# Mild hyperoxia induces moderate pathological alteration in airway epithelium (ultrastructural study)

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**ABSTRACT**: The ultrastructure of the tracheal epithelium in rabbits exposed for 96 hours to 35–37% $O_2$ was studied in our experiments. Due to the influence of mild normobaric hyperoxia, massive differentiation of new secretory elements was initiated and resulted in apparent changes in goblet cells distribution. 60 ± 4% of goblet cells took part in the formation of voluminous intraepithelial mucous glands. Ciliated cells were less damaged than the goblet ones. Tiny signs of pathological alteration of deeper portions of their cytoplasm and apical blebbing accompanied with destruction of some kinocilia were encountered. The ciliary border was slightly impaired. Mild, but significant decrease in the mean number of kinocilia/$\mu m^2$ went along with significant increase in percentage of altered cilia. Among the altered kinocilia, the slightly altered pathological cilia with local swellings of the ciliary membranes or with tiny vacuoles situated in their shafts were the most numerous. Hyperoxia did not influence the process of ciliogenesis in the ciliated cells. As morphological signs of impairment of the vital self-cleaning ability of the airway epithelium, layers of inspissated mucus were encountered in the area of the ciliary border. From morphological point of view, mild hyperoxia caused moderate damage to the airway epithelium.

**Keywords**: trachea; oxygen; ultrastructure; rabbit

A prolonged, slightly increased concentration of $O_2$ is often used in treatment of various respiratory disorders. To our knowledge, the effect of mild hyperoxia on the airway epithelium has not been thoroughly studied. Recently, the reaction of bronchiolar epithelium, especially of the Clara cells, due to the increased $O_2$ concentration was investigated (Johnston *et al*., 1997, 1999; Jean *et al*., 2002). We demonstrated that short exposure to high concentration (90%) of oxygen evoked severe pathological alteration in the tracheal pseudostratified ciliated epithelium. An increase in humidity had only mild effect on the resulting lesion of the epithelium (Konradova *et al*., 1988). We therefore decided to investigate the reaction of the tracheal epithelium exposed to mild hyperoxia for longer period. We performed the experiments in a chamber containing atmosphere with 35–37% $O_2$, with high air humidity and increased temperature 23°C. To demonstrate the isolated effect of normobaric hyperoxia, both untreated animals and also animals exposed to atmosphere with only increased humidity and temperature were used as controls.

## MATERIAL AND METHODS

In our experiments, 9 SPF New Zealand White male rabbits (Charles River Deutschland, Sulzfeld, Germany) of body weight 1 500–3 000 g were used. Three animals served as untreated controls. Three animals (treated controls) were placed for 96 hours in a normobaric chamber containing atmosphere with 100% humidity and temperature 23°C. Three rabbits spent 96 hours under the same conditions, but the atmosphere in the experimental chamber

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was regulated at 35–37% $O_2$ balanced with $N_2$, $CO_2$ was less than 1.5%.

Material for electron microscopic examination was collected immediately after removal of the experimental animals from the chamber. For general anaesthesia, *i.m.* administration of a mixture of ketamine and xylazine according to the certification of the Animals Protection Expert Commission of the Faculty was used. Tiny fragments of the tracheal mucous membrane were removed and processed using standard methods for electron microscopy. The material was fixed for 90 min with 5% glutaraldehyde (Merck, Hohenbrunn bei München, Germany) in cacodylate buffer (pH 7.2) and then for 60 min with 2% $OsO_4$ (JMC, Hertfordshire, United Kingdom) in cacodylate buffer (pH 7.4), dehydrated in graded series of alcohol and embedded in a Durcupan-Epon mixture (Fluka, Buchs, Switzerland). Ultrathin sections were prepared on Ultrotome Nova (LKB, Broma, Sweden), contrasted with uranyl acetate and lead citrate and examined under the JEM 100 C electron microscope (Jeol, Tokyo, Japan).

The ciliary border and the functional state of the goblet cells were evaluated quantitatively. To evaluate the distribution of goblet cells in the epithelium, the isolated elements and the goblet cells arranged in groups were distinguished. The secretory elements were further classified into three categories: (1) mucus-filled, (2) mucus-discharging, and (3) degenerated ones. In untreated controls, in animals exposed only to increased temperature and humidity and in those exposed to normobaric hyperoxia a total of 186, 485 and 330 goblet cells were studied, respectively. In those experimental groups also 1 058 $\mu m^2$, 1 619.5 $\mu m^2$ and 1 789.75 $\mu m^2$ of ciliary border was investigated and 10 252, 8 077 and 13 847 kinocilia were found there, respectively. Four categories of kinocilia were distinguished: (1) intact 9 + 2 cilia, (2) slightly damaged pathological cilia with local swellings of the ciliary membrane or with tiny vacuoles situated in their shafts, (3) degenerating cilia, represented by axonemes incorporated into cytoplasmic blebs or by isolated axonemes, and (4) malformed cilia with either abnormal arrangement or number of microtubules in their axonemes. For statistical evaluation of the ultrastructural findings, relative values of individual categories of goblet cells and of cilia were evaluated by the chi-square test of homogeneity in frequency tables. To specify categories causing deviations from the hypothesis of homogeneity, adjusted standardised deviations were used. Means of cilia/$\mu m^2$ were compared by the one-way analysis of variance (ANOVA). The differences between groups were assessed by the Tukey's test or Bonferroni's method for multiple comparisons. The Levene's test for equal variances was also performed. As a non-parametric analogy of the ANOVA, the Kruskal-Wallis test was used (BMDP New System version 1.0).

## RESULTS

### Tracheal epithelium of untreated control rabbits

In untreated control rabbits, the pseudostratified columnar ciliated epithelium composed of ciliated, goblet, basal cells and a few differentiating elements was observed in their tracheae. In this epithelium, isolated cells of the diffuse neuroendocrine system (DNES) were also encountered. Goblet cells were mostly scattered as isolated elements among the ciliated ones. Only 6 ± 3% of them formed tiny groups, mostly composed of two cells. The proportions of non-stimulated, mucus-discharging and degenerated goblet cells were given in Table 1. Regular ciliary border was developed above the epithelium. The average number of cilia per 1 $\mu m^2$ was 9.7 ± 0.3. The percentage of intact cilia and of individual types of altered cilia illustrates Table 1.

### Tracheal epithelium of rabbits exposed for 96 hours to environment with high temperature and humidity

Due to increase in temperature and humidity, signs of pathological alteration were revealed in the tracheal epithelium, but the intercellular spaces remained narrow and the apical junctional complexes were intact. On the apical portions of the ciliated cells, isolated cytoplasmic blebs were observed. Axonemes of degenerating kinocilia were not encountered inside these cytoplasmic protrusions. Slight increase in number of small to medium-sized vacuoles and of secondary lysosomes, dilatation of the cisternae of granular endoplasmic reticulum, Golgi complex and of perinuclear cisternae were observed in deeper portions of ciliated cells' cytoplasm. Some slightly altered mitochondria were also encountered. Differentiating ciliated cells were not found in the epithelium.

Table 1. Quantitative evaluation of the tracheal goblet cells and ciliary border (CB) of rabbits after 4-day normobaric hyperoxia (relative values)

| | Untreated controls | Treated controls | Hyperoxia |
|---|---|---|---|
| Non-stimulated goblet cells | 97 ± 1% | *4 ± 1% | ⟷ *45 ± 5% |
| Mucus-discharging goblet cells | 3 ± 1% | 4 ± 1% | ⟷ *9 ± 2% |
| Degenerated goblet cells | 0 | *92 ± 1% | ⟷ *46 ± 4% |
| Stimulated goblet cells (total) | 3 ± 1% | *96 ± 1% | ⟷ *55 ± 5% |
| Goblet cells arranged in groups | 6 ± 3% | 13 ± 1% | ⟷ *20 ± 1% |
| Number of cilia per 1 μm$^2$ of CB | 9.7 ± 0.3 | *6.8 ± 0.4 | # 7.7 ± 0.6 |
| Intact cilia | 98.8 ± 0.1% | *96.7 ± 1.3% | *94.9 ± 1.5% |
| Pathological cilia | 0.5 ± 0.2% | *2.4 ± 1.0% | *3.5 ± 1.7% |
| Degenerating cilia | 0.3 ± 0.1% | 0.2 ± 0.1% | 1.1 ± 0.7% |
| Malformed cilia | 0.4 ± 0.2% | 0.7 ± 0.3% | 0.5 ± 0.2% |
| Altered cilia (total) | 1.2 ± 0.1% | *3.3 ± 1.3% | —— *5.1 ± 1.5% |

$n$ = 3, values are expressed as mean ± SD, values designated # differ significantly ($P < 0.05$) from untreated controls, values designated * differ significantly ($P < 0.01$) from untreated controls, values connected by a line differ significantly ($P < 0.05$) from each other, values connected by a double arrow differ significantly ($P < 0.01$) from each other

13 ± 1% of goblet cells were engaged in the formation of small intraepithelial mucous glands. 4 ± 1% of goblet cells were filled with large mucous granules that showed tendency to fuse. No signs of mucus evacuation were observed in those secretory elements. The mucus discharging elements represented 4 ± 1% of goblet cells. Merocrine type of secretion was noticed only exceptionally. Signs of both apocrine secretion and of compound exocytosis were recorded frequently. The exhausted secretory elements prevailed forming 92 ± 1% of goblet cells (Table 1). They gradually degenerated, lost their connections with the basal lamina and appeared in the apical portions of the epithelium. Remnants of their condensed, rather electron dense cytoplasm were frequently observed in the area of altered ciliary border. Only isolated differentiating goblet cells containing small secretory granules of various electron densities were discovered in the epithelium.

In the slightly impaired ciliary border, the mean number of cilia per 1 μm$^2$ decreased to 6.8 ± 0.4. Altered kinocilia represented 3.3 ± 1.3%. The most numerous pathological kinocilia reached 2.4 ± 1.0%, degenerated and malformed cilia 0.2 ± 0.2% and 0.5 ± 0.1%, respectively (Table 1). Remnants of membranes, isolated mucous granules and portions of sloughed off degenerated goblet cells were observed among the kinocilia. In the area of the ciliary border, inspissated secretion was not recorded.

## Tracheal epithelium of rabbits exposed for 96 hours to environment with high temperature and humidity with increased concentration of $O_2$

Tracheae of rabbits exposed to normobaric hyperoxia were lined with an altered pseudostratified ciliated epithelium with narrow intercellular spaces and intact apical junctional complexes.

The ciliated cells revealed only mild signs of pathological alteration. On their apical portions, the process of apical blebbing was recorded. Tiny cytoplasmic blebs were observed on the apical portions of some ciliated cells protruding in the area of the ciliary border. In some of them, a few axonemes of degenerating kinocilia were encountered. The axonemes were either intact or in various stages of disintegration (Figure 1). In deeper portions of ciliated cells' cytoplasm, a slight increase in number of small to medium-sized vacuoles and of secondary lysosomes, slightly dilated cisternae of granular endoplasmic reticulum, Golgi complex and of perinuclear cisternae were recorded. Voluminous intracytoplasmic ciliary vacuoles were frequently

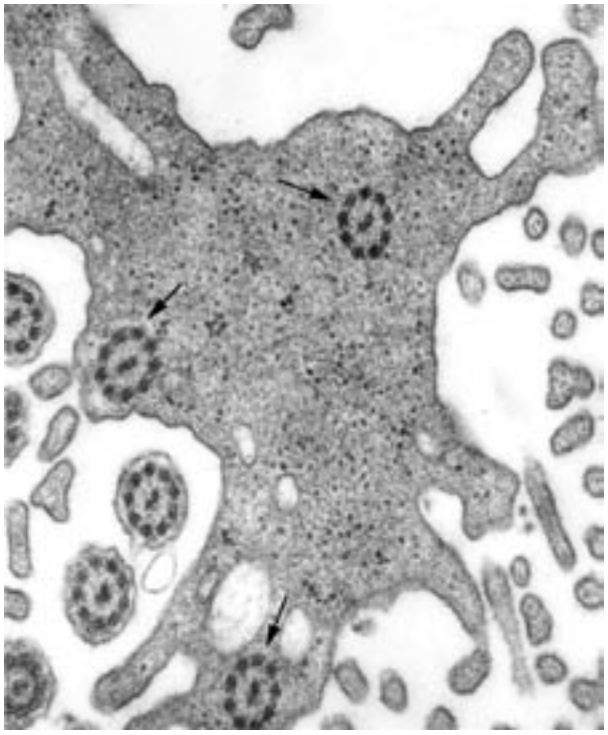

Figure 1. Cytoplasmic protrusion on the apical portions of ciliated cell containing axonemes of degenerating kinocilia (arrows). Rabbit – tracheal epithelium exposed for 96 hours to 35%–37% $O_2$; 50 000×

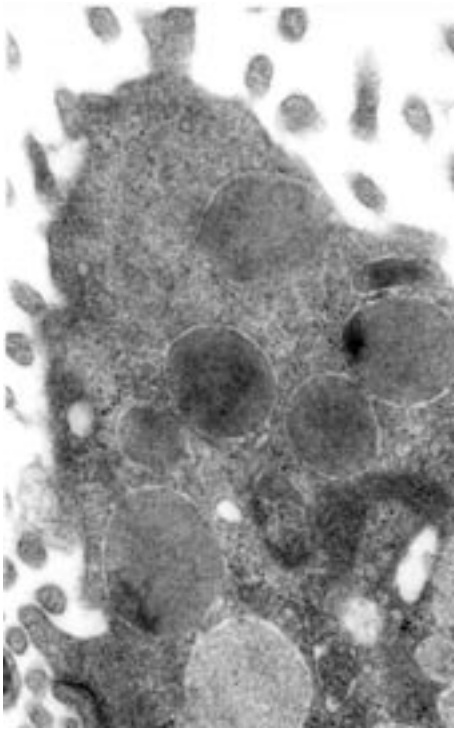

Figure 2. Apical portion of differentiating goblet cell containing isolated small secretory granules of various electron densities. Rabbit – tracheal epithelium exposed for 96 hours to 35%–37% $O_2$; 37 500×

observed in the basal portions of their cytoplasm. As well as in the previous experimental group, differentiating ciliated cells were not recorded.

In the epithelium, 20 ± 1% of goblet cells were arranged in voluminous groups forming thus intraepithelial mucous glands (Table 1). 45 ± 5% of goblet cells did not reveal signs of mucus evacuation. 12 ± 2% of them were filled with large coalescent mucous granules containing light fibrogranular matrix. Among the unstimulated secretory elements, cells containing only isolated secretory granules in their cytoplasm prevailed. They represented 33 ± 4% of goblet cells. In some of them, the tiny granules were highly electron dense, others contained more voluminous granules of various electron densities (Figure 2). Apical portions of cells not entirely filled with mucus protruded above the level of surrounding ciliated cells. Cells filled with small mucous granules separated by voluminous cytoplasmic septa were also encountered in the epithelium.

Goblet cells discharging their secretion represented 9 ± 2% of secretory elements. Evacuation of individual apical mucous granules, detachment of whole packets of granules and also chain exocytosis were encountered.

Exhausted goblet cells were the most numerous in the epithelium. They represented 46 ± 4% of goblet cells. After sloughing off, portions of their degenerated, highly electron dense cytoplasm often appeared above the epithelium (Figure 3).

Regular arrangement of the ciliary border was slightly impaired. The mean number of cilia per 1 $\mu m^2$ decreased to 7.7 ± 0.6, the number of altered elements reached 5.1 ± 1.5%. Among altered kinocilia, the slightly injured pathological cilia were the most numerous (Figure 4). They represented 3.5 ± 1.7% of all kinocilia. The proportions of degenerated and malformed cilia were 1.1 ± 0.7% and 0.5 ± 0.2%, respectively (Table 1).

In some places, the kinocilia were embedded in extensive layers of condensed mucus (Figure 5). In the area of the ciliary border, remnants of membranes, isolated mucous granules, whole apical parts of secretory elements and portions of sloughed off degenerated goblet cells were also encountered.

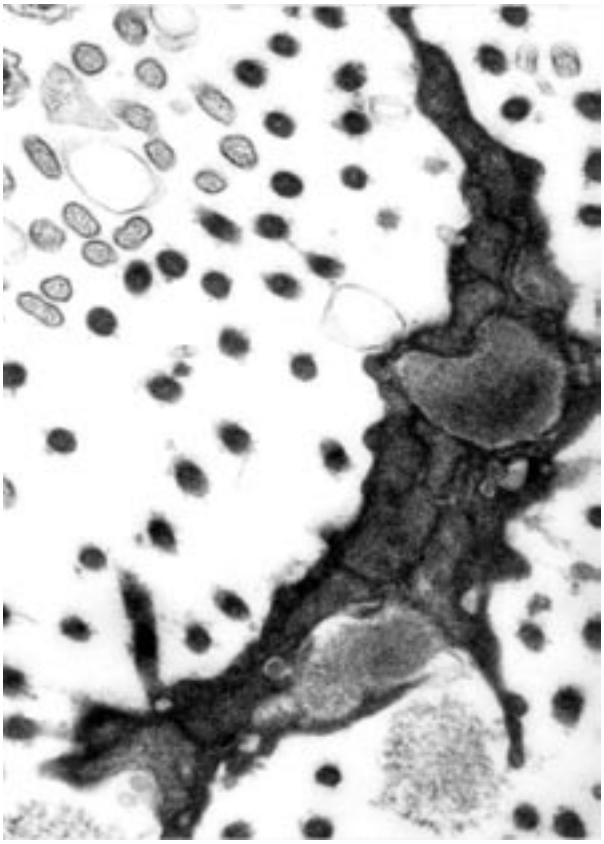

Figure 3. Portion of cytoplasm of sloughed off degenerated goblet cell in the area of ciliary border. Rabbit – tracheal epithelium exposed for 96 hours to 35%–37% $O_2$; 50 000×

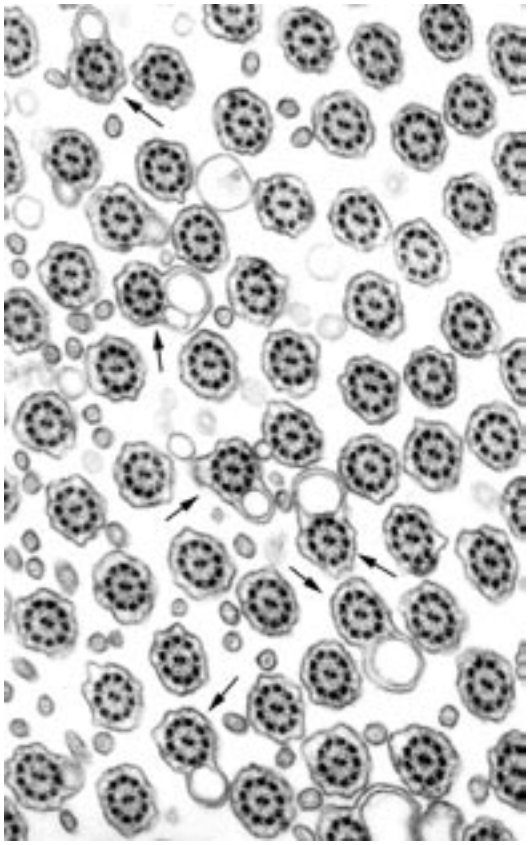

Figure 4. Slightly altered ciliary border containing altered pathological kinocilia with local swellings of their ciliary membrane or with tiny vacuoles in their shafts (arrows). Rabbit – tracheal epithelium exposed for 96 hours to 35%–37% $O_2$; 50 000×

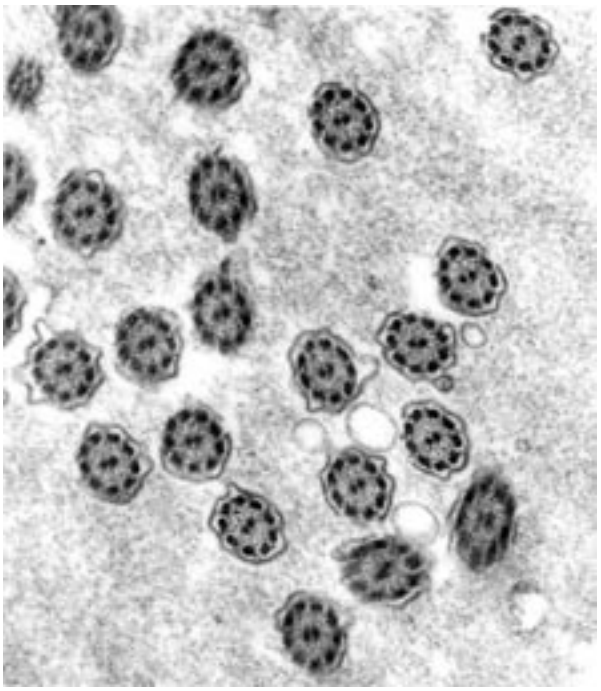

Figure 5. Layer of condensed mucus among kinocilia. Rabbit – tracheal epithelium exposed for 96 hours to 35%–37% $O_2$; 50 000×

## DISCUSSION

After 2-hour exposure to 90% oxygen, the ciliated cells were especially severely injured (Konradova *et al*., 1988). The target cells for the 96-hour action of high temperature, 100% humidity and 35–37% oxygen were the secretory elements in the airway epithelium.

Due to the exposure to atmosphere with increased humidity and temperature, the majority of goblet cells were overstimulated. An increase in number of stimulated cells was highly significant ($P < 0.01$) compared with untreated controls. Mechanism of secretion was accelerated. Signs of both apocrine secretion and of compound exocytosis (Specian and Neutra, 1980; Roumagnac and Laboisse, 1987; Specian and Oliver, 1991; Konradova *et al*., 1996; Newman *et al*., 1996) were frequently recorded. Merocrine type of secretion was noticed only exceptionally. The exhausted secretory elements prevailed in the epithelium. After rapid mucus discharge, the overstimulated goblet cells mostly degenerated and were gradually sloughed off. Remnants of their condensed, highly electron dense cytoplasm were frequently observed among free kinocilia. Compared with untreated controls, the proportion of the degenerated goblet cells differed significantly ($P < 0.01$).

We have demonstrated that high level of stimulation of goblet cells in the tracheal epithelium accompanied with degeneration of about 50% of these secretory elements induced a massive differentiation of new secretory cells (Konradova *et al*., 1990, 1996). As the differentiating goblet cells retained the ability to divide (Becci *et al*., 1978), the result of this process was hyperplasia of secretory elements with changes in their distribution in the epithelium (Konradova *et al*., 1990, 1996). In untreated controls, 6% of goblet cells formed small groups in the epithelium. After 96 hours of exposure to increased temperature and humidity, isolated differentiating goblet cells appeared and incipient changes in goblet cells distribution were recorded in the epithelium. Changes in the secretory cells distribution were still statistically not significant.

In the tracheal epithelium of rabbits exposed to increased temperature, high humidity and 35–37% concentration of $O_2$, acceleration of mucus discharge was also recorded. The percentage of degenerated goblet cells decreased significantly ($P < 0.01$) compared with previous experimental group. They represented less then 50% of all secretory elements in the epithelium. On the other hand, the differentiating goblet cells in various phases of their development represented one third of secretory cells found in the epithelium. Also the changes in the goblet cells' distribution differed significantly ($P < 0.01$) compared with both untreated controls and also with findings in animals exposed to atmosphere with increased humidity and temperature. In the epithelium, rather voluminous intraepithelial mucous glands were encountered.

In our experiment, we thus demonstrated that an increase in $O_2$ concentration accelerated the common reaction of the secretory elements to the injury. After exposure to increased temperature and high humidity, the first phase of common response of goblet cells to injury – represented mostly by degeneration of the exhausted cells – was revealed. After accelerated mucus evacuation, the exhausted goblet cells mostly degenerated. Due to the influence of environment with high temperature, increased humidity and normobaric hyperoxia, the second phase of the goblet cells' reaction was recorded. Massive differentiation of new secretory elements was initiated and resulted in apparent changes in goblet cells distribution accompanied with development of intraepithelial mucous glands. We observed similar reaction in animals exposed to normobaric hypoxia (Konradova *et al*., 2002). Compared with hyperoxia, even more advanced process of secretory cells reaction was encountered. In the epithelium, the most outstanding feature represented voluminous intraepithelial mucous glands in which formation $60 \pm 4\%$ of goblet cells took part.

In contrast to short exposure to 90% oxygen, the ciliated cells were less damaged than the goblet ones after 96-hour exposure to high temperature, humidity and 35–37% oxygen. Due to mild hyperoxia, only tiny signs of pathological alteration of deeper portions of their cytoplasm were noticed, but apical blebbing, accompanied with destruction of some kinocilia, was encountered. The ciliary border was also slightly impaired. Mild, but significant decrease in the mean number of kinocilia/$\mu m^2$ was accompanied by significant increase in percentage of altered cilia compared with both untreated and treated controls. Among the altered kinocilia, the slightly altered pathological cilia with local swellings of the ciliary membranes or with tiny vacuoles situated in their shafts were the most numerous. Low incidence of malformed kinocilia, that did not differ significantly from that encountered in untreated controls, demonstrated that hyperoxia

Table 2. Evaluation of the degree of damage to the airway epithelium of rabbits after 4-day normobaric hyperoxia

| | Control rabbits | Degree of damage | | | Hyperoxia |
|---|---|---|---|---|---|
| | | mild | moderate | severe | |
| | 0 | I | II | III | |
| Stimulated GC | < 3% | 3–50% | 50–90% | > 90% | II (55%) |
| Ratio $\frac{\text{discharging GC}}{\text{degenerated GC}}$ | degenerated GC not found | > 1 | 0.1–1 | < 0.1 | II (0.2) |
| Number of cilia/μm$^2$ | > 9 | 7–9 | 3–7 | < 3 | I (7.7) |
| Altered cilia | <1.2% | 1.2–2.0% | 2.0–10.0% | > 10.0% | II (5.1%) |
| Signs of impairment of the self cleaning ability | 0 | ± | + | ++ | II (+) |

GC = goblet cells

did not influence the process of ciliogenesis in the ciliated cells.

Due to the exposure to mild hyperoxia, morphological signs of impairment of the vital self-cleaning ability of the airway epithelium were recorded (Konradova, 1991; Stratmann *et al*., 1991; Wanner *et al*., 1996; Geiser *et al*., 1997). The disturbance in the mucus flow in airways was responsible for the appearance of layers of inspissated mucus in the area of the ciliary border.

Based on our previous studies, we proposed classification of the airway epithelium injury (Konradova, 1991). We took into consideration the percentage of stimulated goblet cells, the degree of acceleration of mechanism of their secretion, the average number of kinocilia/μm$^2$, the percentage of altered kinocilia and the appearance of the morphological signs of the self-cleaning ability of the airway epithelium. According to this classification, the degree of damage to the tracheal epithelium due to the 96 hours of exposure to mild hyperoxia was moderate (Table 2).

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