

# Cultured Rat Trachea as a Model for the Study of Ciliary Abundance

## The Effect of Oxygen

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THE mucociliary apparatus is the main defense mechanism preventing respiratory infection,<sup>1</sup> removing inhaled debris and pathogens from the airways.<sup>2</sup> Many critical care patients suffer from ciliary loss,<sup>3</sup> reduced cilia beat frequency, and mucus clearance, which may be related to the high incidence of nosocomial respiratory infections within intensive care. It is often necessary to use an increased fraction of inspired oxygen ( $\text{FiO}_2$ ) in patients receiving intensive therapy. High  $\text{FiO}_2$  induces cough and shortness of breath and decreases vital capacity in humans,<sup>4</sup> and causes pathologic alterations within the lungs of many mammals.<sup>5-8</sup>

Several studies have reported that increased  $\text{FiO}_2$  reduces mucus flow<sup>9,10</sup> and cilia beat frequency and can lead to ciliostasis.<sup>11,12</sup> Although oxygen-induced injury in the lung has been morphologically characterized,<sup>5,13</sup> effects of elevated  $\text{FiO}_2$  upon the structure of the mucociliary apparatus have previously been described only qualitatively. These studies suggest that elevated  $\text{FiO}_2$  can cause apical blebbing, increased mucus discharge,<sup>14</sup> and flattening of the epithelium and ciliary denudation.<sup>15</sup> Our study utilized organ culture, in which tissue organization is maintained but systemic influences are eliminated, to investigate the effect of elevated  $\text{FiO}_2$  on ciliary abundance of rat trachea, using a thorough quantitative method.

## Materials and Methods

### Cell Culture

The complete tracheas from 65 male Sprague Dawley rats (240–250 g) were aseptically dissected to produce 390 pairs of matching tracheal half rings. These were placed ciliated surface uppermost, within a cell culture insert upon a 3.0- $\mu\text{m}$ , high-density membrane (Becton Dickinson, Franklin Lakes, NJ). One hundred microliters

of media (M199; Sigma, Poole, United Kingdom) supplemented with penicillin (50 IU/ml), streptomycin (50  $\mu\text{g}/\text{ml}$ ), 10% new born calf serum, and L-glutamine (100  $\mu\text{g}/\text{ml}$ ) was carefully micropipetted around the tissue. A meniscus formed around the tissue, avoiding submersion of the lower ciliated areas while still providing nutrients to the tracheal underside. Supplementary nutrients were absorbed through the insert membrane from the companion plate well below (Becton Dickinson), which contained 1 ml of additional media. The companion plates were placed within specially constructed airtight chambers, with a culture tray in each chamber containing 50 ml of distilled water to maintain humidity. Chambers were flooded with 21, 40, 60, or 100% oxygen, (balance nitrogen), sealed and placed within an incubator set at 37°C. The experiment was repeated at least 6 times for all of the elevated oxygen groups (40, 60, and 100%  $\text{O}_2$ ) and 13 times for the 21% oxygen control group. Media and gas mixture were changed with daily sample collection.

### Scanning Electron Microscopy

After collection, a researcher blinded to the incubation conditions fixed the samples in 2.5% glutaraldehyde, and after dehydration in ethanol, they were dried of liquid carbon dioxide by critical point drying.

The dried specimens were mounted on aluminum stubs and coated with gold (K550; Emitech, United Kingdom) and examined under a Jeol 6100 scanning electron microscope operated at 10 Kv. The complete mucosal surface of each tracheal half ring was contiguously imaged, and digital pictures were collected by a computer (Semaphore; JEOL, United Kingdom) at 500 $\times$  magnification, avoiding areas of mechanical damage.

The images were analyzed using standard graphics software (Adobe PhotoShop v.5.0; Adobe Systems, San Jose, CA) utilizing a series of in built filters and automated actions that can distinguish between the ciliated areas and the background mucosa. This produced a binary image, and through use of the histogram option, which provides a count of black and white pixels, percentage ciliary cover was calculated. The abundance of the matching tracheal half rings was averaged to account for natural variation in dorsal and ventral ciliary coverage.

### Statistical Analysis

Differences in ciliary abundance of the four groups were analyzed for each day using a Kruskal-Wallis test; a

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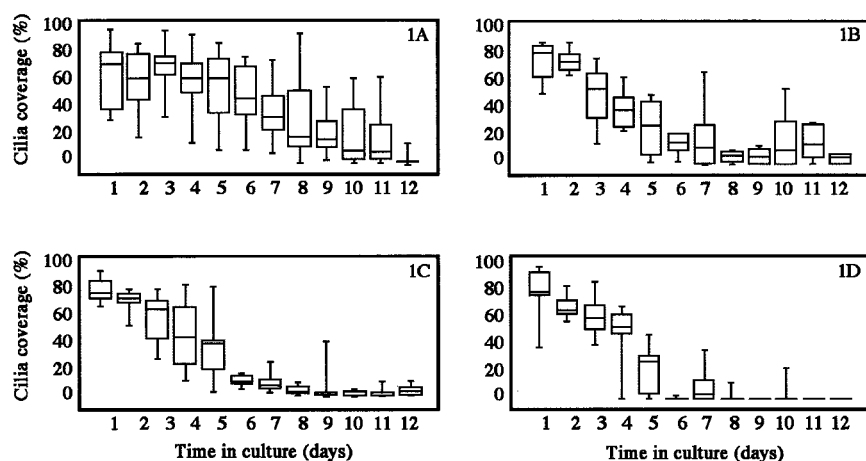


Fig. 1. Box plots to show the effect of (A) 21% O<sub>2</sub>, (B) 40% O<sub>2</sub>, (C) 60% O<sub>2</sub>, and (D) 100% O<sub>2</sub> on ciliary abundance of cultured rat trachea throughout the 12-day experiment.

P value of less than 0.05 was considered to be statistically significant. A follow-up analysis using a multiple range test was used to determine between which groups differences occurred.

## Results

The changes in ciliary abundance over time of the four groups—21, 40, 60, and 100% oxygen—are summarized as box-and-whisker plots in figure 1.

The box represents the interquartile range of the data, which encloses the median, represented by a horizontal line. The graphs show natural variation in ciliary abundance within the first days of culture. The ciliary abundance of the samples decreased over time in all four groups, with marked differences in the rate of decline between the 21% oxygen and the three elevated oxygen groups (40, 60, 100% O<sub>2</sub>).

From day 4 of culture, there was a statistically significant difference ( $P < 0.05$ , Kruskal-Wallis test) developed between the four groups (21, 40, 60, and 100% O<sub>2</sub>), becoming highly significant ( $P < 0.01$ ) from day 5 to the end of the trial. From day 6, the medians of the groups were observed to be within the expected dose-response order. For days 6–12 of the study, median percentage ciliary abundance was calculated for each group. These

values show decreasing ciliary abundance with elevated levels of oxygen (table 1).

## Discussion

The significance of this study, in comparison to previous research, is the robustness of the methodology used. Previous reports have relied upon unblinded, rough grading of ciliary abundance leading to inaccurate measurement and the possibility of observer bias. We overcame this problem by blinding the laboratory researcher to sample identity and by development of an accurate computer-assisted method of quantifying ciliary abundance (fig. 2). The use of computer selection of ciliated and nonciliated areas and calculation of the overall abundance remove any potential problems associated with observer bias.

Using these methods, this study shows a statistically significant relationship between increased oxygen concentration and ciliary loss in cultured rat trachea. A possible mechanism to explain this oxygen toxicity is increased production of reactive oxygen species by the epithelial cells during hyperoxia. Reactive oxygen species, which include the superoxide radical, hydrogen peroxide, and the hydroxyl radical, are mainly derived from a univalent sequential reduction of molecular oxygen. Mitochondria are the main location of intracellular production, which may also result from autooxidation of small molecules or the function of some enzymes.<sup>16</sup>

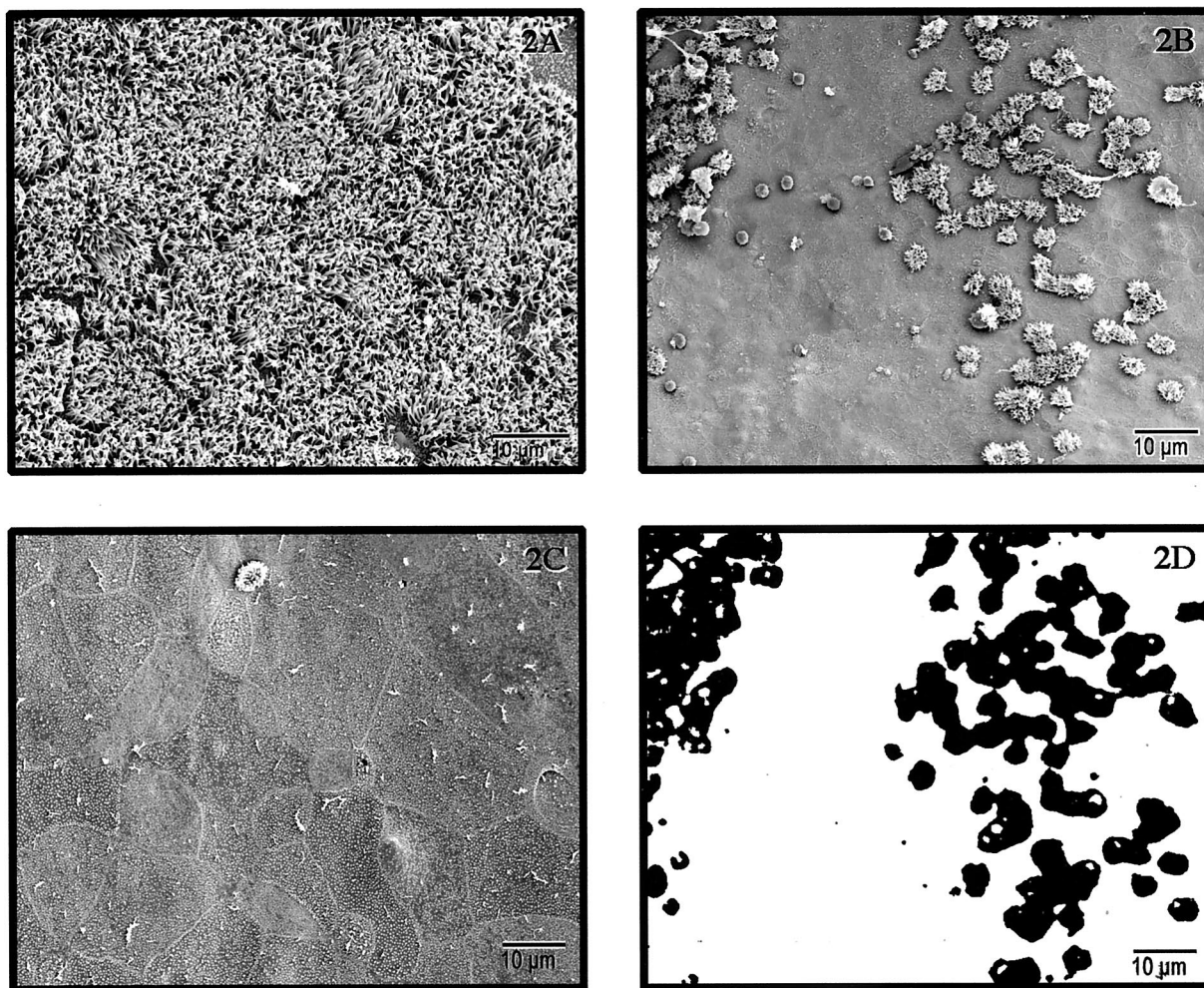
The ciliated epithelium of the upper respiratory tract contains a large number of mitochondria close to the ciliated surface, making it prone to reactive oxygen species production and consequent damage. The new methods developed in this study to culture ciliated epithelium and to measure ciliary abundance should allow future research to assess more accurately potential therapeutic antioxidants and help develop an increased understanding of the process of oxygen stress.

Table 1. Median Percentage Ciliary Abundance of Tracheal Cultures

Group	Ciliary Abundance for Days 6–12	
	Median	90% Reference Range
Air (21% oxygen)	27%	2–70%
40% oxygen	8%	0–31%
60% oxygen	3%	0–15%
100% oxygen	0%	0–15%

Median percentage ciliary abundance is shown for days 6–12 of the study. Reference ranges were calculated from 0.05 and 0.95 percentiles of the data.





**Fig. 2.** Micrographs showing ciliary abundance of rat trachea at day 0 (A), trachea cultured for 6 days in 21% oxygen (B) and 100% oxygen (C), and a binary image (D) produced by PhotoShop from the above micrograph.

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