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Reduction of atmospheric ammonia (NH_3) and incidence of pulmonary lesions in mice kept in plenum chamber microenvironmental ventilation system

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Introduction

Air movement in closed spaces is a specific process due to its requirements. Often, in some industrial processes, the pollutants generated should be immediately withdrawn by exhaustion ventilation systems. In places where the aim is human comfort, with reduction of the CO_2 pressure and maintenance of the adequate pressure of O_2, the process used is the general diluting ventilation (GDV). This has been used in animal facilities for a long time. This process, however, when used in laboratory animal facilities, especially those for small rodents, may not prove efficient enough. This fact led to the development of technological improvements which resulted in ventilation processes specific for animal facilities. These processes had as their priority the internal environment, that is, the cages where animals were, and may be described as microenvironmental processes. One of the first descriptions of this kind of process was by Lane-Petter *(1970)*, who described a shelf provided with an equipment that enabled the flow of filtered air over the cages. Based upon this kind of consideration, Keller et al. *(1983)* and Wu et al. *(1985)*, in order to decrease NH_3 levels inside mouse cages provided with filter-tops, proposed an individual ventilation system for each cage. This system constituted of plastic tubes for air insufflation, which maintained positive pressure in the microenvironment. Later on, Corning and Lipman *(1991)* verified that the use of filter-tops for the infection control in animal facilities increased the differences between micro- and macroenvironment even more, due to the decrease of ventilation rates inside the cages, which led to accumulation of heat, humidity, ammonia and other gases. After that, Lipman et al. *(1992, 1993)* verified not only the reduction of CO_2 and relative humidity in the systems previously described here, but also the efficiency of the system in preventing the dissemination of MHV-Y virus among mice. In spite of filter-top efficiency in preventing infections, these structures tend to block the flow and cause gases to accumulate, even when the number of air changes are increased, as demonstrated by Reeb et al. *(1997)*. On the other hand, attempts to prevent this effect were presented by Ishii et al. *(1998)*, when they demonstrated that, in cages internally ventilated and provided with filter-tops, there was a slower accumulation and lower levels of ammonia, when compared to cages that were not ventilated internally. Lipman *(1999)* presented an excellent review on the intracage ventilation systems available in the market. All systems presented by this author have secondary ducts to supply the air into and to exhaust it from the cages.

An attempt to simplify the intracage ventilation systems was the substitution of insufflation and exhaustion secondary ducts for plenum chambers, with positive pressure for the insufflation of air into the cages and with negative pressure for the exhaustion of the air from them (*Merusse, 1995*). This plenum chamber microenvironmental ventilation system (MEV) is being tested for its capacity of providing adequate ventilation for laboratory rodents, making maximum use of air

speed in order to remove sensible and latent heat, which may reduce cooling costs (*Teixeira et al., 1999*). Reproductive performance tests have shown that animals kept in this system presented a better performance (*Teixeira et al., 2001*). The objective of the present trial was to quantify the levels of ammonia inside cages, as well as to assess the possible correlation of ammonia levels with the type and frequency of lung lesions in the animals kept under the MEV system and compare the results with those obtained with animals kept under the general diluting ventilation system (GDV).

Material and Methods

Animals

A group of 72 female outbred Swiss mice, (Laboratory Animal Facility of Departamento de Patologia da Faculdade de Medicina Veterinaria e Zootecnia da Universidade de São Paulo) of conventional health status, average individual weight of 26.3±2.3 g, were divided into two groups, one with 40 and the other with 32 animals. The first group was placed in five cages with eight animals each, and kept in the MEV system. The second group was placed in four cages with eight animals each, and kept in the GDV system, and used for the NH_3 measurement. 12 other female outbred Swiss mice, of conventional health status, which were born, weaned and kept in the GDV and MEV systems for 56 days after weaning, were divided into two groups of six animals each and used for the lung histopathology and morphological analyses. Mice were fed a commercial diet (Nuvilab-CR1®, Nuvital Ltda., Curitiba, PR, Brazil) and provided with filtered water *ad libitum*. All animals were kept in bedding made of autoclaved pine shavings (manufactured by J.R. Maravalha® Ltda, Conchal, SP, Brazil) about 20mm deep, with 12-hour light-dark cycle. The group of 12 females used for histopathological analysis was submitted to bedding changes every three days. Room temperature was maintained between 16 and 23°C and relative humidity was 45-60%. These management conditions for the animals were in accordance with the recommendations described in ILAR *(1996)*.

Cages

The experimental group was kept in 20 cages made of 2 mm thick transparent acryl, 30 cm long, 20 cm wide and 12 cm high, a non-commercial model specially built for this trial. The back part of the cages presented 68 holes, each of 7 mm of diameter, which was the exhaustion area of the cages. These cages were provided with special lids made of stainless steel, with room for feed and water. In the front part, there were 50 holes, each of 10 mm of diameter, which was the insufflation area of the cages (*Teixeira et al., 1999*). The control group was kept in 20 propylene cages that presented the same dimensions of experimental group ones, with lids made of stainless steel wire, a commercially standardized cage for mice (Anilab®, São Paulo, SP, Brazil).

Ventilation systems

The experimental group was kept in the MEV system, as described by Teixeira et al. *(1999)*, which was constitued by a shelf for 20 cages, distributed in five levels provided with movable axes that enabled the shelf to be placed in a 45° angle in relation to the horizontal plan. In this position, the insufflation area of the cages was exposed all the time. This shelf was assembled inside a closed cabinet. When the shelf was in the 45° angle, two plenum chambers were formed, one of positive pressure in front of the shelf, which performed the insufflation; and the other of negative pressure, in the back of the shelf, which performed for the exhaustion procedure. The control group was kept in a chamber under the GDV system, in a simulation of a normal room in an animal facility. Cages were kept on a shelf made of pine wood, presenting five levels and insufflation was performed by the roof of the chamber, while exhaustion was performed by two vertical ducts, placed behind the shelf. This chamber was a reduction, in a 3:1 scale, of a standard room in animal facilities and was kept in the same room in which the MEV experimental shelf was placed. Air flow was set at 138 m^3/hour, in accordance to the thermal charge (*Besch, 1985*; *Stoecher and Jones, 1982*). For the MEV system,

flow speed of air on the surface of the lids was at 0.5 m/s (*Teixeira et al., 1999*).

NH_3 measurement

Measurements of NH_3 levels in ppm were made with the Drager® (P.Dattler Ind. Com. Ltda., Barueri, SP, Brazil) gas detection system. In the MEV system, air samples were collected in the discharge area of the cages, using the negative pressure plenum (*Teixeira et al., 1999*). In the GDV group, samples were collected in the volumetric center of the cages. Collection was performed at 24-hour intervals, during nine days, before the daily inspection for water and food. Bedding material was not replaced during these nine days. Results were expressed as average concentration of NH_3 of the five cages in the experimental group, and of the four cages in the control group.

Lung Histopathology

Two groups of six females each were randomly chosen from animals that were born, weaned and kept in the MEV and GDV systems for 56 days after weaning. The animals were killed with an overdose of 3% sodium pentobarbital administered by intraperitoneal injection. Sections of the left apical lung lobes were fixed in 10% formalin for 48 hours and routinely embedded in paraffin. Tissue samples were cut in 5-μm thick, approximately 15 mm large and 15 mm long sections, placed on slides and stained by hematoxylin and eosin (HE). Six samples were collected of each group, and a slide was produced for each animal. These samples were evaluated by two methods: a) score evaluation: three microscopic fields of each slide were analyzed, and results were expressed as the average for the three fields observed. Results for the group were expressed as the average calculated for the six animals. Histopathological criteria: Chronic focal pneumonia, that is, foci of inflammatory cells, predominantly mononuclear cells located in different regions of pulmonary parenchyma; chronic peribronchitis, that is, focal inflammatory processes made up predominantly by mononuclear cells, externally involving bronchi and bronchioles; catarrhal bronchitis, that is, sloughing of intrabronchiolar epithelium with a large quantity of mucus and some sparse inflammatory cells: and chronic interstitial pneumonia, that is, diffuse inflammatory process that affects the interstitial region of the lung and in which there is a predominance of mononuclear inflammatory cells. In this evaluation method, the following scores were used according to the intensity of the lesions: 0 - no lesions; 0.25 - low (lesions up to 25% of the field); 0.75 - moderate (lesions up to 75% of the field) and 1 - intense (lesions in 100% of the field); b) morphometric evaluation: quantitative morphological evaluation of the lung was performed by means of a modified point counting technique *(Macchione, 1995)*, using a coherent set of 100 points and 50 lines, attached to the eyepiece of an optical microscope. At the magnification of 1000x, the number of points hitting the epithelium was determined in 20 randomly selected non-coincident microscopic fields. This number of observations was high enough to keep the coefficient of error under 5%. In the same fields, the number of intercepts of the line system of the eyepiece with the nuclei of the respiratory epithelium was determined, and considered as an estimator of the numerical density of nuclei. Again, this procedure was determined in 20 random non-coincident fields and exhibited a coefficient of error below 5%. The values of the morphometric parameters were averaged within each animal to provide a single data point.

Statistical analysis

Student's t test was used to analyze possible correlations between the mean NH_3 levels and morphometry analyses of the experimental and control groups. The hypothesis established expected different mean values for the groups. In these statistic analysis, the GraphPad Instat (version 3.00) for Windows® was the software employed. Mann-Whitney's U test was used for the histopathological evaluation through scores. In this statistic analysis, the SPSS (version 9.0.1) for Windows® was used. The level of significance (p) was set at $P< 0.05$, for both tests.

Results

NH_3 concentration levels

Daily levels of NH_3 are shown in Table 1. The highest concentration observed in the MEV group occurred on the seventh day, and peak was 5.00 ± 2.79 ppm. On the last day of the experiment (ninth day) levels of 2.00 ± 1.77 ppm were detected. In the GDV system, on the first day, a concentration equal to 1.25 ± 1.44 ppm was recorded and the highest levels occurred on the third and fifth day (31.25 ± 12.50 ppm). NH_3 levels detected during the evaluation were significantly lower in the MEV system compared to the levels found in the GDV system.

Table 1. Concentration of NH_3 (ppm) in the MEV and GDV systems, during nine days, without bed changes. Mean ± standard deviation of five and four cages per system, respectively. N = 8 animals per cage

Systems	Days								
	1	2	3	4	5	6	7	8	9
MEV	ND	ND	ND	1.00 ±1.37	1.02 ±1.37	3.00 ±1.12	5.00 ±2.79	4.00 ±3.79	2.50 ±1.77
GDV	1.25 ±1.44	12.50 ±6.45	31.25 ±12.50	25.00* ±17.32	31.25* ±12.50	28.75* ±14.36	22.50* ±18.90	18.75* ±6.29	13.75* ±4.78

ND - no detection of NH_3;
* - $p<0.05$. Student's t test.

Lung Histopathology

In the score evaluation, the following lesions were mainly observed: chronic focal pneumonia (cfp); chronic peribronchitis (cp); catarrhal bronchitis (cb), and chronic interstitial pneumonia (cip). Intensity of lesions varied according to the ventilation system in which animals were kept (Figure 1). Scores for lesions were significant ($p<0.05$) for cfp, cb, and cip lesions; whereas no significant differences were observed for the cp lesions (Table 2).

The morphological study using morphometric analyses (Table 3), revealed that animals in the GDV system presented a significant increase ($p<0.05$) in the volume fractions of the epithelium (ev), when compared to the animals in the MEV system (24.50 ± 5.60$\mu m^3/\mu m^2$ and 19.70 ± 4.90$\mu m^3/\mu m^2$, respectively). Also, a significant increase in the numerical density of nuclei was found in animals from the GDV system, compared to animals from the MEV system (14.60 ± 3.00 and 10.40 ± 3.00 respectively).

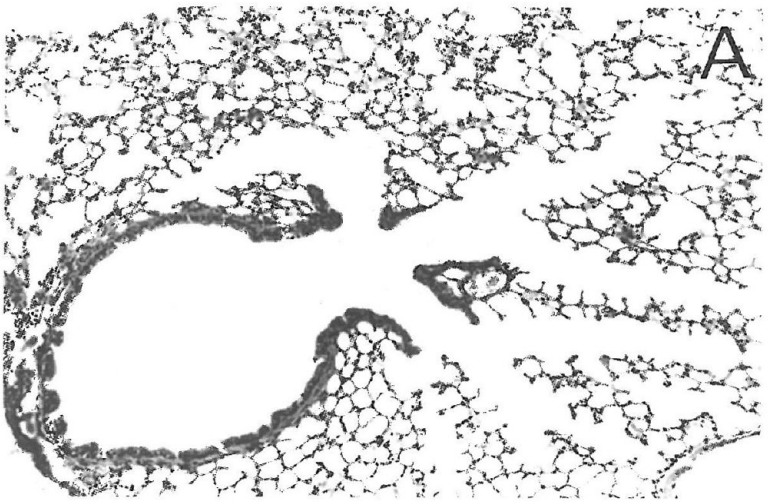
Discussion

Large differences in NH_3 levels between the two systems were recorded. After 24 hours in the GDV system (Table 1), the presence of NH_3 has already been observed, whereas no NH_3 was detected in the first three days in the MEV system. Though the maximum level observed in the MEV system (5.00± 2.79 ppm, on the seventh day) has been reported to cause pathologic lesions in the respiratory system (*Serrano, 1971*), it is believed that the concentration found in this trial was even lower than levels found in other reports (*Gambel and Clough, 1976; Eveleigh, 1993*). Further studies should be performed in order to verify the intensity of lesions in the respiratory system of animals kept under NH_3 levels as low as 5.00± 2.79 ppm.

NH_3 concentration in the cages in the GDV system (Table 1), which is detectable by humans, was

Figure 1. Photomicrographs of histological sections of the lungs of rats kept: (A) in the microenvironmental ventilation system (MEV) bronchiolar tree and pulmonary parenchyma without significant changes; (B) in the general diluting ventilation system (GDV) mononuclear inflammatory infiltrate and bronchiolar epithelium sloughing (larger arrow); several areas of alveolar emphysema (smaller arrow) and focal inflammatory infiltrate composed of mononuclear cells (arrow head). Hematoxylin and eosin.

A



B

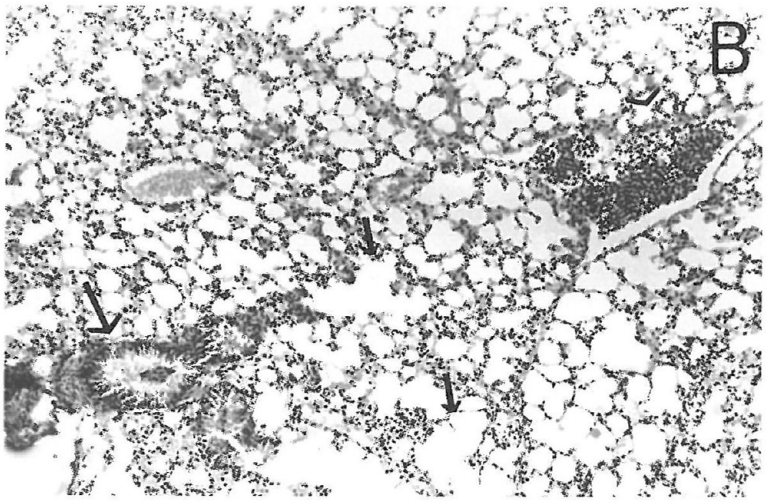


Table 2. Values obtained in the histopathological evaluation of the lung of mice kept under the microenvironmental ventilation system (MEV) and the general diluting ventilation (GDV). N = 6.

Lesions	Systems	Mean±Std Deviation	Median	Percentile 25	Percentile 75	p
Chronic Focal Pneumonia	MEV	0.01±0.03	0.00	0.00	0.00	0.049
	GDV*	0.23±0.22	0.25	0.00	0.33	
Chronic Peribronchitis	MEV	0.07±0.03	0.08	0.08	0.083	0.862
	GDV	0.17±0.24	0.04	0.00	0.33	
Catarrhal Bronchitis	MEV	0.15±0.16	0.12	0.00	0.25	0.007
	GDV*	0.50±0.09	0.50	0.42	0.58	
Chronic Interstitial Pneumonia	MEV	0.15±0.16	0.12	0.00	0.25	0.007
	GDV*	0.50±0.91	0.50	0.42	0.58	

*p < 0.05 Mann-Whitney's U-test

Table 3. Mean ± standard deviation in morphometry analyses of the bronchial epithelium of mice kept in the MEV and GDV systems.

Systems	Volume fractions of the Epithelium #	Numerical density of nuclei §
MEV	19.70±4.90$\mu m^3/\mu m^2$	10.80±3.00
GDV	24.50± 5.60$\mu m^3/\mu m^2$	14.60±3.00*

- volume fractions of the epithelium / μm^2 of basal membrane;
§ - number of intercepts with nuclei / 100μm linear of epithelium;
* - p<0.05. Student's t test.

observed on the second day, and the concentration measured on the third day may cause eye irritation *(Osweiler et al., 1985)*. Moreover, levels below those observed on the third day in the GDV system are related to higher occurrence of *Mycoplasma pulmonis* respiratory infections in rats (*Broderson et al., 1976*). Thus, NH_3 levels observed in the cages of the GDV system are in accordance with what was proposed by Besch *(1985)*, who stated that the use of ventilation speeds over 20 air changes / hour present little contribution to the reduction of the balance between the production and removal of ammonia. As the temperature and humidity levels that are considered comfortable for animals favor the generation of NH_3 *(Gamble and Clough, 1976; Eveleigh, 1993)*, as well as NH_3 concentration is inversely related to the frequency of bed change (*White and Mans, 1984*), the MEV system conciliates comfortable temperatures with low levels of NH_3 inside cages, due to the continuous air flow.

The lower levels of NH_3 observed in the MEV system (Table 1) may explain the differences observed between the groups of animals in the histopathological analyses of the lungs. Animals kept under the GDV system (Table 2 and Figure 1) showed higher incidence of focal pneumonia, chronic interstitial bronchitis, and catarrhal bronchitis when compared to the animals in the MEV system. The most serious case in the lesion found in animals kept under the GDV system may be a result of *M. pulmonis* action, which is frequently associated to Sendai virus, promoting an inflammatory and hyperplastic process in the proximal airways and in the terminal bronchioles,

respectively *(NRC, 1991)*. In the specific case of *M. pulmonis*, the lungs present, grossly, hepatization areas and the airways contain highly viscous exudates. Microscopically, the spectrum of pathologic changes may include suppurative bronchitis, bronchiectasis and alveolitis (*NRC, 1991; Percy and Barthold, 1993*). Natural infection of laboratory rats and mice could seriously impair research efforts investigating a variety of body systems, primarily the respiratory, reproductive, and immune systems (*Baker, 1998*). Evaluation through scores was confirmed by the morphological study, and revealed a significant increase in the volume fractions of the epithelium volume and in the numerical density of nuclei in the bronchi of the animals kept in the GDV system, what is in accordance with previous data obtained by Gamble and Clough *(1976)*, who observed hyperplasia in the trachea of rats. Animals kept in the GDV system presented significantly higher incidence of airway epithelium hyperplasia than the animals kept in the MEV system (Table 3), what may be a consequence of the continuous inhalation of higher concentrations of NH_3

Although several authors report the relationship between exposure to NH_3 and development of several pathological signs *(Serrano, 1971; Gamble and Clough, 1976; Lindsey and Conner, 1978; Cassel et al., 1981; Targowski et al, 1984)*, no data were found to be compared to results presented here, as these reports refer mainly to trials performed under the GDV system. Recent trials, performed in the Laboratório de Ventilação Experimental do Departamento de Patologia [*Experimental Ventilation Laboratory in the Pathology Department*] da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, may be used in the comparison with data presented here. In these trials, the objective was to simplify the MEV technology, with modifications in the design and position of plenum chambers, placing them in a horizontal position, above the cages (MEV-H). Chaguri *(1998)*, in a trial with the MEV-H system, using different air speed levels, observed that the lowest incidence of pulmonary lesions in rats mated and kept in the MEV-H system occurred in those animals submitted to the highest air speed level (0.52 to 0.80 m/s), and that chronic intersticial pneumonia was absent in speed levels over 0.34 m/s. Carissimi *(1998)*, who also worked with the MEV-H system and mean air speed levels equal to 0.54 m/s, in order to verify which was the best interval between bed changes, observed that the group kept in this system presented a lower intensity of pulmonary lesions. This fact was confirmed by the smaller alteration in transepitelial permeability in epiglottis and trachea, when compared to those kept in the GDV system (*Carissimi et al., 2000*).

Although filter-tops were not used in the flow of air into and out of the cages, the plenum chamber system enables this adaptation. The only change to be performed is the use of fans with more pressure capacity.

Low ammonia levels, and the better pulmonary health condition may be corroborated by the histopathological and morphological data described in the present trial, as well as by those found in the trials by Chaguri *(1998)*, Carissimi *(1998)* and Carissimi et al. *(2000)*. These data, together with the observations on reproductive performance of mice kept under the MEV system (*Teixeira et al., 2001*), and rats (*Chaguri et al., 2001*) are an incentive for the improvement of plenum chambers MEV systems and its use in laboratory animal facilities due to the advantages it presents.

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Summary

In the plenum chamber microenvironmental ventilation system (MEV) for laboratory animal housing, air exchanges are made directly inside animal cages. In this study we measured the daily levels of ammonia (NH_3) in cages without bedding changes and made comparative histopathological analyses of mice born and kept in two different systems. Mice were kept under the MEV (n= 40,

in five cages) and general diluting ventilation (GDV) (n= 32, in four cages) systems for nine days. In the MEV system, NH_3 was not detected in the first three days. The highest concentration occurred on the seventh day (5.00 ± 2.90ppm). On the ninth day, a level of 2.50 ± 1.70 ppm was measured. In GDV, NH_3 was detected from the first day, and the highest levels were observed on the third and fifth day (31.20 ± 12.50 ppm), respectively. From the fourth to the ninth day, the GDV system presented higher concentrations of NH_3 than the MEV system ($p< 0.05$). Histopathological analyses of lungs of six female mice from each group were performed after keeping mice in the two systems for 56 days. In the score evaluation, the incidence of chronic focal pneumonia, catarrhal bronchitis, and interstitial pneumonia was significantly higher ($p< 0.05$) in the GDV group. Using morphometry, it was observed that animals from the GDV system showed a significant increase ($p<0.05$) in the volume fractions of the epithelium, when compared to the MEV system (24.50 ± 5.60 $\mu m^3/\mu m^2$ and 19.70 ± 4.90$\mu m^3/\mu m^2$, respectively). An estimator of the numerical density of nuclei over 100 µm of basement membrane was significantly higher ($p<0.05$) in animals from the GDV system, when compared to animals from the MEV system (14.60 ± 3.00 and 10.84 ± 3.00, respectively). It was shown that animals kept in the MEV system presented better health condition than animals kept in the GDV system.

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