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Age and experimental obstructive emphysema. A morphometrical study on the rat

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Summary. Age, as a risk factor in the development of experimental obstructive emphysema, is proposed as the hypothesis of this study.

Ninety-two Wistar rats were organized into two age groups: adult (16 weeks) and middle-aged (56 weeks). Each age group was subdivided into three groups: a control group, consisting of unmanipulated animals; a «cannula» group consisting of animals into whose trachea a cannula was implanted; and a «valve» group, consisting of animals into whose trachea a valve had been implanted. The survival was one month. A histomorphometric study was performed on the lungs and the results were compared statistically. Throughout the experiment the amount of food consumed by each animal and the variations in weight were monitored. After sacrifice, the lungs were processed for light microscopy. Thirteen histomorphometric variables were quantified and subsequently systematized into three groups: those which quantified the size of the distal airspace («area of the alveolar section», «alveolar chord» and «mean linear intercept»); those which quantified the tissue («wall thickness», «tissue density», «internal perimeter of each alveolar section», «internal alveolar perimeter per field» and «alveolar section/section perimeter»); and those which quantified the elastic fibre («elastic fibre area», «elastic fibre perimeter», «elastic fibre area/elastic fibre perimeter», «elastic fibre density» and «elastic fibre density per tissue density»). The results were compared statistically and the sensitivity, specificity and misclassification indices were calculated, as well as the attributable and relative risk.

From the results, it was observed that, in general, the animals of the valve and cannula groups in both age groups displayed a decrease in food intake and a body weight loss. The middle-aged animals were the only group which displayed significant differences in all the morphometric variables except wall thickness, when the cannula and valve groups were compared with the control group. In both the cannula and valve groups, the values of the variables which quantified the distal airspace increased, while the values of the variables which quantified the lung tissue and the elastic fibre decreased. In the manipulated middle-aged group, the attributable risk of developing emphysema was 56.66% and the relative risk 5.55; in the group of manipulated adult animals, the attributable risk was 23.55% and the relative risk 1.66.

The results of this study lead us to propose that the middle-aged rats with experimental airflow obstruction displayed a greater risk of developing emphysema than the adult rats which were subjected to the same procedure.

Key words: Morphometry, Rat, Emphysema, Elastic fibre, Airflow obstruction, Age

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Introduction

Emphysema is defined as a permanent enlargement of the airspace distal to the terminal bronchiole, accompanied by the destruction of their walls and without obvious fibrosis (Snider et al., 1985). Laenec (Boren, 1965) was the first author to propose that obstruction of the airways could cause emphysema. This hypothesis has given rise to a series of experimental studies, the results of which are difficult to compare at present, since the methodology used in them is not described with sufficient precision, or the terminology is ambiguous. We would underline the experiences of Frayser, who implanted a balloon valve in the airway of the dog (Frayser, 1963), Cosio (1979), who introduced small steel balls into the airway of the rabbit, and Reichart and Martin (1984), who reduced the air passage by means of external constriction of the trachea in the cat (Reichart et al., 1975; Reichart and Martin, 1989). The results of the studies carried out with the object of demonstrating the hypothesis of obstruction are very imprecise. The majority of these authors describe alveolar enlargement and, less frequently, tissue destruction. These experiments are not conclusive for the

demonstration of the obstruction hypothesis but neither do they refute it. We believe that one of the keys for the experimental demonstration of the hypothesis that obstruction of the airways can produce emphysema might lie in the age of the animals. All the authors used adult animals. Boren (1965) proposed that emphysema could be related to the ageing process. However, Goldstein (1982) administered elastase to young and old hamsters and found a greater emphysematous lesion in the young animals. This experiment has not been repeated, since all the models of emphysema caused by elastase have been developed on adult animals (Snider et al., 1986; Eidelman et al., 1990; Cantor et al., 1993).

The hypothesis which propose elastic fibre destruction as the cause of emphysema has been demonstrated experimentally in numerous studies, and for this reason the rupture of elastic fibre has been considered as the most important factor in producing emphysema (Boren, 1965; Snider et al., 1986; Eidelman et al., 1990; Cantor et al., 1993). It has been described in studies on humans and on laboratory animals that in emphysema the elastic fibre can undergo qualitative and quantitative variations (Boren, 1965; Niewoehner et al., 1975; Wright and Churg, 1990; Shapiro et al., 1991; Cardoso et al., 1993); the study of the elastic fibre thus being of great importance in all the emphysema studies performed.

In this research, the proposed working hypothesis is that age is a risk factor in the production of experimental emphysema by airflow obstruction. In order to demonstrate this, the airflow was obstructed in animals of different ages; a higher incidence of emphysema should be found in older animals. Emphysema will be considered as having occurred when there are simultaneous signs of distal airspace enlargement and tissue destruction. Moreover, these changes in the alveolar architecture can be accompanied by modifications in the elastic fibre.

Materials and methods

Animals

92 Wistar rats were used. They were disease-free and chosen at random from our animal unit. The animals were divided into two groups: 47 adult animals (mean age 16 weeks ± 6) and 45 middle-aged animals (mean age 56 weeks ± 4). Each age group was subdivided into two groups: 1) unmanipulated control animals (17 adult animals and 14 middle-aged ones); and 2) manipulated animals. The latter were in turn divided into: 2.1) animals into whose trachea a cannula was implanted (14 adult animals and 15 middle-aged ones); and 2.2) animals into whose trachea a valve was implanted (16 adult animals and 16 middle-aged ones).

Methods

The cannula which was implanted consisted of an 8 mm-long segment of a number 14 abbocat in the case of the adult animals and of an 8 mm.-long segment of a number 12 braunula in the case of the middle-aged animals. The valve was made with the same cannulas, into which a thin, malleable, triangular sheet of goretex was fixed. This was held at three points with the aim of limiting the air outflow (Fig. 1).

Intervention: In order to implant the cannula or valve in the trachea, the animals were anaesthetized intraperitoneally with ketamine (50 mg/ml), diazepan (5 mg/ml) and atropine (1 mg/ml) in a solution mixture (0.5-1.5 ml in relation to the body weight). Once the skin had been sectioned and the muscular planes separated, access to the trachea was gained. A longitudinal incision was made from the second sublaryngeal cartilage and the cannula or valve was introduced and later fixed by means of a stitch in the trachea.

Survival: Throughout the duration of the experiment, the animals were housed in cages, with a filter cover in order to avoid contamination. To avoid infection, ciprofloxacine was administered to all the animals in their drinking water (15 mg/Kg body weight per day). The survival time of one month was set at the beginning of the experiment. The food intake of the animals was monitored throughout the experiment. Only the animals which did not die spontaneously were used.

Sacrifice: The animals were anaesthetized with sodium pentobarbital (0.10 mg/gr body weight) intraperitoneally. A thoracotomy was performed and the lungs were washed with saline solution, applied through the right ventricle. Then they were fixed in 10% formalin with a positive transtracheal pressure of 25 cm of H_2O.

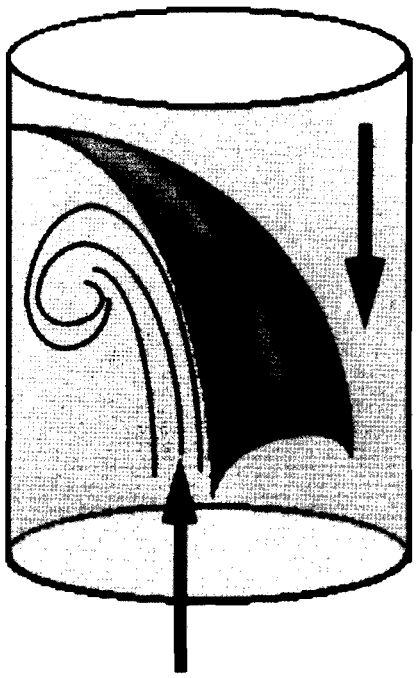

Fig. 1. Schematic representation of the valve model used. The air enters easily in inspiration (descending arrow) and exits during expiration with greater difficulty (ascending arrow).

Histological processing

From each animal, a block which was perpendicular to the main axis was chosen from each lung. In the left lung, the block was taken from the area immediately below the main bronchus where it becomes intra-parenchymatous. In the right lung, it was taken from the basal lobe (Fig. 2). The blocks were dehydrated in alcohols of increasing degree and were embedded in paraffin. 5 µm sections were cut and two sections were studied per block and staining. These were separated by more than 200 µm. Two stainings were used: methylene blue; and Weigert's resorcin-fuchsin. In the case of Weigert's resorcin-fuchsin, background staining was not made.

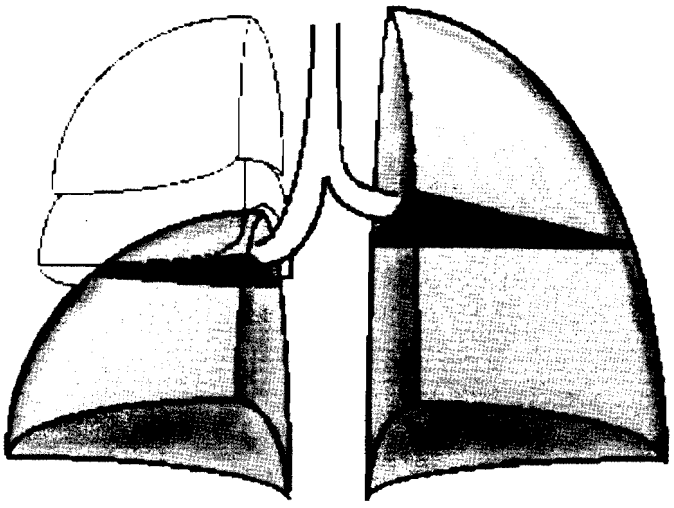

Fig. 2. Schematic representation of the areas of the lung from which the histological sections which were studied were taken. The accessory lobe is not represented.

Analysis

The variables studied were divided into morphometric and non-morphometric variables.

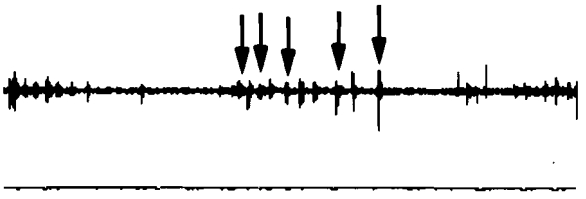

Fig. 3. Graphic recording of the respiratory sound. The lower line is the sound recording of an animal from the adult control group. The upper recording is that of an animal from the middle-aged valve group. The arrows indicate some expiratory sounds.

Fig. 4. Image of a histological field stained with methylene blue, captured by a computer in 256 grey levels 384 x 288 pixels. x 100

Non-morphometric variables:

1. Signs of ventilatory insufficiency: Audible stridor and wheezes, tachypnea, tirage and cyanosis. The respiratory sound emitted by the animals was recorded in the following manner: before the sacrifice, each animal was placed in a hermetic soundproofed chamber in which the respiratory sound was recorded by means of a Macintosh IIcx computer, using the SoundEdit™ programme, which also enabled the sound recording to be visualized (Fig. 3).
2. Food intake of the animals. Grams per day.
3. Variations in weight in the animals. This is the weight in grams lost or gained by the animal from the beginning to the end of the experiment.
4. Cardiac weight. This is described as the relation between the weight of the two ventricles and the weight of the right ventricle.

Morphometric variables:

Pulmonary volume (Pv): This was calculated by the displacement of the water volume.

A morphometric lung study of the histological sections was performed according to the technique already described (Escolar et al., 1994), the process of which was divided into three phases: capture; treatment; and quantification.

Capture: All the sections studied were divided into thirteen areas, of which seven were always chosen (Escolar et al., 1991). From each of these areas a field which had no substantial airway or blood vessel was taken at random; a total of 2,576 fields per staining were studied. The histological fields were captured in 256 grey levels by a computer (Macintosh IIcx). The size of the images captured was of 384 x 288 pixels. The sections stained with methylene blue were captured in one hundred amplifications (Fig. 4) and those which were stained with resorcin-fuchsin in two hundred amplifications (Fig. 5). A Nikon® microscope was used for the capture, together with a Hitachi® camera (Kp-110E/K). The capture programme (QuickImage®) automatically corrected the grey level.

Treatment: In order to transform the images captured in 256 grey levels into binary images, the threshold option was used. The images of the sections stained with methylene blue were processed with dilatation and erosion filters and with the use of the pen and scissors tool options. The resulting image was artefact-free; the tissue corresponded to the colour black and the air to the colour white (Fig. 6). The images of the sections stained with resorcin-fuchsin were not filtered; the colour black corresponded to the elastic fibre and the colour white to the remaining tissue and the air (Fig. 7). The programme used was Image 1.26®.

Quantification: In the images of the sections stained

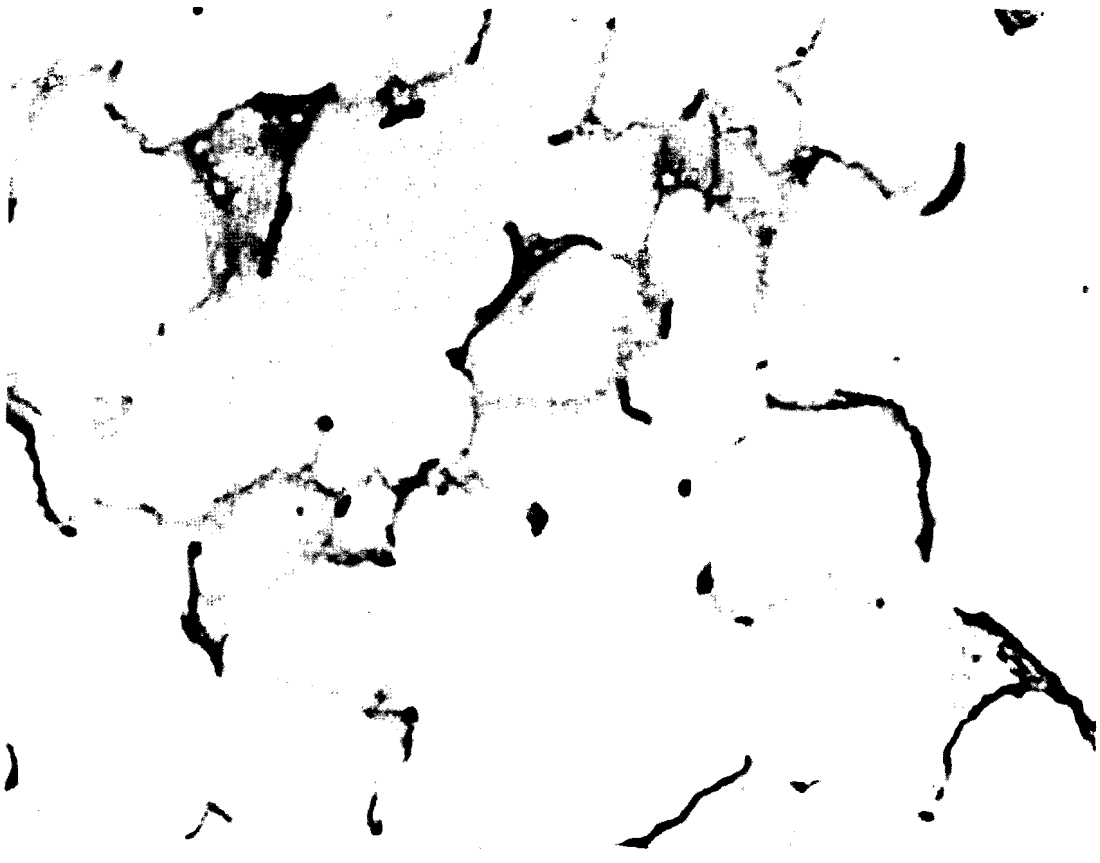

Fig. 5. Image of a histological field stained with resorcin-fuchsin and captured in 256 grey levels 384 x 288 pixels. x 200

with methylene blue (Fig. 6), the following variables, which have been named alveolar architecture variables, were quantified:

1. Alveolar chord: Distance taken at random between the two walls of a single alveolus (Lum et al., 1990; Escolar et al., 1991), expressed in μm.

2. Wall thickness: Expressed in μm.

These two variables were quantified by means of a programme designed by ourselves, according to the criteria of Lum (Lum et al., 1990). For the quantification, the programme drew 192 horizontal, parallel, equidistant lines on which the alveolar chord and the wall thickness were measured.

3. Mean linear intercept (Lm) (Dunnill, 1962): This was deduced by means of the following formula:

$Lm = \sum L/n$; $Lm = (\sum A + \sum W)/n$; $Lm = \bar{X}A + \bar{X}W$.

Where: $\sum L$= total length of all the lines in a microscope field; n= number of measurings; A: alveolar chord; W: wall thickness.

4. Area of the alveolar section (ALVEOLAR SECTION) (Reichart and Martin, 1989): Area of a distal airspace. This is represented in Figure 6 by each surface in white, expressed in μm^2.

5. Tissue density (TD): Tissue area in relation to total field area. The percentage of the colour black (Fig. 6) in relation to the total area of the histological field was calculated.

6. Internal perimeter of each alveolar section (ALVEOL. SECTION PERIMETER): Expressed in μm. In Figure 6 it corresponds to the white/black interphase of each alveolar section.

7. Internal alveolar perimeter per field (IAP): Length of the air/tissue interphase in a histological field, expressed in μm.

8. Alveolar section/alveolar section perimeter (ALVEOL. SECTION/ALVEOL. SECTION PERIMETER): This variable is presented as a geometry factor. The circle is the geometrical figure which occupies the greatest area while at the same time offering the smallest perimeter.

The variables numbered 4, 5, 6, 7 and 8 were quantified with the Image® 1.26 programme.

Using the data obtained by computer and once the lung volume had been established, the following data, which we have called volumetric variables, were obtained.

Lung tissue volume (Tv). This was obtained using the following formula:

$$Tv = \frac{Pv \cdot TD}{100}$$

Internal alveolar surface area (IAS). This variable was calculated by means of two methods:

The method proposed by Dunnill (IASd) (Dunnill, 1962), which is obtained by means of the formula:

$$IASd = \frac{4 \cdot Pv}{Lm}$$

Fig. 6. Figure 4 converted into a binary image and filtered. The quantifications relating to the alveolar architecture were made on this image.

Our own method, which uses the internal alveolar perimeter (IAS*) as a starting point. By multiplying the surface (S) (600 x 450 µm) of each field studied (S) by 5 µm, it was transformed into volume; the internal alveolar perimeter per field (IAP) was transformed into internal alveolar surface per field when multiplied by 5 µm.

$$\frac{0.5 \cdot IAP}{IAS^*} = \frac{0.5 \cdot S}{Pv} \qquad IAS^* = \frac{0.5 \cdot IAP \cdot Pv}{0.5 \cdot S}$$

$$IAS^* = \frac{IAP \cdot Pv}{S}$$

The percentage of the difference which existed between the two IAS was calculated and called «error».

The images taken of the sections stained with resorcin-fuchsin (Fig. 5) were quantified using the programme Image 1.26®. The following variables related with the elastic fibre were quantified (Fig. 7):

1. Elastic fibre area (ELAST. SECTION): Mean of the sections in black. This is expressed in μm^2.
2. Elastic fibre perimeter (ELAST. PERIM): Mean of the perimeters of the sections in black, expressed in µm.
3. Elastic fibre area/Elastic fibre perimeter (ELAST. SECTION/PERIM).
4. Elastic fibre density (EFD): Percentage of black in relation to the total of the histological field.
5. Elastic fibre density per tissue density/100 (EFD/TD).

Statistical study

The values are presented as mean ± one standard deviation. The Kurtosis and Skewness indices were found. When the results were close to normal distribution, they were compared using the Anova and Student t tests. When this was not the case, they were compared using the non-parametric Kruskal Wallis and Mann-Whitney's U tests. Values were considered to be significant when $p<0.05$. The means of the values obtained in each histological section were used for the statistical study (368 sections per staining). The so-called volumetric variables were compared for each case and a correlation study was made between them. In each age group the sensitivity, specificity and overall misclassification indices of the morphometric variables were calculated. The criterion «manipulated» and «unmanipulated» was established as the gold standard. The specificity index corresponded to the proportion of histological sections studied in the control group, whose morphometric results corresponded with those which were expected of their group. The sensitivity index corresponded to the proportion of histological sections studied, in the cannula and valve groups, whose morphometric results corresponded with those which were expected of their groups. The misclassification

Fig. 7. Figure 5 converted into a binary image. The quantifications relating to the elastic fibre were made on this image.

index (or rate) is defined as the proportion of histological sections studied which are incorrectly classified, resulting from the quotient False positives + False negatives/total number of sections.

The threshold or cut-off point to differentiate probable «lung sections of unmanipulated animals» and «lung sections of manipulated animals» was calculated in the following manner (Logstreth et al., 1987; Escolar et al., 1994):

$$\overline{XP} = \frac{\overline{XC} + (\overline{XV} + \overline{XCa})/2}{2}$$

$\overline{XC}$: mean of the values obtained in the control group; $\overline{XV}$: mean of the values obtained in the group of valve animals; $\overline{XCa}$: mean of the values obtained in the group of cannula animals. Emphysematous lungs were considered to be those in which at least three of the variables had deviated from the cut-off point towards emphysema. The three variables taken as a whole should define alveolar enlargement and tissue loss.

In each age group, both the attributable risk and the relative risk of the manipulated animals developing emphysema, together with their confidence intervals (level 95%), were calculated (Fletcher et al., 1988). The significance was found by means of the test χ^2.

Results

The mortality related to the surgical act was 16.77%. 11% of the adult animals upon which surgery was performed died spontaneously between the second and fourth postoperative weeks. None of these animals was included in the study. All the lungs studied were free from inflammatory morphological lesions.

Non-Morphometric variables (Table 1)

Signs of respiratory insufficiency: All the animals on which surgery was performed displayed tachypnea, wheezings, stridor, tirage and cyanosis. All these signs appeared at least one week before the sacrifice. No wheezings sounds could be recorded in the control animals, whereas all the animals which had been operated upon emitted wheezing sounds both on inspiration and expiration (Fig. 3).

Food intake: in the group of treated animals, a decrease in food intake took place, which, with the exception of the group of adult animals with valve, was significant ($p<0.05$) when compared with the control group.

Body weight: The reduction in the food intake was accompanied by a weight loss in the manipulated animals, when these were compared with the animals of the control group. This weight loss was significant ($p<0.05$) except in the case of the adult animals with cannula.

Heart weight: The relation between the weight of the cardiac ventricles and the weight of the right ventricle displayed a decrease in all the animals treated, but this decrease was only significant in the group of middle-aged animals with valves ($p<0.05$).

Table 1. Non-morphometric variables.

	FOOD INTAKE	BODY WEIGHT	VENTRICLES/ RIGHT VENT.
Adult control	17.84±1.21	26.47±23.1	5.18±1.79
Adult cannula	15.73±1.92**	4.29±42.37	4.6±1.55
Adult valve	15.39±3.92	-4.37±34*	4.99±1.95
Middle-aged control	20.48±5.81	6.43±32.61	4.91±0.75
Middle-aged cannula	15.87±5.41*	-54.11±38.13***	4.41±0.62
Middle-aged valve	16.29±4.66*	-56.25±39.26***	4.27±0.58*

Mean ± one standard deviation. The food intake is expressed in grams per day. The body weight are the grams gained (+) or lost (-) by the animal from the beginning of the experiment until the sacrifice. Ventricles/right vent.: relation between the weight of the two cardiac ventricles and the weight of the right ventricle. *: $p<0.05$ when compared with the control group. **: $p<0.01$ when compared with the control group. ***: $p<0.001$ when compared with the control group.

Morphometric variables

The lung volume did not display significant variations in either of the two age groups when the manipulated animals were compared with those of the control group.

Alveolar architecture: From the morphological study of the histological sections stained with methylene blue (Tables 2, 3) we would point out that the majority of the animals which were subjected to manipulation displayed morphological alterations which were qualitatively similar, although only the middle-aged animals displayed differences which were quantitatively significant.

The variables related with the alveolar size: ALVEOLAR SECTION, «alveolar chord» and Lm were found to have increased in all the groups of manipulated animals, but the values were only significant in the middle-aged animals ($p<0.001$). The same applied to the remaining variables. The ALVEOL. SECTION PERIMETER increased significantly in the manipulated middle-aged animals ($p<0.01$). The variables related to

Table 2. Morphometric variables. Adult animals.

	CONTROL	CANNULA	VALVE
Alveolar section	4002±869	4620±1399	4550±1495
Lm	69.20±7.95	71.74±8.88	72.38±12.58
Alveolar chord	54.6±7.97	57.43±9.24	57.6±12.86
Wall thickness	14.6±1.80	14.32±1.15	14.79±1.93
TD	25.23±3.85	23.59±2.81	25.16±5.77
ASP	375±47.36	407±99.45	402±62.39*
IAP	95237±6861	91917±8583	92145±11146
Alv. Sect./ASP	10.56±1.2	11.23±1.52	11.07±2.03

Mean ± one standard deviation. *: $p<0.05$ when compared with the control group. ASP: alveolar section perimeter.

the possible tissue destruction, the IAP and the TD were found to have decreased significantly ($p<0.001$) in the manipulated middle-aged animals; the relation ALVEOL. SECTION/ALVEOL. SECTION PERIMETER displayed a significant increase ($p<0.05$) in the manipulated middle-aged animals; the «alveolar wall thickness» did not undergo significant modifications when compared with the different groups of animals used.

The so-called volumetric variables did not display significant differences in any of the age groups (Tables 4, 5). We would point out that the IASd tended to decrease in the manipulated animals. The result of the IASd displayed very marked differences when compared with the method proposed by ourselves (IAS*). The error varied between 16% in the case of the cannula group of adult animals and -33% in the control group of old animals (check). When the volumetric variables IASd and IAS* were related to one another (Table 6), it was observed that the correlation coefficient was very high (r= 0.911). The IASd obtained by means of the Dunnill method gave very high indices when related with the Pv (r = 0.926). The IAS calculated according to our method was related with the Pv (r=0.906) and the tissue volume (r= 0.864). The IAS error related better with the IAS obtained by Dunnill's method (r=0.9) than by ours (r= 0.645).

The results of quantifying the elastic fibre in the sections stained with resorcin-fuchsin were not found to be uniform in the adult animals. The most interesting of them (Table 7) was the significant decrease ($p<0.01$) in the proportion of EFD in the cannula group (0.94±0.12) when this group was compared with the control group (1.02±0.14) and the valve group (1.06±0.17).

The «elastic fibre perimeter» of the manipulated middle-aged animals (Table 8) decreased significantly ($p<0.001$) in relation to the control group. The values of the remaining variables which quantified the elastic fibre - «elastic fibre area», «EFD», «EFD/TD» and «Elastic

Table 3. Morphometric variables. Middle-aged animals.

	CONTROL	CANNULA	VALVE
Alveolar section	4889±1100	6975±1610***	6821±1144***
Lm	81.38±10.23	94.54±10.26***	94.17±8.64***
Alveolar chord	68.17±10.23	81.19±10.29***	81.06±8.43***
Wall thickness	13.2±1.27	13.35±1.14	13.11±0.85
TD	19.89±2.45	17.55±2.00***	17.23±1.29***
ASP	401.98±49.5	484.89±57.14***	479.24±43.64**
IAP	89633±8232	77978±6379***	78509±5574***
Alv. Sect./ASP	12.06±1.5	14.24±1.6*	14.15±1.23*

Mean ± one standard deviation. *: $p<0.05$ when compared with the control group. **: $p<0.01$ when compared with the control group. ***: $p<0.001$ when compared with the control group.

Table 5. Volumetric variables. Middle-aged animals.

	CONTROL	CANNULA	VALVE
Pv	13.50±2.74	14.14±2.54	13.37±2.12
Tv	2.69±0.58	2.46±0.55	2.30±0.39
IASd	6738±1570	6005±1239	5706±956
IAS*	4492±994	4063±818	3882±617
Error	-33.05±2.01	-32.25±1.68	31.79±1.87

Mean ± one standard deviation.

Table 7. Variables which quantify the elastic fibre. Adult animals.

	CONTROL	CANNULA	VALVE
Elast. section	11.59±1.34	11.43±1.23	11.15±1.56
Elast. perim.	13.49±1.1	13.43±1.03	13.17±1.27
Elast. section/perim.	0.86±0.04	0.85±0.05	0.84±0.05
EFD	1.02±0.14	0.94±0.12**, ##	1.06±0.17
EFD/TD	4.09±0.69	4.07±0.83	4.36±0.85

Mean ± one standard deviation. **: $p<0.01$ when compared with the control group. ##: $p<0.01$ when the cannula group was compared with the valve group.

Table 4. Volumetric variables. Adult animals.

	CONTROL	CANNULA	VALVE
Pv	8.82±1.45	8.86±1.23	9.06±1.18
Tv	2.25±0.53	2.10±0.32	2.27±0.49
IASd	2575±518	2521±380	2566±494
IAS*	3119±579	3004±403	3088±489
Error	17.62±3.3	16.05±6.5	17.34±4.97

Mean ± one standard deviation.

Table 6. Correlation test. Volumetric variables.

VARIABLE	TD	IAP	Lm	Pv	Tv	IASd	IAS*
IAP	0.87						
Lm	0.88	0.96					
Pv	0.57	0.47	0.55				
Tv	0.219	0.255	/	0.66			
IASd	0.52	0.31	0.44	0.93	0.63		
IAS*	0.25	/	/	0.91	0.86	0.91	
Error	-0.67	-0.51	0.62	0.77	0.31	0.9	0.64

The indices which did not reach statistical significance have been excluded.

Table 8. Variables which quantify the elastic fibre. Middle-aged animals.

	CONTROL	CANNULA	VALVE
Elast. section	12.53±1.1	10.69±1.72***	11.50±1.61***, ##
Elast. perim.	14.18±0.83	12.96±1.39***	13.43±1.29***
Elast. section/perim.	0.88±0.03	0.82±0.05*	0.85±0.04*, #
EFD	1.54±0.26	0.95±0.18***	1.12±0.23***, ###
EFD/TD	7.82±1.33	5.43±1.08***	6.51±1.43***, ###

Mean ± one standard deviation. *: $p<0.05$ when compared with the control group. ***: $p<0.001$ when compared with the control group. #: $p<0.05$ when the cannula group was compared with the valve group. ##: $p<0.01$ when the cannula group was compared with the valve group. ###: $p<0.001$ when the cannula group was compared with the valve group.

fibre area/Elastic fibre perimeter» - as well as decreasing significantly in the group of manipulated animals compared with the control group ($p<0.05$), displayed significant differences ($p<0.05$) between the cannula and the valve animals, the values of the latter group being higher.

Sensitivity, specificity and misclassification indices

Since this is an experimental study, we believe that

Table 9. Sensitivity, specificity and misclassification indices obtained in adult animals.

	SPECIFICITY CONTROL	SENSITIVITY CANNULA	SENSITIVITY VALVE	MISCLASSIFICATION
Alveolar section	68.18	51.79	53.12	42.3
Lm	63.64	50	54.69	43.89
Alveolar chord	63.64	46.15	56.25	44.65
Wall thickness	46.97	69.23	54.69	43.03
TD	50	66.07	59.38	41.52
Alv. sect. perim.	65.15	39.29	54.69	46.96
IAP	60.61	50	59.38	43.34
Alv. sect./alv. sect. Perim.	62.12	48.21	56.25	44.47
Pv	58.82	50	37.5	51.22
Tv	52.94	35.75	43.75	55.86
IASd	47.06	57.14	37.5	52.76
IAS*	52.94	57.14	43.75	48.72
Elast. section	52.94	51.79	57.81	45.82
Elast. Perim.	54.41	53.57	51.56	46.82
Elast. section/perim.	53.85	55.36	59.02	43.92
EFD	45.31	72.22	45.9	45.52
EFD/TD	56.06	42.86	51.56	49.69
Emphysema	64.44	58.97	59.26	39.11

The specificity index corresponds to the proportion of histological sections studied in the control group whose morphometric results correspond with those which were expected of their group. The sensitivity index corresponds to the proportion of histological sections studied, in the cannula and valve groups, whose morphometric results correspond with those which were expected of their groups. The misclassification index is the proportion of histological sections in the three groups for which the results fell outside the expected values. Emphysema specificity: percentage of histological sections of the control group whose results for the three variables (area of alveolar section, internal alveolar perimeter per field and TD) are not compatible with emphysema. Emphysema sensitivity: percentage of the sections of the cannula and valve groups whose results were compatible with emphysema.

Table 10. Sensitivity, specificity and misclassification indices of the morphometric results obtained in middle-aged animals.

	SPECIFICITY CONTROL	SENSITIVITY CANNULA	SENSITIVITY VALVE	MISCLASSIFICATION
Alveolar section	82.14	78.33	84.38	18.38
Lm	76.79	76.67	78.12	22.80
Alveolar chord	73.21	76.67	81.25	22.95
Wall thickness	42.86	48.33	53.12	51.90
TD	73.21	75	85.94	21.91
Alv. sect. perim.	83.93	76.67	81.25	19.38
IAP	76.79	90	84.38	16.28
Alv. sect./alv. sect. Perim.	82.14	85	82.81	16.89
Pv	57.14	57.14	43.75	47.32
Tv	64.29	57.14	75	34.52
IASd	57.14	71.43	73.33	32.7
IAS*	57.14	64.29	62.50	38.69
Elast. section	73.21	71.67	56.25	32.96
Elast. Perim.	76.79	71.67	60.94	30.2
Elast. section/perim.	74.07	78.33	53.12	31.49
EFD	82.14	98.33	81.25	12.76
EFD/TD	73.21	90	67.19	23.2
Emphysema	87.5	63.33	75	24.72

The specificity index corresponds to the proportion of histological sections studied in the control group whose morphometric results correspond with those which were expected of their group. The sensitivity index corresponds to the proportion of histological sections studied, in the cannula and valve groups, whose morphometric results correspond with those which were expected of their groups. The misclassification index is the proportion of histological sections in the three groups for which the results fell outside the expected values. Emphysema specificity: percentage of histological sections of the control group whose results for the three variables (area of alveolar section, internal alveolar perimeter per field and TD) are not compatible with emphysema. Emphysema sensitivity: percentage of the sections of the cannula and valve groups whose results were compatible with emphysema.

the most interesting of the three indices is the misclassification index. For purposes of simplification, only the results of the misclassification indices of the middle-aged animals will be exposed here. In Tables 9 and 10 all of the indices of the morphometric variables are shown. Five variables presented misclassification indices of less than 20%: EFD (12.76%), IAP (16.28%), ALVEOL. SECTION/ ALVEOL. SECTION PERIMETER (16.89%), ALVEOLAR SECTION (18.38%) and ALVEOL. SECTION PERIMETER (19.38%). Three variables gave a misclassification index of between 21% and 23%: TD (21.91%), Lm (22.8%) and «alveolar chord» (22.95%). Of the remaining variables, we would point out that the wall thickness gave a very high misclassification index (51.90%).

The presence of emphysema was established when the value of the ALVEOLAR SECTION of a single histological section was greater than the cut-off point and the values of the IAP and TD were smaller. In the group of adult animals, the results were not significant in the variables which define emphysema and for this reason the possibility of the small differences found being due to chance cannot be discarded. In the group of middle-aged animals 63.33% of the sections belonging to cannula animals were classified with emphysema as were 75% of the sections of the valve animals. The specificity index was considerably higher (87.5%) and the misclassification index low (24.72%).

Risk

The attributable risk of developing emphysema after the placing of an intra-tracheal device was 56.66% (confidence intervals 54-58) in the middle-aged group and 23.55% (confidence intervals 14.4-28.5) in the adult group. The relative risk of developing emphysema in the manipulated middle-aged animals was 5.55 (confidence intervals 5.1-2.9) times greater than that of the control animals, while in the adult animals it was only 1.66 (1.06-2.59) times greater. Significant differences according to the χ^2 test were found between the two age groups.

Discussion

Our results lead us to consider that the implantation of a cannula or a valve in the trachea gives rise to alterations of greater intensity in the lung of animals of middle-age than in that of adult animals. However, the clinical manifestations observed in both groups are similar; these clinical manifestations are included among the so-called non-morphometric variables.

Non-morphometric variables

The first question to be solved is whether or not the devices placed in the tracheas of the manipulated animals are capable of producing ventilatory insufficiency. Frayser (1963), after inserting a ball valve in the right lobar bronchus of the dog, detected a low HbO_2 percentage and high carbon dioxide tension in the arterial blood. Eiseman et al. (1959) report that, after inserting a Venturi valve in the dog trachea, alterations which are compatible with emphysema took place. In various experiments, Reichart's research group describes that after stenosizing the trachea of the cat externally, the animal displayed wheezings, tirage and alterations of the gases in the blood (Reichart et al., 1975; Reichart and Martin, 1984). Our results coincide with those described by these authors, since our animals presented wheezings, tirage and cyanosis. These results lead us to consider that the insertion of a cannula or a valve in the trachea of the rat gives rise to the appearance of easily detectable signs of ventilatory insufficiency.

All the treated animals displayed a loss in body weight and/or a decrease in food intake. There is no evidence that these two variables are not interrelated and so we accept that the decrease in food intake is the cause of the weight loss. We consider this to be important for two reasons: 1) The weight loss could be linked to this model of experimental emphysema. The cats with external trachea obstruction studied by Reichart and Martin displayed lower weight than that which corresponded to their age (Reichart and Martin, 1989). We report here that, in a study which preceded this one, a very aggressive surgical technique which separated the trachea from the oesophagus was used to insert the valve in the trachea of 15 rats. The presence of abundant food in the oesophagus of these rats was a frequent finding in the autopsy performed on the animals. This discovery led us to adopt a less aggressive attitude, with the aim of respecting the nervous plexus of the oesophagus. For this reason, only the trachea was manipulated. We believe, that the food intake reduction and the subsequent weight loss are due to the debility generated by the respiratory difficulty and not to the alterations in swallowing originated by the accidental surgical section of any vegetative nerve. In this sense significant weight losses have been described in models of experimental fibrosis (Last et al., 1993). 2) Emphysema which is secondary to malnutrition is an experimental model described in animals, with a loss of over one third of body weight (Sahedjami, 1993). The data presented by other authors in relation with experimental lung fibrosis (Last et al., 1993) or nutritional emphysema (Sahedjami, 1993), together with our own results, lead us to consider that the animals in which experimental pathological models which hamper the respiratory activity are developed can experience body weight loss.

In all the manipulated animal groups the proportion «weight of the cardiac ventricles/weight of the right ventricle» was found to have decreased, but only in one group were significant differences reached. Experimental models of emphysema describe right ventricular hypertrophy after eight weeks of development (Icochea et al., 1982; Martorana et al., 1990).

Morphometric variables

Our results lead us to observe that in the lungs of all the manipulated animals there is a tendency towards an increase in size of the distal airspace, but only the groups of manipulated middle-aged animals displayed significant differences. Significant differences were not found in the values obtained for the group of adult animals. They are, therefore, not going to be discussed, since we cannot discard the possibility of the differences being due to chance.

The majority of obstructive experimental emphysema models have been developed without the use of morphometric methods and only alveolar enlargement and, on rare occasions, alveolar wall rupture have been described in them (Frayser, 1963; Boren, 1965; Reichart et al., 1975; Cosio, 1979; Reichart and Martin, 1984, 1989; Snider et al., 1985). Cosio (1979) demonstrated that in the groups of rabbits in which he had obstructed the small pulmonary airways with small steel balls, the Lm was found to have increased significantly. The Lm is a variable which is only related with the alveolar size (Dunnill, 1962), and so does not provide information regarding lung tissue destruction. Reichart and Martin (1989), after stenosizing the cat trachea, took as emphysematous the lungs in which the increase of the Lm and of the alveolar section was greater than the mean + two standard deviations in relation to the control group (Reichart and Martin, 1989). These authors did not describe any variable related to lung destruction. The only indicator of lung tissue destruction which was proposed was the decrease in the internal alveolar area (Thurlbeck, 1967) and the relation «airspace wall surface area per unit volume of lung tissue» has also been proposed for the study of alveolar enlargement (Gillooly and Lamb, 1993). The fact that the results of obtaining the IAS using two different methods were considerably different lead us, in principle, to doubt the validity of at least one of the methods used. The high correlation index found on relating the two IAS leads us to propose that there is great dependency between the two variables and that a problem of gauging could exist. The calculation of the error percentage is very different, depending on whether we are dealing with the middle-aged group, in which case it is negative, or the adult group, in which it is positive. The latter are the animals which have the least Tv, the least Pv and the smallest alveolar size. The error percentage gave a higher correlation index when related with the IASd (r =0.9) than with IAS* (r=0.64). The IASd is based on shape factors (Weibel and Knight, 1964) which do not coincide exactly with the reality of the lung tissue. The results obtained from the variable «ALVEOL.SECTION/ALVEOL. SECTION PERIMETER» lead us to propose that the alveolar section of the middle-aged animals is more circular in shape than that of the adult animals; we consider that the IASd would be indicated by alveoli with a specific shape, with a sphericity index at an intermediate distance between the two groups of rats studied. The variable «airspace wall surface area per unit volume of lung tissue» proposed by Gillooly and Lamb (1993) is comparable to our IAP variable for the following reason. If the IAP variable and the surface area of a field are each multiplied by the thickness of the section, the first becomes surface and the second, volume. We consider that, although our manipulated animals did not display significant differences in the variables Tv and IAS, our quantification system is very sensitive, since it detected differences with regard to the control group of diseased middle-aged animals with other variables such as the IAP, the decrease in which suggests loss in the alveolar walls (Gillooly and Lamb, 1993).

The morphometric variables quantified in this study are grouped into variables which measure the size of the alveolus and those which are related with possible lung tissue destruction. The values of the variables which measure the size of the alveolus («area of the alveolar section», «alveolar chord» and Lm) showed a significant increase in the groups of manipulated middle-aged animals. From these variables, we have chosen the «area of alveolar section» since its misclassification index is the lowest of the three in this experiment. The variables which measure the degree of lung destruction have different meanings: the decrease in the TD suggests that the tissue has disappeared; the decrease in the «internal alveolar perimeter per field», suggests destruction of alveolar walls (Gillooly and Lamb, 1993). The increase in the relation «alveolar section/alveolar section perimeter» lead us to propose that the section of the distal airspaces of the manipulated middle-aged animals presents a more circular morphology than that of the control group, since the increase of the airspace areas was proportionally greater than the increase of its perimeter; the circle is the geometrical figure which presents the greatest area while at the same time offering the smallest perimeter. The fact that significant differences in the wall thickness variable could not be demonstrated is of great interest as it leads us to consider that the tissue destruction is not due to a progressive thinning of the alveolar wall.

According to our study, 75% of the histological sections of the middle-aged valve animals and 63.33% of those of the middle-aged cannula animals displayed emphysema. We would point out that there was a smaller number of cases of emphysema among the cannula animals and their results were the furthest from those of the control group. Since no significant differences were found between the two groups of manipulated middle-aged animals, we consider that the differences existing between them could be due to chance.

Our method for studying the sample according to histological sections and not in terms of individual animals is similar to that used by Cardoso et al. (1993) on the human. These authors demonstrated that in a single lung different types of emphysema could be found. We are conscious of the fact that the sample of the results obtained from histological sections is four

times greater than the sample obtained from examining animals and that, therefore, the possibility of finding significant differences is also greater. However, the size of the sample has no influence on the specificity and sensitivity indices which were used in this study for the calculation of risk, which was the principal aim of the study. The method used by ourselves for the differentiation of non-emphysematous lung tissue from emphysematous tissue, consisted in establishing the sensitivity and specificity indices, using the threshold option or cut-off point determined from the mean values. This method has been proposed in the clinical study in order to find the cut-off point which would separate healthy groups from diseased ones (Logstreth et al., 1987). Another system for separating the diseased animals from the healthy ones is that used by Reichart and Martin (1989), which establishes diseased animals as being those in which the results are distanced from the mean value of the control group ± two standard deviations. The method used by Reichart and Martin (1989) implies a normality criterion in the distribution of the results (95% of the control group), which means that the use of this method would only be advisable when the distributions of the results were close to normal distribution.

The length of the elastic fibre has been measured by Niewoehner et al. (1975) in the normal and emphysematous human lung, and was found to have been significantly shortened from mild emphysema onwards. Experimentally, in guinea pigs, secondary emphysema to the inhalation of tobacco smoke was developed and significant shortening of the elastic fibre was observed (Wright and Churg, 1990). For Snider (1992) the biochemical studies, in which the elastic fibre in emphysematous lungs has been quantified, are not conclusive. Cardoso et al. (1993) quantified, using radio-immunoassay techniques, desmosine from blocks of human lungs bearing different types of emphysematous lesions. These authors have described significant decreases in the amount of elastic fibre in all the degrees of panacinar emphysema and the severe degree of centriacinar and distalacinar emphysemas. Our results suggest that in the two groups of treated middle-aged animals, the elastic fibre has decreased in size and that it has become thinner and shorter («the elastic fibre area» is smaller and the «elastic fibre area/elastic fibre perimeter» has decreased). As well as the qualitative changes in the elastic fibre, a quantitatively considerable decrease took place in the elastic fibre, which was greater than the decrease of the remaining pulmonary tissue.

The data obtained from the study of the lung architecture suggest that the emphysema which was produced in our animals is not of a severe degree, since the difference between the results of the animals of the control group and those which were manipulated, while significant, was not very striking. The possibility of panacinar emphysema having occurred cannot be verified from our results. Any attempt to homologize our obstructive emphysema model with one of the types of emphysema described in the human would lead us away from the initial objective of the study.

What is striking is that of the two groups of manipulated middle-aged animals, more significant values were found in the variables which define emphysema in the cannula group, and more damage was found to have been done to the elastic fibre. These results, together with the observations of Niewoehner et al. (1975) and Cardoso et al. (1993) in emphysematous human lungs, suggest that the decrease in the elastic fibre could be related to the degree of emphysema.

We wish to state that emphysema was defined (Snider, 1992) in relation with techniques which enabled different variables to be quantified and qualified. At present, morphometric techniques are far more accessible and sensitive, both for the measurement of the alveolar airspace, the lung tissue and the elastic fibre.

The hypothesis of this study proposes age as a risk factor in the genesis of emphysema. Taking our results as a basis, the manipulated animals of the middle-aged group ran an additional risk of developing emphysema (attributable risk) of 56.66%, while the probability of their suffering from it (relative risk) increased 5.55 times in comparison with the group of unmanipulated animals. These data were statistically higher than those obtained for the adult animals, whose results did not show significant differences in comparison with the unmanipulated group. The possibility of the morphological changes detected in the adult animals being due to chance cannot, therefore, be discarded. In conclusion, we propose that in our experimental model of airflow obstruction in the rat, age is a risk factor in the development of emphysema.

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