

## *Intravenous Bleomycin Does Not Alter the Toxic Effects of Hyperoxia in Rabbits*

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The purpose of this study was to test the hypothesis that bleomycin administration enhances the toxic effects of oxygen on the respiratory system. Twenty-one rabbits, with no evidence of respiratory disease, received intravenous injections of 45 units of bleomycin (Blenoxane®), twice a week, for a total dose of 300 units. Fifteen rabbits received an equal volume of saline and served as controls. Treatment with bleomycin resulted in failure to thrive, weight loss, and 30% mortality from nonpulmonary causes, as indicated by the lack of respiratory distress or cyanosis, during or shortly after the injection period. The remainder of the animals were allowed to recover for 21 days following the last injection. At that time, no differences were found between the experimental and the control groups with respect to arterial blood gases, total lung capacity, compliance, and hydroxyproline content. Histologic examination of lung tissue revealed normal lung architecture. When exposed to 100% O<sub>2</sub>, bleomycin-treated rabbits developed arterial hypoxemia and died from respiratory failure at the same rate as the controls. It was concluded that pretreatment of healthy rabbits with 300 units of intravenous bleomycin did not result in the development of significant amounts of lung fibrosis or enhance the toxic effects of oxygen on the respiratory system. (Key words: Lung: compliance; fibrosis; oxygen toxicity. Oxygen: toxicity. Toxicity: bleomycin; oxygen.)

BLEOMYCIN IS A POTENT antitumor agent that is particularly effective for the treatment of squamous cell and testicular carcinomas.<sup>1,2</sup> Its effectiveness, however, is limited by its lung toxicity. Pneumonitis and pulmonary fibrosis, along with lung histologic changes and impairment of pulmonary function, have been reported in patients who have received more than 300 units of this drug.<sup>3-5</sup>

Because injury may change the response of the pulmonary system to a variety of agents, a number of investigators questioned whether bleomycin treatment renders patients more susceptible to the toxic effects of oxygen. This is an important clinical question because patients who receive bleomycin may be exposed to higher-than-ambient oxygen concentrations during and following lengthy surgical procedures.

At present, this question remains unanswered. An earlier report indicated an unusually high incidence of adult respiratory distress syndrome (ARDS) in bleomycin-treated patients following exposure to 40-50% oxygen during surgery for the removal of retroperitoneal lymph nodes or esophageal resection.<sup>6</sup> These levels of oxygen have been thought to be innocuous in the mammalian respiratory system, even when administered for long periods of time.<sup>7</sup> More recent reports, however, have disputed these findings and indicate that bleomycin treatment does not increase the risk of developing ARDS.<sup>8,9</sup>

Several factors may account for these contradictory findings. The bleomycin-induced lung injury depends on a number of factors including patient age, previous existence of lung disease, renal dysfunction, the concomitant administration of other antineoplastic agents or radiotherapy, and the use of crystalloid rather than colloid solutions for volume replacement.<sup>4,6,10</sup> Thus, patient studies contain too many uncontrolled variables and cannot provide definitive answers to this question.

The purpose of this study was to determine whether pretreatment with bleomycin modifies the appearance and time course of pulmonary damage due to oxygen toxicity. This was accomplished by measuring a number of physiologic, histologic, and biochemical variables throughout the oxygen exposure and shortly after death. To circumvent the previously mentioned modifying factors and isolate the effects of bleomycin on the respiratory system, we conducted these experiments in healthy, unanesthetized rabbits, an animal whose response to oxygen has been previously reported in detail.<sup>11-13</sup> The mode of administration and the dosages used were designed to mimic the clinical situation as closely as possible.

### Materials and Methods

Experiments were performed on New Zealand White rabbits with initial weights of 1.9 to 2.5 kg. All animals were active, eating and drinking normally, and had white blood cell counts of less than 12,500 cells/ml at the time they were included in this study. The bleomycin, contained in sealed sterile vials (Blenoxane®; Bristol Laboratories, Syracuse, NY), was past its expiration date by about 1 yr. It has been shown that this does not diminish its lung toxicity.<sup>14</sup> Forty-five units of bleomycin were dissolved in 5 ml of sterile saline and injected in an ear vein

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twice a week. Twenty-one animals received a total intravenous dose of 300 units during a 24-day period. The control group consisted of 15 animals that received an equal volume of sterile saline.

All animals were allowed to recover for 21 days after the last injection. At this time, a 1-ml sample was drawn from an ear artery, and the  $\text{Pa}_{\text{O}_2}$ ,  $\text{Pa}_{\text{CO}_2}$ ,  $\text{pH}$ , and hematocrit were determined with an Acid-Base Laboratory Analyzer® (Radiometer, Copenhagen, Denmark). Seven of the experimental animals and six of the controls were anesthetized with an intravenous injection of 6 mg ketamine (Ketalar®, Parke-Davis, Detroit, MI) and 6 mg promazine (Sparine®, Wyeth Laboratories, Philadelphia, PA) and killed by an intracardiac injection of KCl. Static lung pressure-volume relationships were successfully measured in three control and six experimental rabbits. Lung hydroxyproline content and wet and dry weights were measured in all animals. In addition, tissue samples were taken from the lower ventral margin of the upper, middle, and lower lobes of each lung and fixed by immersion in 10% neutral buffered formalin. The quantity of tissue taken was approximately 10% of the lung mass. They were then processed according to standard histologic techniques, sectioned at a thickness of 5  $\mu\text{m}$  and stained with hematoxylin and eosin and Masson's trichrome stains. Lung dry weights were measured after the tissue had remained in an oven at 70° C for at least 1 week.

Immediately after death, the chest was opened, and the lungs were allowed to collapse to their resting volume. The trachea was exposed and cannulated with a small endotracheal tube that was attached to a 50-ml syringe and a water manometer. The lungs were then inflated by 10-ml increments, and the equilibrium water pressure was recorded. This pressure was reached within 10–25 s from the end of each air injection. Inflation was continued until the equilibrium pressure reached 40  $\text{cmH}_2\text{O}$ . The lungs were then deflated in the same step-wise manner. In each experiment, we fitted the following exponential function through the deflation pressure-volume ( $P$ ,  $V$ ) data:

$$V = \text{TLC} * (1 - \exp(-K * P)) \quad (1)$$

where TLC is the Total Lung Capacity, defined as the static lung volume at 40  $\text{cmH}_2\text{O}$ , and  $K$  is the calculated rate constant. The half-inflation pressure ( $h$ ) was then computed from the following relationship:

$$h = 0.693 / K. \quad (2)$$

This variable has been shown to be a sensitive indicator of the lung recoil force and to be independent of the lung volume at which it is measured.<sup>15</sup> We also calculated the value of the lung compliance by measuring the slope of the steep part of the deflation limb of the pressure-volume curve.

The amount of hydroxyproline in lung tissue, a variable indicating the amount of collagen present, was measured by the method of Woessner<sup>16</sup> using the variation that utilizes benzene extraction of the chromogen. Recovery of known amounts of hydroxyproline, added to the tissue as an internal control, exceeded 90%.

The six surviving bleomycin-injected rabbits and the nine saline-injected controls were exposed to 100%  $\text{O}_2$  until death in individual environmental chambers. Oxygen was continuously circulated through them at a flow rate of 6 l/min. Oxygen and carbon dioxide concentrations, checked periodically with a mass spectrometer, were found to be higher than 99% and lower than 0.2% respectively. Administration of food and water and daily removal of wastes did not alter the gas compositions. Indwelling Teflon® catheters (20-g cathlon IV; Criticon, Inc., Tampa, FL), were inserted and secured in an ear artery of all animals. One-milliliter blood samples were drawn periodically throughout the exposure for the measurement of  $\text{Pa}_{\text{O}_2}$ ,  $\text{Pa}_{\text{CO}_2}$ ,  $\text{pH}$ , and hematocrit. The catheters were filled with a solution of 100 U/ml of heparin and 10 mg/ml of chloramphenicol (Chloromycetin®; Parke-Davis, Detroit, MI) and remained patent in six of the control and five of the experimental rabbits. Immediately after death, the lungs were removed, weighed, and processed for lung histology, and measurement of hydroxyproline content and dry weight was performed as described previously. All measurements and procedures have been previously described in detail.<sup>17</sup>

#### STATISTICAL ANALYSIS

All results are expressed as means plus or minus one standard error of the mean ( $\bar{X} \pm \text{SEM}$ ). Significant differences among means were determined as follows: (1) one independent variable, two groups: Student's  $t$  test for equal or unequal variances; (2) one independent variable, multiple groups: one-way analysis of variance and the Bonferroni modification of the  $t$  test for multiple comparisons.<sup>18</sup> Values were considered significantly different from each other if the two-tailed  $P$  value was less than 0.05 ( $P < 0.05$ ). Linear regression lines and the 95% confidence intervals were calculated using the method of least squares.

#### Results

Rabbits that received bleomycin ate considerably less and failed to thrive (see fig. 1). Seven animals died either during or shortly after the injection period and were not included in this study. None of the animals showed any signs of respiratory distress or cyanosis. On the other hand, saline-injected rabbits remained active, eating and drinking normally, and continued to gain weight throughout the injection and recovery periods. In both

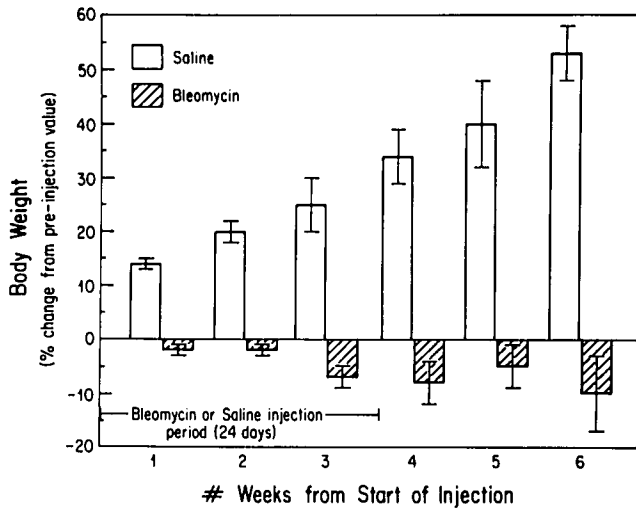


FIG. 1. Per cent change in rabbit body weights from their preinjection values. Means  $\pm$  1 SEM.

groups of animals, arterial blood gases, measured while breathing air just prior to death or exposure to 100% O<sub>2</sub>, were within the normal range. These measurements, along with the corresponding values from a group of uninjected rabbits, are shown in table 1.

Values for total lung capacity, half-inflation pressure, and static lung compliance for six experimental animals and three controls are summarized in table 2. Figure 2 shows a typical pressure-volume curve in a bleomycin-treated animal. There were no significant differences in any of these variables between the two groups using Student's *t* test for unpaired samples ( $P > 0.05$ ). Furthermore, the control values were similar to those we have reported previously.<sup>17</sup>

Figure 3 shows a plot of total lung hydroxyproline content versus the weight of the animals at the time of death. In addition to the bleomycin and saline groups, we have included values from another group of five animals that did not receive any injections. There is a significant correlation between lung hydroxyproline content and the body weight of the control animals. Because rabbits continue to gain weight as they grow older, we believe that our data indicate that the amount of lung hydroxyproline increases with age. Sahebajami and MacGee found a similar relationship when they compared the levels of lung

hydroxyproline in young and mature rats.<sup>19</sup> This explains the differences between the uninjected and saline groups: the former were lighter and thus had a lower lung hydroxyproline content than the latter (see fig. 3 and table 3).

Rabbits in the bleomycin and saline groups had similar weights at the onset of injections, but the former were considerably lighter at the time of death, as a result of the treatment (see fig. 1). Thus, at death, their body weight did not reflect their age. To account for this difference, the weight of the bleomycin-injected rabbits at the time of death was computed from the following expression:

$$W_f = W_i + (0.065 + 0.007 \cdot D + 0.0002 \cdot D^2) \cdot W_i \quad (3)$$

where  $W_f$  and  $W_i$  are the final and initial weights (kg), respectively, and  $D$  is the number of days from the onset of injections. This expression was calculated by a nonlinear least-squares fit of the rate of weight change versus time of the saline-injected animals. When the levels of lung hydroxyproline are plotted versus the calculated body weights, the values fall within the normal range (see fig. 3).

The mean lung wet to dry weight value of the bleomycin group was 13% higher than the corresponding saline value. This difference is too small to indicate any significant amount of lung edema.

Histologically, the lungs of bleomycin and saline-injected animals were similar in appearance. The capillary bed was uncongested, the alveolar septae were thin, and there was no significant amount of lung edema, excess collagen, or increased cellularity.

The mean survival times of rabbits exposed to 100% O<sub>2</sub> were  $81 \pm 4.3$  h (mean  $\pm$  1 SEM; range 68–95 h) and  $78 \pm 5$  h (range 66–96 h) for the bleomycin and saline groups, respectively. Mean values for arterial gas tensions, pH, and hematocrit, obtained at different intervals during the oxygen exposure, are shown in table 4. Both the length of survival and the pattern of blood gas changes are similar to those obtained previously in untreated and saline-injected animals.<sup>12,17</sup>

## Discussion

Our data indicate that intravenous injection of 300 units of bleomycin over a period of 3 weeks did not sig-

TABLE 1. Arterial Blood Gases in Control and Bleomycin-treated Rabbits Breathing Air (means  $\pm$  1 SEM)

Group	n	PaO <sub>2</sub> (mmHg)	Paco <sub>2</sub> (mmHg)	pH	BE (mEq/l)	Hct (vol %)
Uninjured controls*	5	84 $\pm$ 1	33 $\pm$ 1	7.48 $\pm$ 0.01	1.5 $\pm$ 1	36 $\pm$ 1
Saline controls†	5	83 $\pm$ 3	31 $\pm$ 1	7.46 $\pm$ 0.01	0 $\pm$ 1	37 $\pm$ 1
Bleomycin†	2	86 $\pm$ 3	32 $\pm$ 2	7.42 $\pm$ 0.02	-4 $\pm$ 1	36 $\pm$ 2

\* Measured just prior to death.

† Measured 21 days after the last injection.

TABLE 2. Variables Calculated from Static Pressure-Volume Curves in Control and Bleomycin-treated Animals (means  $\pm$  1 SEM)

	No. of Measurements	C ml/cmH <sub>2</sub> O	H (cmH <sub>2</sub> O)	TLC (ml)
Saline	3	8.0 $\pm$ 2	6.0 $\pm$ 1	94 $\pm$ 10
Bleomycin	6	12 $\pm$ 2	4.5 $\pm$ 0.5	82 $\pm$ 9.5

C = static lung compliance; H = half-inflation pressure; TLC = total lung capacity.

nificantly alter either the length of survival or the response of rabbits to 100% O<sub>2</sub>. Despite the fact that the dose of bleomycin used in this study (expressed per kilogram of body weight) is much greater than what is normally administered to humans, the level of lung hydroxyproline content, measured three weeks after the last injection, was similar to that found in saline-injected rabbits. This finding, along with the normal arterial blood gas values and lung volumes and the lack of histologic changes at the light microscopic level, are consistent with the notion that no significant amount of lung fibrosis had developed at that time. Larger doses of bleomycin (400-450 units) were associated with a 70-90% mortality rate during the injection period.<sup>1</sup> On the other hand, a single intratracheal injection of this drug (5 U/kg) resulted in the development of pulmonary fibrosis.<sup>17</sup> Thus, our inability to produce significant pulmonary pathology using intravenous injection of bleomycin cannot be dismissed as being due to species differences.

Studies in experimental animals indicate that intravenous or intraperitoneal administration of bleomycin results in only a mild degree of fibrosis. Schlein *et al.*<sup>20</sup> injected mice with 144 U/kg of bleomycin or saline intraperitoneally during a 4-week period, using a protocol similar to ours. Lung hydroxyproline content in bleomycin-treated animals was 20% higher than controls a month after injections were stopped and did not increase any further in subsequent weeks. We believe that this difference is too small to be physiologically significant. Collins *et al.*<sup>21</sup> injected baboons with 280 units of bleomycin intramuscularly over a period of 22 consecutive weeks. Three months after the cessation of treatment, physiologic and histologic measurements were consistent with only minimal amounts of lung fibrosis. Large increases in lung hydroxyproline content have been reported following administration of this agent in amounts that are associated with significant mortality (432 U/kg; Sikic *et al.*<sup>22</sup>).

Bleomycin-induced lung damage in patients has been

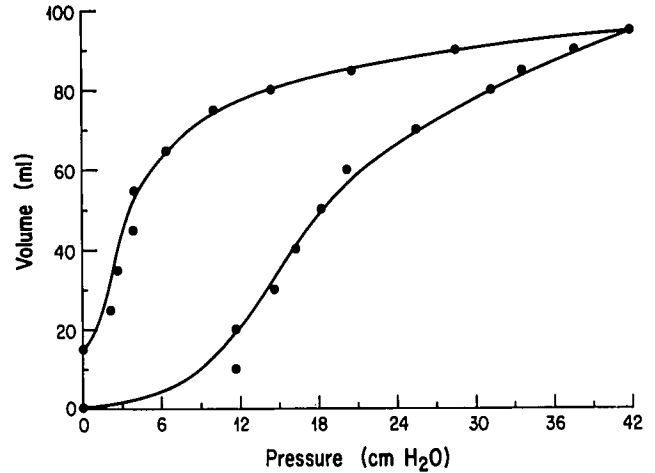


FIG. 2. Inflation and deflation pressure-volume curves for a bleomycin-treated rabbit. The solid lines are the exponential functions fitted through the data (see text for details). The compliance was the slope of the deflation pressure-volume curve in the 20- to 50-ml volume range.

reported following injections of doses higher than 360 U/m<sup>2</sup>. Light microscopy studies of the lungs of patients that received bleomycin (400-1400 U iv) and developed respiratory distress indicated diffuse alveolar damage progressing to interstitial pneumonia.<sup>5</sup> Electron microscopy studies revealed interstitial edema, collagen accumulation, and damage to the type I alveolar cells. The capillary endothelium seemed intact with only minor damage.<sup>3</sup>

There are, however, large variations in the response of patients to bleomycin. Comis *et al.*<sup>23</sup> reported that al-

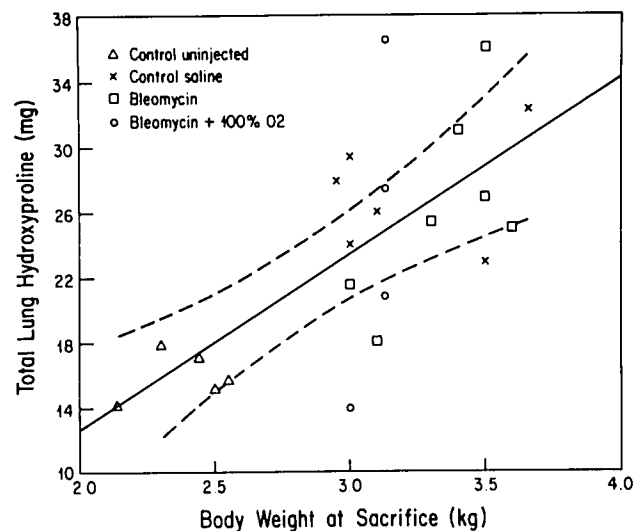


FIG. 3. Lung hydroxyproline versus rabbit body weight at death. The solid line is the best line of fit through the two control groups. [ $Y = -9.1 * X + 10.8 * X$ ;  $r^2 = .74$ ;  $P < 0.01$ ]. For details, see text.

<sup>1</sup> Harper, WV: Bleomycin lung disease in the rabbit and its effects on pulmonary oxygen toxicity (master's thesis). Buffalo, State University of New York, 1983.

TABLE 3. Lung Weights and Hydroxyproline Contents in Control and Bleomycin-treated Animals (means  $\pm$  1 SEM)

Group	n	Bwfin* (kg)	Lung		
			W/D	OH-Pro † (mg/g)	OH-Pro ‡ (mg)
Uninjured controls	5	2.4 $\pm$ 0.07§	5.02 $\pm$ 0.33	4.7 $\pm$ 0.5	16 $\pm$ 0.7§
Saline controls	6	3.21 $\pm$ 0.15	4.62 $\pm$ 0.1	8.8 $\pm$ 0.63	27 $\pm$ 1.43
Experimental	14	3.3 $\pm$ 0.064¶	5.23 $\pm$ 0.22§	8.65 $\pm$ 0.75	26 $\pm$ 2.1

\* Body weight just prior to death.

† Values expressed in mg of hydroxyproline per g of dry lung weight (mg/g).

‡ Total amount of hydroxyproline in lung tissue (mg).

§ Significantly different from the control value ( $P < 0.05$ ) using the unpaired student's *t* test.

¶ Represents calculated value; see text for details.

though one group of patients developed a linear fall in the diffusion capacity of carbon monoxide with the administration of increasing doses of bleomycin, another showed no change in this variable with doses up to 370 units. The reason for this discrepancy is not clear. However, Crooke *et al.*<sup>24</sup> have reported a significant increase in the elimination half-time of this drug when creatinine clearance was below 25–35 ml/min. The increased residence time in the circulation may enhance both the therapeutic and the toxic effects of this agent.<sup>16</sup> Furthermore, Klein and Wilds<sup>4</sup> reported that patients most at risk for the development of pulmonary fibrosis following injection of bleomycin were those who were 65 yr of age or older, those with preexisting lung disease, or those receiving radiotherapy.

The evidence from existing studies indicates that high doses of bleomycin may result in pulmonary fibrosis. This damage, however, may only be seen in the higher risk group mentioned previously. Furthermore, our data indicate a significant correlation between lung hydroxyproline and body weight, which is most likely due to the normal aging process.

The results of our study indicate that pretreatment with intravenous bleomycin does not predispose rabbits to the

toxic effects of oxygen. In this respect, our findings are contrary to the conclusions of Goldiner *et al.*<sup>6</sup> and Nygaard *et al.*<sup>25</sup> but corroborate more recent findings.<sup>9,10</sup> Goldiner *et al.* studied two groups of patients with testicular carcinoma that had received the same amount of bleomycin and undergone the same surgical procedure for the removal of retroperitoneal lymph nodes.<sup>6</sup> The two groups had similar preoperative pulmonary functions but the second group, consisting of five patients, received higher amounts of crystalloids (5.86 *vs.* 3.87 ml  $\cdot$  kg<sup>-1</sup>  $\cdot$  h<sup>-1</sup>) and breathed a higher fraction of inspired oxygen (0.39 *vs.* 0.24) than the first group during surgery. All patients in the second group developed severe respiratory distress 3 to 5 days after the operation and died from pulmonary complications. In contrast, no deaths or pulmonary complications were seen in the first group. The authors concluded that treatment with bleomycin predisposes the lungs to the toxic effects of oxygen. However, interpretation of these results is made difficult by the fact that the pulmonary complications may have been caused, at least in part, by the higher levels of crystalloids administered to the nonsurviving group. Finally, Nygaard *et al.*<sup>25</sup> observed pulmonary complications in patients that underwent radical esophageal resection following a course of

TABLE 4. Serial Arterial Blood Gases in Rabbits That Received 300 Units of Intravenous Bleomycin (Bl) or Saline (Sal) and Exposed to 100% O<sub>2</sub> 3 Weeks after Last Injection (means  $\pm$  1 SEM)

Hours in 100% O <sub>2</sub>		n	Paco <sub>2</sub> (mmHg)	PaO <sub>2</sub> (mmHg)	pH	BE (mEq/l)	Hct (%)
Air	Bl	5	32 $\pm$ 4	85 $\pm$ 4	7.47 $\pm$ 0.01	0 $\pm$ 2	32 $\pm$ 1
	Sal	8	26 $\pm$ 2	84 $\pm$ 6	7.49 $\pm$ 0.01	-2 $\pm$ 1	35 $\pm$ 1
0–40	Bl	5	37 $\pm$ 3	437 $\pm$ 43*	7.45 $\pm$ 0.02	4 $\pm$ 3	34 $\pm$ 2
	Sal	5	33 $\pm$ 1	521 $\pm$ 11*	7.45 $\pm$ 0.00	0 $\pm$ 1	37 $\pm$ 1
40–60	Bl	4	29 $\pm$ 2	453 $\pm$ 24*	7.44 $\pm$ 0.01	-4 $\pm$ 2	35 $\pm$ 4
	Sal	6	24 $\pm$ 2	500 $\pm$ 26*	7.44 $\pm$ 0.01	-6 $\pm$ 2	35 $\pm$ 3
60–80	Bl	4	27 $\pm$ 1	329 $\pm$ 105*	7.35 $\pm$ 0.03	-5 $\pm$ 1	30 $\pm$ 3
	Sal	5	27 $\pm$ 5	284 $\pm$ 118*	7.41 $\pm$ 0.03	-7 $\pm$ 1	35 $\pm$ 2
80–100	Bl	3	41 $\pm$ 3	167 $\pm$ 46*	7.24 $\pm$ 0.02	-10 $\pm$ 2*	29 $\pm$ 4
	Sal	6	42 $\pm$ 3*	118 $\pm$ 29*	7.33 $\pm$ 0.006	-9 $\pm$ 2*	37 $\pm$ 2

BE = base excess; Hct = hematocrit.

\* Significantly different from the air value ( $P < 0.05$ ).

bleomycin treatment. A control group that underwent exploratory surgery without resection after bleomycin treatment showed no abnormalities. The authors concluded that the observed complications were due to the higher degree of surgical trauma and not the oxygen concentration, *per se*.

Bleomycin administration during exposure to oxygen has led to increased mortality in rats.<sup>26</sup> This is due to the increased production of free radicals by these two agents. On the other hand, a single intratracheal injection of bleomycin resulted, 35 days later, in a significant increase of lung superoxide dismutase and catalase levels.<sup>17</sup> Rabbits exposed to 100% O<sub>2</sub> 35 days postinjection survived 50% longer than saline controls.

In conclusion, our animal studies support the findings of LaMantia *et al.*<sup>9</sup> that, in humans, intravenous treatment with bleomycin does not make the pulmonary system more susceptible to the toxic effects of oxygen. Thus, reducing the oxygen concentrations during anesthesia in patients who have received bleomycin may not be of any benefit to them. On the contrary, it may render the patients hypoxemic and result in undesirable systemic side effects.

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