EFFECT OF TOBACCO SMOKE ON CILIATED LUNG EPITHELIUM OF *RANA PIPIENS*[1]

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ABSTRACT

KIKTA, MARILYN COLLINS, JOHN M. HOLSER AND MILTON A. LESSLER. Effect of Tobacco Smoke on Ciliated Lung Epithelium of *Rana pipiens*. Ohio J. Sci. 76(1): 27, 1976.

The effects of cigarette smoke and other related ciliary toxicants, i.e., tar and nicotine, on ciliated epithelium of lung tissue from the frog, *Rana pipiens*, were studied. It was found that after solubilization of filtered cigarette smoke, by bubbling through a frog-Ringer solution, the ciliary beat and oxidative metabolism of the lung tissue decreased an average of 35% and 37%, respectively. The smoke from filtered cigarettes decreased ciliary beat 11% less than that of unfiltered cigarettes. Smoke first passed through an Aquafilter was shown to have less ciliotoxic effect with a reduction of only 12% in rate of ciliary beat as compared to the untreated control. It also was shown that cigar smoke and pipe tar residues inhibited ciliary beat to about the same degree as 0.1% nitocine.

Cilia lining the respiratory tract serve as a protective mechanism by cleaning the airways of foreign material. Foreign particles and soluble substances are adsorbed on the mucus which covers the cilia. Beating in a concerted manner, the cilia move the mucus, along with the entrapped material, towards the oral cavity. It has been found that most cilia beat between 600 and 2400 times per minute and the energy source for this is ATP (Satir, 1974).

Studies have shown that tobacco smoke contains many H_2O soluble components that are ciliotoxic (Walker and Kieffer, 1966; Weiss and Weiss, 1967). One would expect the water soluble components to affect the cilia because, in solution, ciliotoxic substances can diffuse through the protective mucus coat (Wynder and Hoffman, 1967). Our work, was concerned with those soluble components of smoke contained in a solution through which smoke had been bubbled. Included in this suspension would be some of the gas and particulate phase. The latter two have been shown to have ciliotoxic elements (Weiss and Weiss, 1967; Gray and Kennedy, 1974). Of the hundreds of smoke components, those most frequently studied are nicotine, tar, cyanide, phenols, acids and aldehydes. The mode of action of toxic products seems to involve a toxic effect on the mitochondria, which decreases the ATP production required for ciliary movement (Satir, 1974). Kennedy and Elliot (1970) correlated ciliotoxicity with mitochondrial alterations and found general disruption of internal mitochondrial structure associated with the particulate phase of cigarette smoke. Based on their evidence, ciliated tissues treated with soluble components of cigarette smoke should exhibit reduced oxidative activities. Our studies were designed to assay oxidative activity and ciliary rate effects of the soluble constituents of smoke derived from different tobacco sources.

METHODS

Ciliated lung epithelial tissue was obtained from the frog, *Rana pipiens*. The animals were double pithed, the lungs excised, dissected, and placed in a dish of ice cold frog-Ringer solution. Thin slices of lung were cut off with a sharp razor blade, and mounted inner surface up on a clean slide. Each slice of lung tissue was placed in a large drop of frog-Ringer solution (pH 7.4) containing 112.1 mM NaCl, 0.8 mM $CaCl_2$, 2.4 mM $NaHCO_3$, 0.1 mM NaH_2PO_4, 1.9 mM KCl, and 5.5 mM glucose. The preparations where kept viable by regularly replenishing the solution under the cover slip. The lung tissue and solutions were kept on ice to preclude any loss of activity and were exposed to room temperature only during the test period.

An optical system was set up so that the

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light emitted by a calibrated Strobatac (General Radio, Model 1532A) was projected though a Beseler condesnor lens and focused by a plano-convex lens onto the mirror of a Nikon binocular microscope. Thinly sliced lung tissue wet-mounted in frog-Ringer solution, was observed at 400×magnification, using the stroboscope as a light source. Exposure of the tissue slices to the various experimental agents was accomplished by adding drops of the solution to one side of the coverslip and then drawing the frog-Ringer solution to the other side of the coverslip with bibulous paper. One experimenter would slowly adjust the speed of the Strobotac while the other experimenter observed the cilia. The two then changed positions for a second determination. When approximately 90% of the cilia were bent in one direction and showed minimal movement, they were considered stopped. The strobe reading at this point was recorded. This procedure was done with the tissue in untreated frog-Ringer solution as a control, followed by lung tissue from the same frog after exposure to the various smoke-saturated solutions. Determinations were obtained by taking readings 3, 6 and 9 minutes after addition of the test compound. Experimental bias was reduced by not allowing the two independent observers to see the Strobotac dial. The ciliary beating rates prior to and after exposure to the potential ciliotoxic agents were compared by *t*-test.

It has been determined that the volume of smoke inhaled in an average cigarette puff is 40 ml (Frances *et al.*, 1970). Our smoking machine was devised from an ordinary kitchen roast-baster to simulate this volume. A total of 10 puffs were bubbled through 10 ml of frog-Ringer solution from each of the following smoke sources: pipe, cigar, cigarette with a cellulose acetate filter, filterless cigarette of the same brand, and filter cigarette with an Aquafilter (Aquafilter Corp.). A tar solution was obtained by collecting the damp residue (0.98 mg) from several pipes and mixing with 1 ml of frog-Ringer solution. A 0.1% nicotine solution in frog-Ringer was prepared and applied directly to ciliated lung epithelium in several experiments.

Oxidative activity was studied using a micromodification of the YSI Model 53 Biological Oxygen Monitor and a Moseley 680 potentiometric recorder. Pieces of lung tissue were minced and the oxidative activity of nine paired samples of tissue from 7 frogs was measured for fifteen minutes (at 32°C) in both frog-Ringer solution and in frog-Ringer solution through which filtered cigarette smoke had been bubbled. After the oxygen uptake measurement, each sample was carefully pipetted into a tared weighing dish, dried for 24 hrs at 110°C, cooled in a desiccator, and weighed on a semimicro balance. The oxygen uptake of each sample was calculated in μl O_2/g dry wt/hr, by using straight line segments of the oxygen uptake versus time record, as described by Lessler and Brierley (1969). Love's (1924) tables were used to determine the significance of the difference between control and experimental oxygen uptake and ciliary rate data.

RESULTS AND DISCUSSION

The average ciliary beating rate of untreated lung tissue from 5 individual control frogs was 1222 ± 50 (mean $\pm$ SD) beats/min (fig. 1). The lung tissue exposed to pipe or cigar smoke-treated frog-Ringer solution averaged 836 ± 74 and 803 ± 44 beats/min, respectively, a reduction of approximately one third from the mean value obtained for untreated tissue. The lung tissue exposed to unfiltered cigarette smoke-treated frog-Ringer solution had an average ciliary beat of only 657 ± 78 beats/min, a 46% decrease below the mean of the untreated control lung tissue. Cilia exposed to frog-Ringer solution treated with filtered cigarette smoke had an average beat of 790 ± 53 beats/min only a 35% decrease in activity. Tissue exposed to frog-Ringer solution treated with smoke from Aquafiltered cigarettes had an average ciliary beat of 1075 ± 32 beats/min, a much smaller decrease (12%) compared to the untreated tissue. Slowing of ciliary beat with all treatments was found to be highly significant, ($P < 0.001$) when compared to control values. The average change in ciliary rate in duplicate determinations done during the 9 minute experimental period ranged from 0 to 5%.

The mean oxygen consumption of 9 individual samples of lung tissue in untreated frog-Ringer solution (control), was 1753 ± 139 μl O_2/g dry wt/hr (table 1). Nine pieces of tissue in the same solution treated with filtered cigarette smoke had an average O_2 consumption of 1107 ± 120 μl O_2/g dry wt/hr. This difference, as calculated from 9 paired determinations, was significant at the 95% level.

In most cases it was not possible to observe all the cilia completely stopped using the Strobotac as a microscope illuminator (Dalhamn, 1956). Apparently some cilia beat at different rates and exposure to the treatment solutions cause the cilia to lose coordination (Iravani and Melville, 1974). In our experiments we chose the point of least movement, where alteration of the strobe rate by 100 in either direction caused little or no change in ciliary movement. Outside this range,

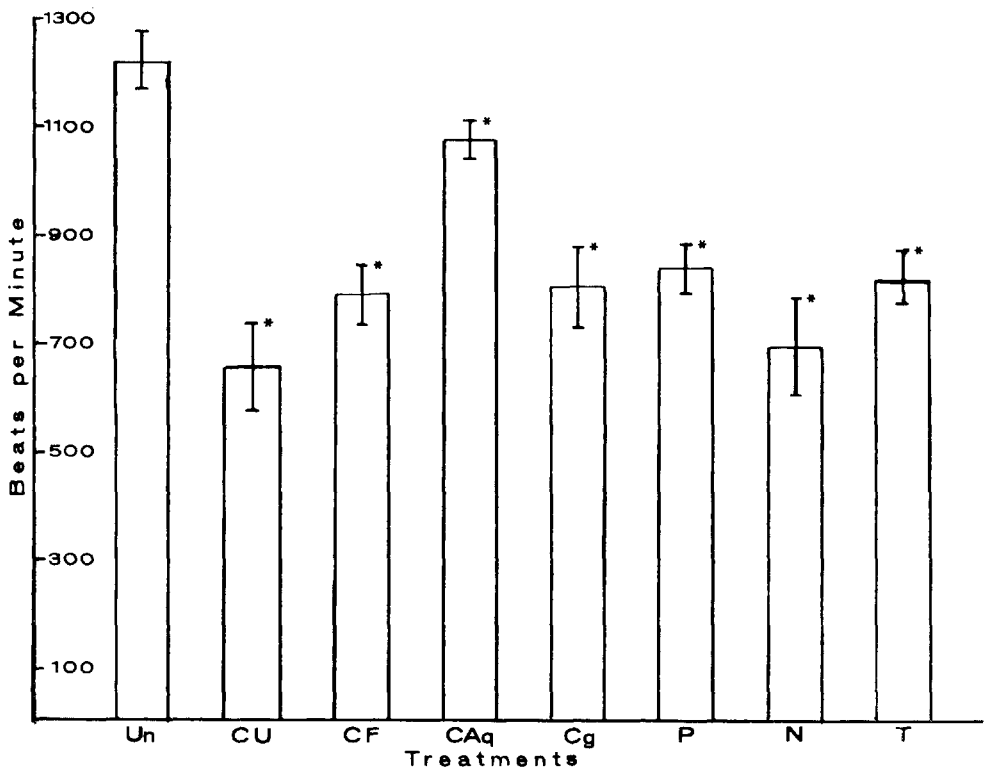

FIGURE 1. Ciliary rate of untreated and treated frog lung tissue. Bars indicate standard deviation of the mean. Asterisks indicate significant decrease below untreated rate ($P < .001$). Un = untreated; CU = cigarette, unfiltered; CF = cigarette, filtered; CAq = cigarette, with Aquafilter; Cg = cigar; P = pipe; N = 0.1% nicotine; T = tar.

TABLE 1
Oxygen uptake of lung tissue exposed to filtered cigarette smoke treated frog-Ringer solution.

Control	Smoke-treated
μl O_2/g/hr	μl O_2/g/hr
2431	1731
1612	1107
1424	1451
2106	1503
1010	1068
2100	814
1604	606
2040	730
1448	949
1753 ± 139*	1107 ± 120**

*Mean ± standard error of the mean.
**Statistically different from control ($P < 0.05$).

however, the ciliary movement would increase greatly. The middle point of this range was chosen as the record point for each determination.

Variables are inherent in any biological system because of small differences in treatment solutions, changes in temperature, length of exposure, and normal animal to animal variations. Replication of experiments appears to be the best, if not the only method one can employ to reduce variables inherent in ciliary studies (Donnelly *et al.*, 1974). Each of our experiments was repeated twice in two different years during which animal to animal variations were observed but the general results remained consistent in that cigarette, pipe, or cigar smoke tended to materially reduce the observed rate of ciliary beat.

The soluble components of cigarette

smoke were apparently toxic to lung epithelium of *Rana pipiens*, because significant changes in ciliary beating rate were always noted on the addition of smoke to the suspending medium. Pipe, cigar, and cigarette tobacco smoke showed similar ciliotoxic effects; unfiltered cigarette smoke depressed the ciliary beat more than cigar, pipe, or filtered cigarette smoke (fig. 1). Cytotoxic effects on cilia were expected since pipe and cigar tobaccos are similar to cigarette tobacco except for the addition of sweeteners, flavorings, and scents (Wynder and Hoffman, 1967). Since cigar and pipe smoke are usually not inhaled, their actual effect on lung cilia may be less than the effect of cigarette smoke which is inhaled. Our results comparing filtered and unfiltered cigarette smoke confirm the findings of Dalhamn (1964) and others who showed a decrease in ciliotoxicity with filtered smoke compared with unfiltered smoke. Our data indicates that unfiltered smoke was 11% more ciliotoxic than filtered smoke, indicating that the dry cellulose-acetate filter removed some of the soluble components. Wynder and Hoffman (1967) reported that water moistened cellulose-acetate filters were more effective than dry filters. This finding is in agreement with our results which showed that an Aquafilter was 20 to 25% more effective than the dry cellulose-acetate filter. The Aquafilter apparently reduced the soluble components of smoke, as indicated by the relatively small change (12%) in ciliary beat noted (fig. 1).

The tar and nicotine solutions, in the concentrations used, also showed significant ciliotoxicity (fig. 1). Rakieten *et al.* (1952) showed that 2% nicotine stopped cilia in rat respiratory epithelium in 5–10 minutes. Our results showed a reduction of 43% in ciliary beat using 0.1% nicotine (less than the amount reported for an individual cigarette) and a 33% decrease in ciliary beat for the tar containing solution. These decreases in ciliary beat were similar to those found for the treatment solutions from the various smoke sources and may indicate that these compounds (tar and nicotine) are the major ciliostatic agents for frog lung epithelium. Meyer *et al.* (1971) found that nicotine is very soluble in membrane lipid and we suggest that this may partly explain its ciliostatic effect.

There was a mean decrease of 45% in oxygen uptake due to the water soluble components of filtered cigarette smoke (table 1). A decrease was expected since smoke components have been shown to cause mitochondrial alterations. Kennedy and Elliot (1970) described changes within the internal mitochondrial membrane and destruction of inner tubular membrane with continued exposure to cigarette smoke. The change in oxygen consumption we observed had a relatively large standard error, as expected from this type of experiment, but there was a significant difference ($P<0.05$) between experimental and control tissue (table 1). In our results, decreased oxygen consumption was invariably accompanied by a decrease in ciliary rate. We found that unfiltered cigarette smoke caused a complete loss of oxidative activity by the minced lung tissue in two to three minutes. Therefore, oxygen uptake studies of lung epithelium were reported only for filtered smoke preparations and demonstrated that the soluble components of cigarette smoke substantially decreased both the ciliary beating rate and oxidative activity of frog lung tissue.

LITERATURE CITED

Dalhamn, T. 1956. Mucus flow and ciliary activity in the trachea of healthy rats and rats exposed to respiratory irritant gases. Acta Physiol. Scand. 36(Suppl. 123): 1–158.

——— 1964. Studies on tracheal ciliary activity. Am. Rev. Resp. Dis. 89: 870–877.

Donnelly, G. M., H. E. McKean, C. S. Heird and J. Green. 1974. Ciliostasis as a bioassay. Arch. Environ. Health 28: 350–355.

Frances, R., D. Allessandro, M. Lippmann, D. E. Proctor and R. E. Albert. 1970. Effect of cigarette smoke on particle transport on nasociliary mucosa of donkeys. Arch Environ. Health 21: 25–31.

Gray, J. P. and J. R. Kennedy. 1974. Ultrastructure and physiological effects of non-tobacco cigarettes on *Tetrahymena*. Arch Environ. Health 28: 283–291.

Iravani, J. and G. N. Melville. 1974. Long-term effect of cigarette smoke on mucociliary function in animals. Respiration 31: 358–366.

Kennedy, J. R. and A. M. Elliot. 1970. Cigarette smoke: the effect of residue on mitochondria structure. Science 168: 1097–1098.

Lessler, M. A. and G. P. Brierley. 1969. Oxygen electrode measurements in biochemical analysis. *In* Methods of Biochemical Analysis, (D. Glick, editor) 17: 1–29.

Love, H. H. 1924. A modification of Student's table for use in interpreting experimental results. J. Am. Soc. Agron. 16: 68–73.

Meyer, D. H., C. D. Cross, A. B. Ibrahim and M. G. Mustaja. 1971. Nicotine effects on alveolar macrophage respiration and adenosine triphosphatose activity. Arch. Environ. Health 22: 362–365.

Rakieten, N., M. L. Rakieten, D. Feldman and M. J. Boykin, Jr. 1952. Mammalian ciliated respiratory epithelium. AMA Arch. Otolaryngol. 56: 494.

Satir, P. 1974. How cilia move. Sci. Amer. 231: 44–52.

Walker, T. R. and J. E. Kieffer. 1966. Ciliostatic components in the gas phase of cigarette smoke. Science 153: 1248–1250.

Weiss, W. and W. A. Weiss. 1967. The gas phase toxicity of cigarette smoke for paramecium. Arch Environ. Health 14: 682–686.

Wynder, E. L. and D. Hoffman. 1967. Tobacco and tobacco smoke. Academic Press, New York, (pp. 253–256, 538–545).