of animals used in both groups showed increase during the experiment (fig 1), though significantly less in the bleomycin treated group than in the control group. The reduction in weight gain in bleomycin-treated animals may be due to the direct cytotoxic action on cell growth. Bleomycin has been demonstrated to inhibit DNA and protein synthesis in a variety of cell systems *(Tom & Montgomery,1980).* Anorexia, as experienced by most bleomycin-treated patients, could also be another explanation for reduction in body weight gain *(Tom & Montgomery,1980).*

As expected, cellular infiltration and consequent fibrosis were observed in the present study in the of mice lungs due to bleomycin treatment. The increase in the hydroxyproline content of the lung in this group became statistically significant after the 6th week of the experiment (Fig 2). Since hydroxproline is an amino acid and found in collagen fibres, an increase in collagen fibre bundles would lead to the increase seen in this amino acid. This was confirmed by structural examination of the tissue samples of the same animals. This association was shown also by many other investigators previously *(Goto et al., 1984; Schrier et al., 1984; Lindenschmidt et al., 1986; Tomioka et al., 1989; Wang et al., 1990).*

As in the changes of lung hydroxyproline levels, our light and electron microscopic findings were also parallel to those of the previous workers *(Cross et al., 1985; Shen et al., 1988; Grant et al.,* 1991). Thickening of the alveolar septum, increase in the type II pneumocytes and mixed cellular infiltration (lymphocyte, monocyte and macrophage) were brief summarized findings. A striking feature at electron microscopic level was the presence of membrane - bound rod - shaped

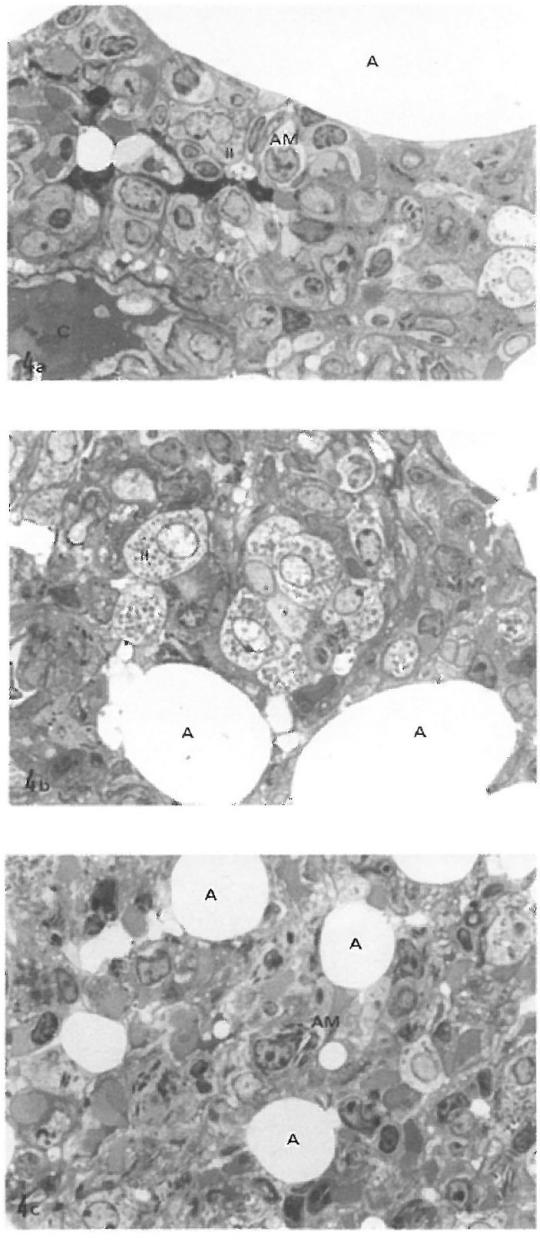

Fig. 4a,b,c: Light micrographs of bleomycin treated groups (10 weeks). Interalveolar septum was observed to be thickened in all of the micrographs due to type II pneumocyte (II) hyperplasia and mononuclear and polymorphnuclear cell infiltration. C:Capillary; A:Alveolar air space; Am: Alveolar macrophages, methylene blue-Azur II, x1200.

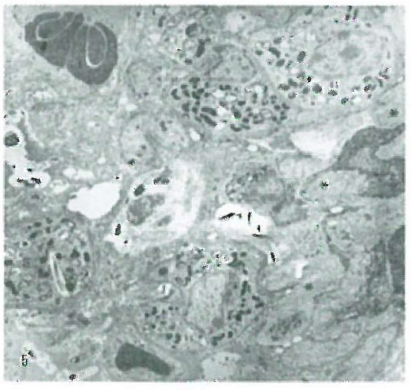

Fig. 5: A transmission electron micrograph of a mouse interalveolar septum of the bleomycin treated group (10 weeks), (Original magnificationx5600). Uranyl acetate and Reynold's lead stain. Note the moderate accumulations of collagen fibre bundles (asteriks). II: Type II pneumocytes; E: Erytrocyt.

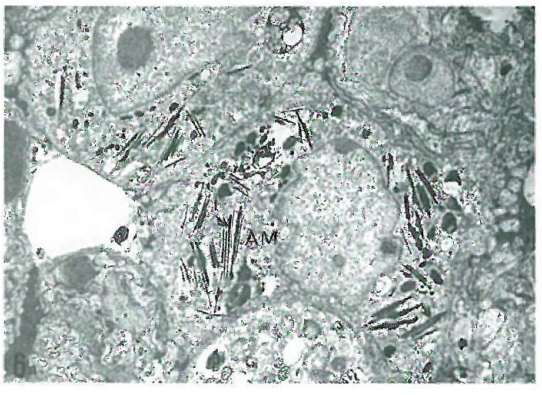

Fig. 6a

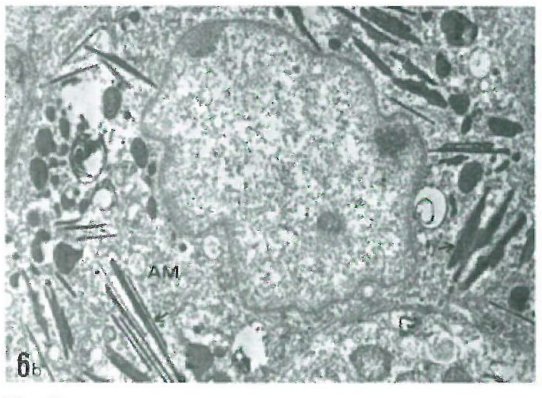

Fig. 6b

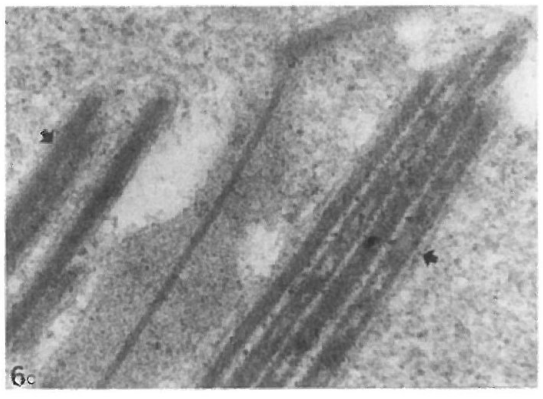

Fig. 6: (a,b) Electron micrographs of mice lungs 10 weeks after bleomycin administration. (c) Higher magnification electron micrograph of the rod shaped amorphous structures in the cytoplasm of the alveolar macrophages. These structures were observed to be membrane bound (arrows), (Original magnification, (a)x5600, (b) 19500, (c) 87.500). Uranyl acetate and Reynold's lead stain. II: Type II pneumocyte. Am: Alveolar macrophages.

amorphous structures observed in the alveolar macrophages. In addition, it was observed that the alveolar macrophages and membrane-bound rod-shaped amorphous structures in their cytoplasm were increased significantly. These structures were considered to be representing microbodies related to increased oxygen radical metabolism caused by the bleomycin treatment.

It is concluded that, bleomycin treatment may produce fibrosis due to increases in interstitial connective tissue, hydroxyproline levels and the number of alveolar macrophages. However, more studies are needed to demonstrate whether there is a relationship between the increased membrane-bound rod shaped amorphous structures and occurrance of fibrosis.

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