Ideal transpulmonary pressure for excised lungs. Morphometric study of the rat

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SUMMARY

We carried out this study to propose a transpulmonary pressure (TPP) which will permit the utmost fixation of a lung in which artefacts are produced. 80 rats were used divided into two age groups: adults and older animals. These groups were then organised into four sub-groups according to theTPP used for the fixation: 20, 25, 30, and 35 cm H_2O. They were fixed by airway with formalin at 10%. Lung volume was measured and a descriptive histological and morphometric study was made. The results obtained in the adult animals reveal a direct relation with the TPP. As the TPP was elevated, the pulmonary aerial volume and the internal alveolar surface increased significantly in the lungs fixed at 25, 30, and 35 cm H_2O cm as compared to those fixed at 20 H_2O. The alveolar cord diminished significantly in the three groups fixed at the higher pressure as compared with that of the 20 cm H_2O group. The number of alveoli and the tissue volume increased significantly when the TPP was raised from 20 to 30 cm H_2O and from 30 to 35 cm H_2O. Only the older animals showed significant differences in the alveolar cord when the TPP was raised from 20 to 25 cm H_2O.

We propose that in the adult animals the TPP of 25 cm H_2O is the most adequate for lung fixation; the TPP of 35 cm H_2O is that which most distended the lung. However, the increase in tissue volume suggests the possibility that tissue edema has occurred. In the lungs of older animals, the slight differences found suggest that any of the TPP utilised may be used since the lungs were barely modified by them.

Key Words: Alveolar chord - Internal alveolar surface - Total lung capacity - Alveolar recruitment

INTRODUCTION

The techniques used in morphological fixation are intended to preserve the tissue architecture and tissue elements. Pulmonary fixation is effected by maintaining the lung distended in its "physiological" volume, which, in most cases, is related to the total lung capacity (TLC). The lung is considered to be in the TLC volume when the pressure-volume (PV) curve has reached inflexion in the plateau phase (Gil et al., 1979; Lum and Mitzer, 1985).

It has been proposed that in excised lungs, the adequate transpulmonary pressure (TPP) for reaching the TLC volume is 30 cm H_2O (Glaister et al.,1973). Nevertheless, Lum et al. (1990) consider 30 cm H_2O to be a high TPP. D'Angelo (1972) proposes one somewhat lower: 25 cm H_2O for achieving TLC. Gil et al. (1979) and Bachofen et al. (1982) maintain the lung within a pressure range of 25-30 cm H_2O for achieving TLC, while the range of pressure used by Forrest and Weibel (1975) is lower, 20-25 cm H_2O. Higher transpulmonary pressures have also

been used: 32-36 cm H_2O (Forrest, 1976). These experiments were performed in air-filled lungs.

In most morphological studies, the fixing liquid was introduced through the airway, obtaining a result different from that of air-filled lungs. Actually, experiments performed on lungs filled with liquid, i.e., with physiological serum, have shown the TPP necessary for distending the lung to TLC to be much less: around 10 cm H_2O (Bachofen et al., 1970; Gil et al., 1979). This has been explained as follows: in gas-pulmonary distension different forces are at work, among them, interfacial tensions, while in liquid ones, this activity is minimal, the tissue tensions predominating (Gil et al., 1979). In aerial pulmonary fixation, Lum and Mitzer (1975) have proposed that TLC is achieved with 20 cm H_2O, although fixed with 25 cm H_2O. The different authors consulted used a very wide TPP range for pulmonary fixation by airway, oscillating between 20 to 30 cm H_2O, 25 cm H_2O being the most frequent pressure.

The different TPP used may be related to the species of animal used: dog (Bachofen et al., 1970; Bachofen et al., 1982), monkey (Glaister et al., 1973), gerbil (D'Angelo, 1972), rat (Forrest, 1976; Pinkerton et al., 1982; Cheng et al., 1996), rabbit (D'Angelo, 1972; Gil et al., 1979), guinea pig (Forrest, 1970; Forrest and Weibel, 1975). Nevertheless, it is considered that all species need the same transpulmonary pressure to achieve TLC (Lum and Mitzer, 1985, 1987). In all these experiments the weight of the animal has been taken into consideration, but not the age. We believe age to be a significant factor since the lungs of older animals are more distendable and reach TLC before those of adults (Verbeken et al., 1992).

For a correct morphological study it is necessary that the lung be as distended as much as possible since otherwise tissue folds may form which hide zones of emphysema or can be confused with false zones of inflammation. Following the P-V curve, when the TPP rises, the lung increases its volume and modifies its microscopic structure until it reaches the plateau phase, TLC. From this point on, the increase in TPP does not produce substantial modifications in the lung volume, but at the microscopic level, the lung tissue continues to distend (Assimacopoulos et al., 1976; Lum et al., 1990).

Functional studies have shown how the lung is modified as the TPP varies. However, there are no studies in which a TPP value has been suggested for distending the lung correctly without producing artefacts. Following the most used TPP, in liquid-filled excised lungs, the ideal TPP for fixation should be between 20-30 cm H_2O. This study has as its aim the proposal of an ideal TPP for fixation in lungs excised via the trachea. To achieve this, we used lungs with different distension capacities, adult and older, to which distinct TPP was applied, ranging between 20 and 35 cm H_2O.

Materials and Methods

Eight Fischer rats were used. They were divided into two age groups, 5 and 18 months, which were referred to as adult and older animals, respectively. Each group, in turn, was divided into four groups, each containing ten animals, according to the intratracheal pressure with which lungs were inflated during fixation. Each group contained equal numbers of male and female rats. One month prior to sacrifice, the rats were placed in cages with cover filters.

The animals were placed lying sideways in order to perform a middle thoracotomy incision. The trachea was then cannulated and the lungs were removed from the thoracic cavity. The lungs were fixed with 10% formalin at four different pressures: 20, 25, 30 and 35 cm H_2O. Four similar fixation devices, differing only in the fixing liquid column height, were constructed with the aim of achieving homogenous inflation in all lungs (Fig. 1). After 48 hours' inflation with formalin they were immersed in the same fixing liquid for 15 days.

According to a technique already described (Escolar et al., 1997), three 3 mm-thick blocks —apical, medial and basal— were chosen. 7μm sections were made and stained with methylene blue. Seven zones were systematically chosen from each section and a randomly selected field from each of them was studied. Forty-two fields were studied per animal. A descriptive and morphometric histological study was performed.

Morphometric study: This was performed according to a previously described technique (Escolar et al., 1994). Lung volume was measured by water displacement. For the quantitative study of lung tissue, the process was systematised in three phases: capture; image treatment; and quantification of variables. The images were captured in 256 shades of grey (Figs. 2 and 3). A Nikon® microscope, a Hitachi® videocamera, a Quick-Image® capture card and a Macintosh IIcx® computer with 8 megabytes of RAM were used. Treatment of the images consisted in turning the 256 shades of grey into binary images (Fig. 2). This was done on a Power Macintosh® 6100/60 computer with 16 megabytes of RAM, using the program Image 1.37. The following variables, also described previously (Escolar et al., 1994), were quantified:

Variables quantifying alveolar architecture: obtained from sections stained with methylene blue; planimetric variables.

– Alveolar chord: random distance between two walls of a single distal air space. Expressed in mm.

The following variables were quantified in order to calculate volumetric variables and for this reason the results are not presented:

– Tissue density: percentage of tissue in relation to a studied field.

– Internal alveolar perimeter: length of air/tissue interface.

Volumetric variables:

– Lung volume. Expressed in cm^3.

– Lung tissue volume. Expressed in cm^3 and calculated from tissue density and lung volume (Escolar et al., 1994).

– Lung air space volume. Expressed in cm^3. Obtained by subtracting lung tissue volume from lung volume.

– Total number of air spaces. This is the total number of distal air spaces in the lung. Calculated by dividing lung air space volume by mean air space size. The result was multiplied by 10^{-7}. In order to find the air space volume, a regular dodecahedron alveolar model was employed (Linhartova et al., 1986; Kimmel et al.,

1995; Gil et al., 1979) because it was most similar to the sphere whose radius equals that of the alveolar chord (Escolar et al., 1994).

– Internal alveolar surface. Expressed in m^2. Calculated from lung volume and internal alveolar perimeter (Escolar et al., 1997).

Statistical study: Data are presented as their values ± one standard deviation. When the results approached normal distribution, they were compared using the ANOVA test, and otherwise, with the Kruskall-Wallis non-parametric test. Results in which $p < 0.05$ were considered significant.
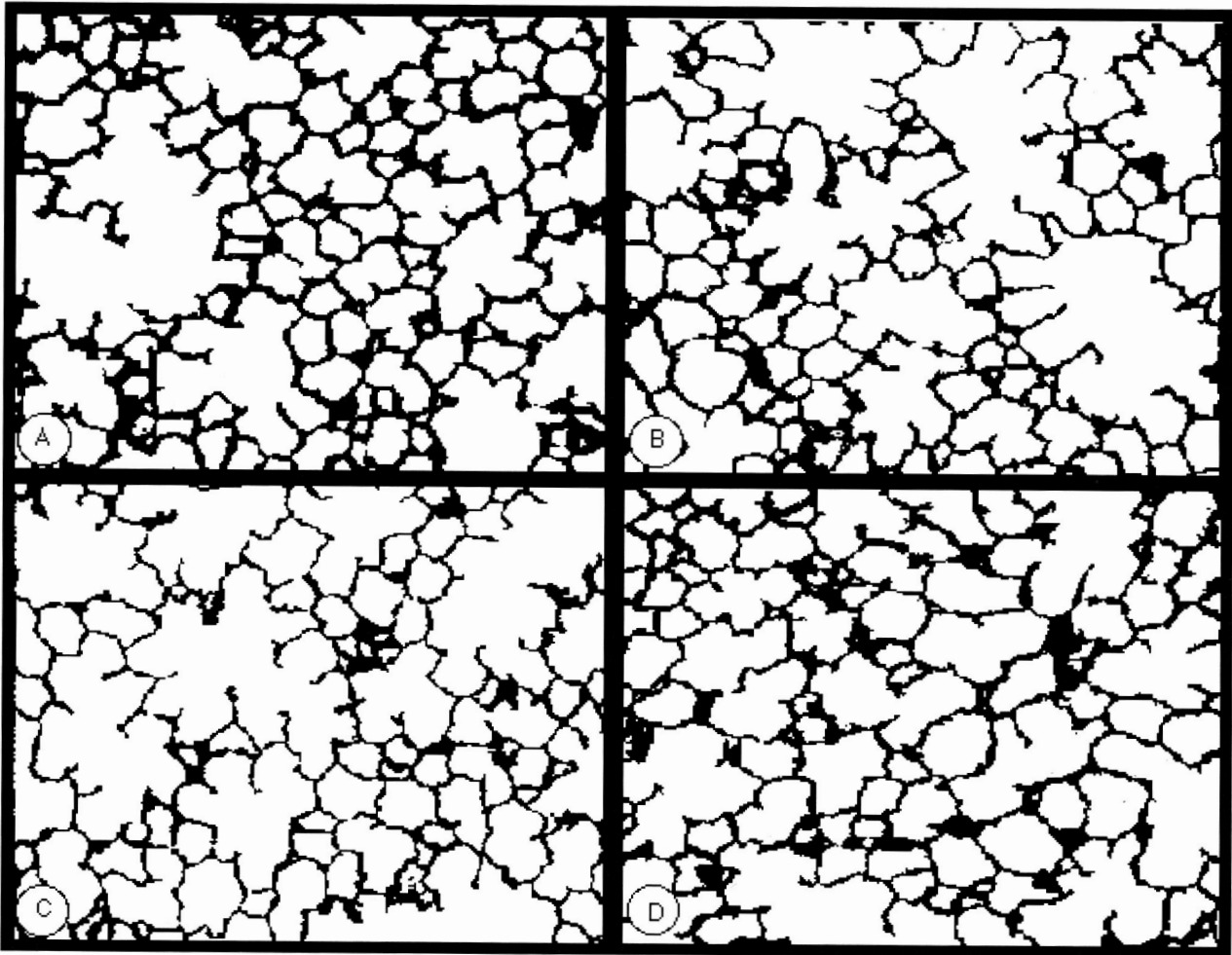
RESULTS

Adult animals

Since we were aware of the difficulties that exist in differentiating, in a histological section, the alveolar ducts, the following systemisation was formed: We noted in lungs fixed at 20 cm
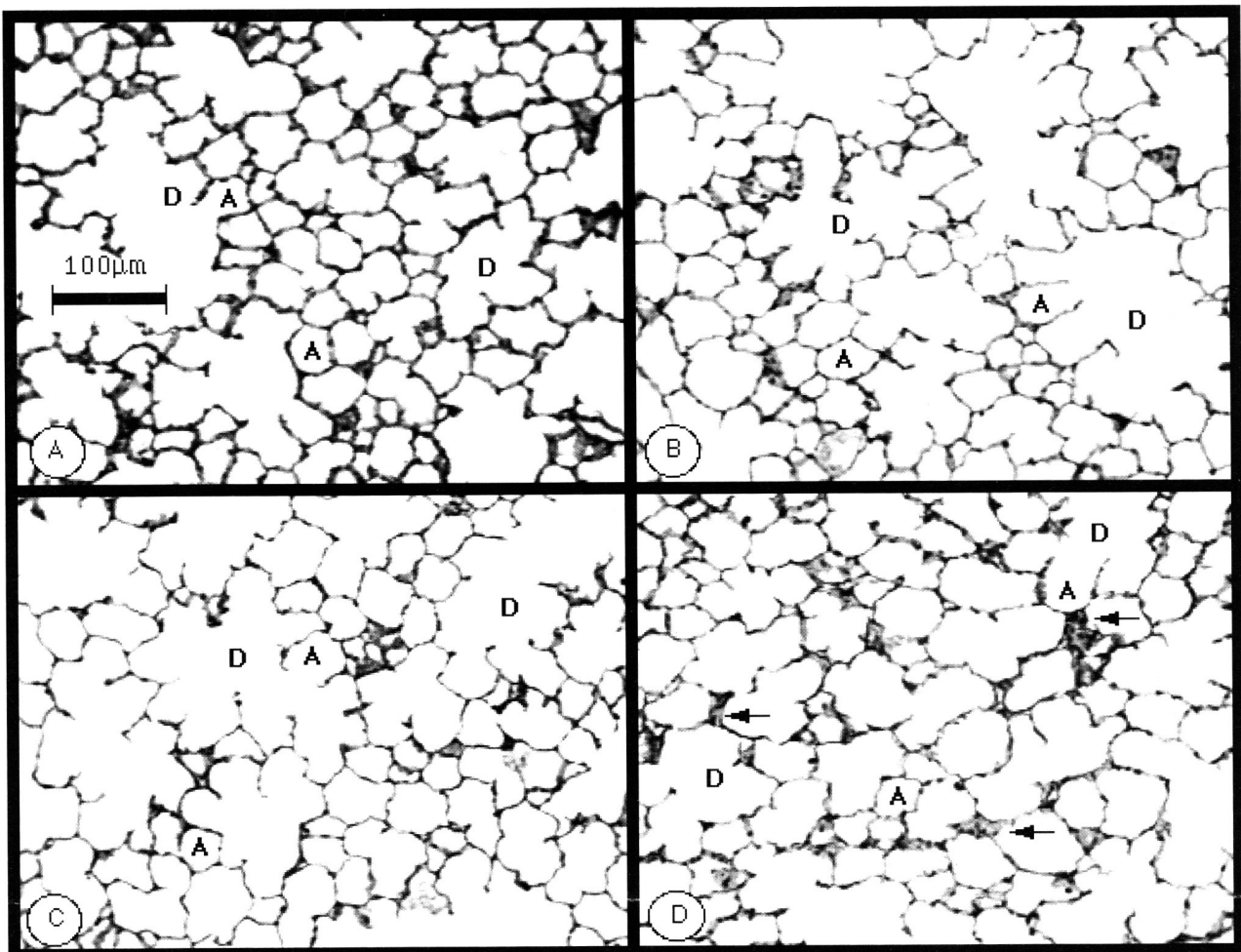
Fig. 1.- Lungs in adult animals. Digitalised images of left medial lung sections, stained with methylene blue. **A**: Lung fixed at 20 cm H_2O. Note the large aerial spaces we have denominated ducts (D) and small aerial space which we have called alveoli (A). Swollen alveolar walls. **B**: Lung fixed at 25 cm H_2O. There is a smaller number of large aerial spaces. **C**: Lung fixed at 30 cm H_2O. The image is very similar to the previous figure; **D**: Lung fixed at 35 cm H_2O. The size of the distal aerial spaces is more uniform than in previous images. The alveolar tissue is swollen in areas of septal intersection (arrows).

Fig. 2.- Binary image resulting from the images in figure 1.

H_2O (Fig. 1A) the presence of large distal aerial spaces, which we denominated ducts; the alveolar ones are smaller aerial distal spaces, and we have divided them into two types: 1) Those that emptied into the ducts, and 2) closed aerial spaces smaller in size. An alveolus could be discovered instead of a duct because the section was tangential to the duct. However, we considered it more probable that it corresponded to an alveolus. The lungs fixed at 25 and 30 cm H_2O (Figs. 1B and C) showed images similar to one another. Compared to the animals fixed at 20 cm H_2O they displayed the following: a reduction in the number of ducts, which showed a larger number of walls of aerial spaces in general. In lungs fixed at 35 cm H_2O (Fig. 1D) the distal aerial spaces showed, in general, a more homogenous size due to the disappearance of the ducts, which were smaller size. The alveolar walls displayed a peculiar morphology since they were imbedded in septal intersection zones (Fig. 1D, arrows).

The results of the morphometric variables, presented in figures 4, 5 and 6, were linearly related to the increase in the TPP since there was an increase in pulmonary volume, pulmonary air

volume, pulmonary tissue volume, the number of alveoli and IAS, while the alveolar cord diminished. The degree of significance reached in each variable were differentiated thus: The increase in lung pressure (Fig. 4) showed significant difference as the TPP increased from 20 to 25 cm H_2O and from 25 to 35 cm H_2O. Lung tissue (Fig. 4) increased significantly from 25 to 30 cm H_2O and from 30 to 35 cm H_2O. The internal alveolar surface (Fig. 5) showed significant differences between the 20 cm H_2O group and the rest of the groups. The increase in the number of alveoli (Fig. 6) showed significant differences between the groups fixed and 20 and 30 cm H_2O and the one fixed at 35 cm H_2O and the rest of the groups. The alveolar cord significantly diminished in the three groups of greater pressure respecting the lungs fixed at 20 cm H_2O (Fig. 6).

Older animals:

No morphological differences could be established between the lungs of older animals fixed at different TPP (Fig. 3).

The results of the morphometric study are shown in figures 7, 8, and 9. Pulmonary volume,
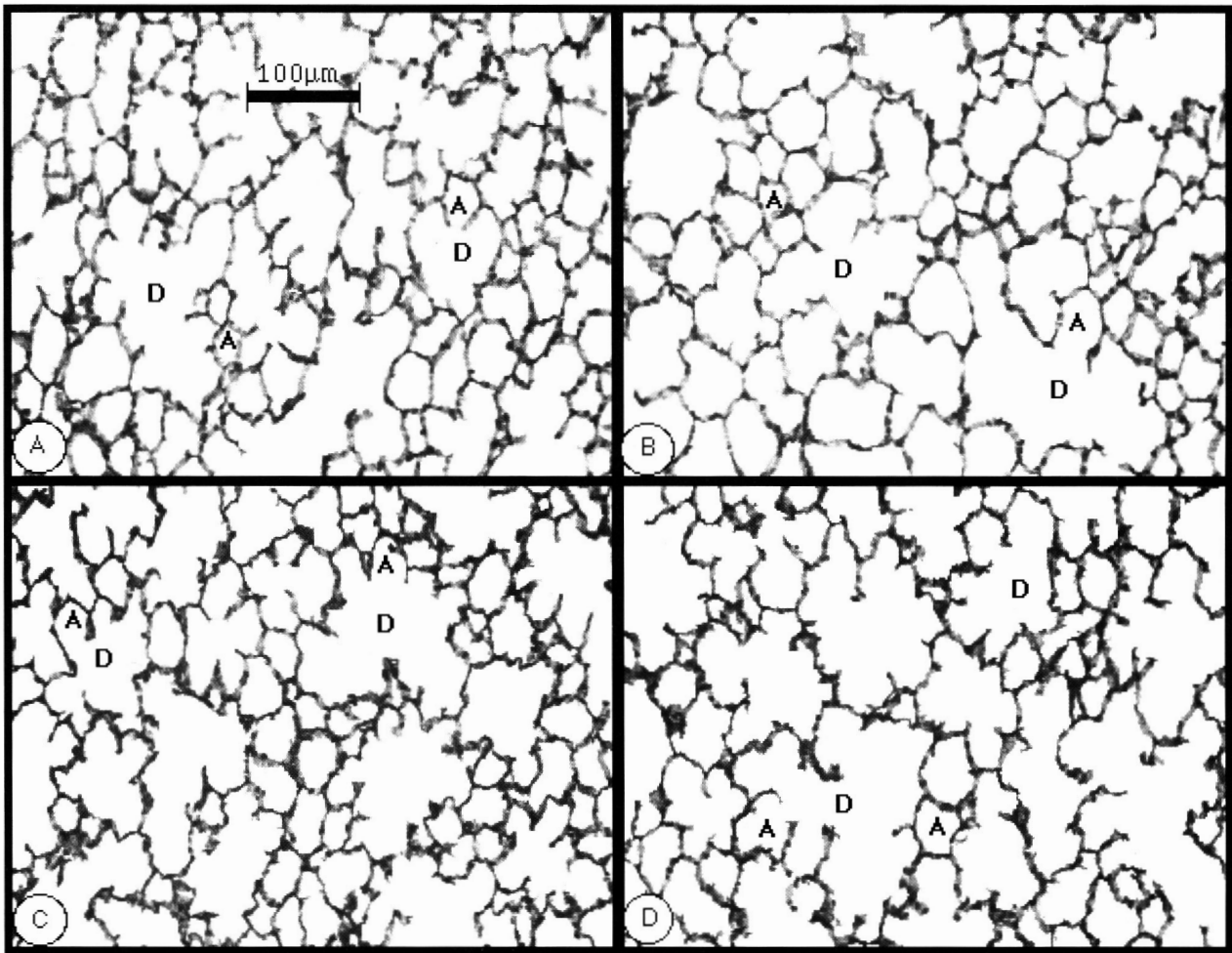
Fig. 3.- Lungs of older animals. Digitalized images of left medial lung sections, stained with methylene blue, applying to each of the insufflation pressures used. **A**: 20 cm H_2O; **B**: 25 c . H_2O; **C**: 30 cm H_2O; **D**: 35 cm H_2O.
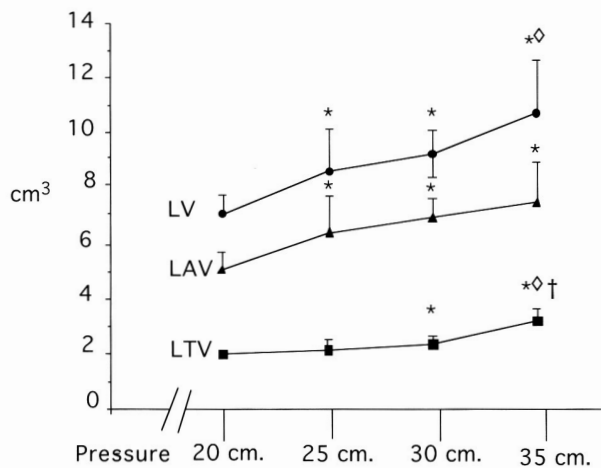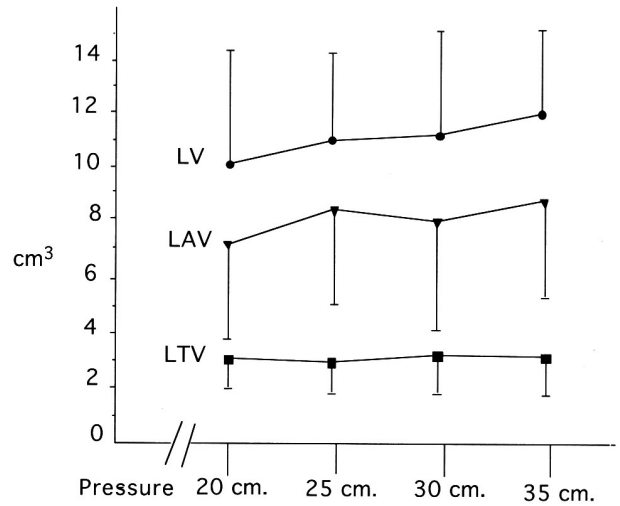

Fig. 4.- Lungs of adult animals. Representation of means-plus-one standard deviation of the results obtained of the variables lung volume (LV, circles), lung air volume (LAV, triangles) and lung tissue volume (LTV, squares).

    *: $p < 0.05$ with respect to the group perfused at 20 cm. H_2O.

    ◊: $p < 0.05$ with respect to the group perfused at 25 cm. H_2O.

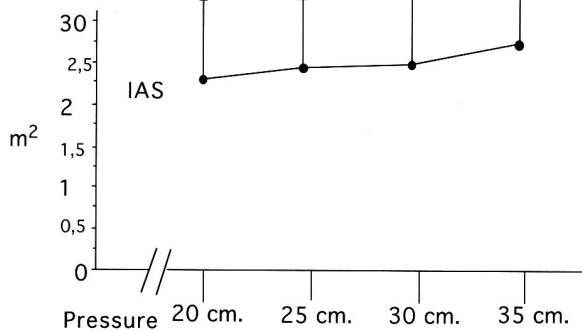   †: $p < 0.05$ with respect to the group perfused at 30 cm. H_2O.
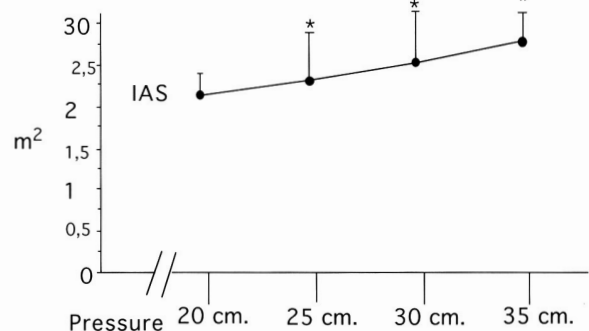
Fig. 5.- Lungs of adult animals. Representation of the means-plus-one standard deviation of the results of the variable internal alveolar surface (IAS).

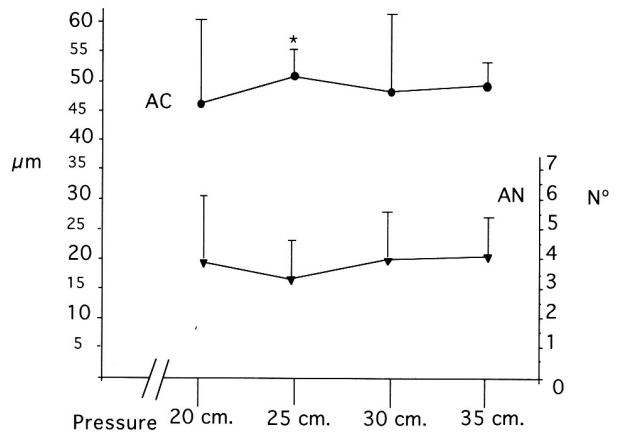   *: $p < 0.05$ with respect to the group perfused at 20 cm H_2O.
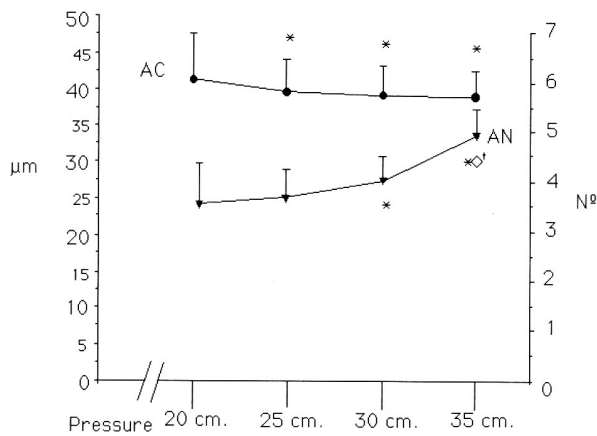
Fig. 6.- Lungs of adult animals. Representation of the means-plus-one standard deviation of the results obtained in the variables alveolar chord (AC, circles) and number of air-spaces (AN, triangles).

*: $p<0.05$ with respect to the group perfused at 20 cm. H_2O.

◊: $p<0.05$ with respect to the group perfused at 25 cm. H_2O.

†: $p<0.05$ with respect to the group perfused at 30 cm. H_2O.

Fig. 7.- Lungs of older animals. Representation of means-plus-one standard deviation of the results obtained of the variables lung volume (LV, circles), lung air volume (LAV, triangles) and lung tissue volume (LTV, squares).

Fig. 8.- Lungs of older animals. Representation of the means-plus-one standard deviation of the results obtained of the variable internal alveolar surface (IAS).

Fig. 9.- Lungs of older animals. Representation of the means-plus-one standard deviation of the results of the variables alveolar chord (AC, circles) and number of air-spaces (AN, triangles).

*: $p<0.05$ with respect to the group perfused at 20 cm H_2O.

pulmonary air volume, lung tissue volume and the IAS increased with TPP, but in no case were there significant differences. The TPP did not linearly influence the results of the alveolar cord and the number of alveoli. The only significant result obtained was the increase of alveolar cord in the 25 cm H_2O group with respect to that of 20 cm H_2O.

DISCUSSION

Our results lead us to believe that the lungs of both rat groups behave differently. The significant increase in lung volume produced in the adult animals after reaching a TPP of 20 to 25 cm H_2O and from 25 to 35 cm H_2O raises the question of whether or not the TPP which produces the PV curve inflexion is included within the range used.

It has been proposed that liquid-filled lungs reach TLC at TPP's lower than 25 cm H_2O. Gil et al. (1979) and Bachofen et al. (1970) inflated the lungs with physiological serum and the plateau phase near 10 cm H_2O. There are less clear authors who, in principal, propose that the TPP for reaching TLC is 30 cm H_2O, and then consider that with 20 cm H_2O the plateau phase is reached in the pressure/volume curve and finally are fixed at 25 cm H_2O (Lum and Mitzer, 1975). While generally the pressure most used for aerial fixation is 25 cm H_2O, the use of 20 cm H_2O is frequent (Forrest and Weibel, 1975; Pinkerton et al., 1982). In this study we were able to split the pulmonary volume into two components: lung tissue volume and pulmonary air volume. These two variables showed significant differences: when the TPP increased from 20 to 25 cm H_2O the aerial volume increased, just as the tissue volume did between 20 and 30 and between 30 and 35 cm H_2O. These results lead us to make two proposals: first, the volume of liquid introduced into aerial spaces was not significantly modified above 25 cm H_2O. And second, the differences in lung volume found in lungs fixed at 35 cm H_2O are the consequence of the increase in pulmonary tissue. We therefore propose that TLC was reached at 25 cm H_2O.

The total volume of lung tissue should have remained constant. Nevertheless, the tissue augmented in the lungs perfused at 30 and 35 cm H_2O. It has been demonstrated that the use of fixatives with different osmotic pressures on the one hand, and the increase in perfusion pressure on the other, can produce tissue edema (Bachofen et al., 1982; Conhaim et al., 1989; Wu et al., 1995). We therefore consider the production of tissue edema to be due to an increase in TPP. We observed the edema tissue as an increase of the tissue. We were able to demonstrate this increase morphometrically and believe it to coincide with the widening of the septal intersection (Fig. 1D).

The morphological modifications which occur with the volumetric variations of the lung have been studied by several authors. Dunnill (1967) described that the percentage of lung volume occupied by the alveoli rises with an increase in lung inflation. This increase in alveolar volume, it was proposed, was produced by the increase in size of the distal aerial spaces (Storey and Staub, 1962; D'Angelo, 1972). Nevertheless, at present, it is accepted that as the TPP increases it produces an alveolar recruitment (Assimacopoulos et al., 1976; Smaldone et al., 1983; Lum et al., 1990; Okada, 1992; Cheng et al., 1995; Kimmel et al., 1995). That is to say, when the TPP increases, aerial units open, which increases their number, while at the same time they become smaller. Lum et al. (1990) studied alveolar recruitment and proposed that at low transpulmonary pressures the alveoli are collapsed, with their walls flattened

together permitting the observation of numerous ducts. Upon increasing the TPP the alveolar walls open up: alveoli that were closed open. The quantifiable result of the alveolar recruitment is that the aerial units increase in number while the average size of the air spaces diminishes. This corresponds with the interpretation of figure 1D. The result of this recruitment is that the volumetric fraction of the ducts does not vary, although the total volume of the alveoli increase (Forrest, 1970).

With the opening up of the walls the IAS gaseous interchange surface increases. The increase in the IAS in lung insufflation is well documented (Storey and Staub, 1962; Dunnill, 1967; Forrest, 1970; D'Angelo, 1972; Gil et al., 1979). In the lungs of adult animals the TPP increase tends to increase the IAS and to produce alveolar recruitment. Attending to only the indices of significance: The IAS only increased until TLC was reached. Alveolar recruitment was only produced when the pressure was raised from 20 to 30 cm H_2O. If we attend separately to the two variables which constitute the alveolar recruitment, size and number, the significances are different: the size of the alveoli is not modified once the PV curve plateau phase has been reached. The number of alveoli, increasing significantly from 20 to 30 cm H_2O and at 35 cm H_2O, shows very important differences with all the other groups. Therefore, in the adult animals there are important structural modifications until TLC is reached. At 35 cm H_2O TPP substantial changes are produced. On the other hand, in the first plateau phase of between 25 and 30 cm H_2O there are no structural modifications.

In the lungs of the older animals the structure and behaviour differ from those of adults. Indeed, the pulmonary interstitial matrix increases with age and with it the collagen (Pinkerton et al., 1982) and the elastin (Escolar et al., 1994) contents. These characteristics suggest that the non-fibrous component most abundant in the extra-cellular matrix, glycosaminoglycans, may have been modified. The glycosaminoglycans are closely related to the fixation of the tissues of elastin fibres and collagen (Erlinguer, 1995; Van de Lest et al., 1995), have the property of retaining water (Negrini et al., 1996; Negrini et al., 1998), and influence the properties of tissue pressure (Negrini et al., 1996). The modifications of the extra-cellular matrix of the lung may be involved in the distendability of the lung since the senile lung reaches TLC earlier (Verbeken et al., 1992) and in the retention of liquids, as the lung tissue volume was not modified in the older animals. It has also been described that the older lung presents increases in distal aerial spaces (Pinkerton et al., 1982; Escolar et al., 1994; Escolar et al., 1997) which may condition pulmonary distension.

In conclusion, the age of the animal used may be a very important factor when selecting the ideal TPP for lung fixation. In our experiments, the two

samples of rats used followed distinct behaviours. In the older animals, there were no differences in the volume fixed with different pressures; therefore, the TLC volume should be reached at the TPP of 20 cm H_2O or less. Nor were important modifications detected at microscopic level when TPP was increased. In adult animals the pulmonary volume increased from 20 to 25 cm H_2O and underwent no modifications from 25 cm H_2O onwards. Therefore, we consider that at this point TLC has been reached. The increase in the number of alveoli found at 35 cm H_2O leads us to think that these lungs would be the best distended, but the possible presence of edema obliges us to discard this TPP for fixation. Although not all aerial units were opened with the TPP of 25 cm H_2O, we consider it ideal for intra-tracheal fixation since it presents a more reduced possibility of tissue edema.

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