

Structure and Concentration of Elastic System Fibers in the Trachea of the Rat

Estructura y Concentración del Sistema de Fibras Elásticas en la Tráquea de la Rata

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SUMMARY: The extracellular matrix is a key element in the tracheal morphology and physiology, both in normal and pathological states, yet little is known regarding its composition and organization. Herein we carried out a detailed study of the morphological organization and volumetric density of elastic system fibers in the trachea of Wistar rats. Six Wistar rats at the age of 45 days were used. The trachea was excised following anesthesia with Thiopental and perfusion through the left ventricle with buffered saline followed by formalin solution. Samples were fixed in formaline, embedded in paraffin, and histologically processed. The elastic system fibers were evaluated at light microscopy by using Weigert's resorcin-fuchsin technique after oxidation with oxone. Morphometric studies were performed by the point-counting method. Quantities were expressed (% , mean) as volumetric densities and were determined on 25 random fields for each animal. Elastic system fibers were identified beneath the tracheal mucosa showing mainly a longitudinal profile. There were also some bundles of oblique fibers in this region, forming an irregular network of elastic tissue. Close to the inner surface of the cartilaginous ring, a well defined circular layer is present. The volumetric density of the elastic system fibers in the trachea of Wistar rats is $2.46 \pm 0.99\%$. The data in the present study provides original information regarding the elastic system fibers of the rat trachea, which might be used as a model for studying human major airway morphology. The results reported herein provide the basis for continuous investigations on tracheal extracellular matrix by stereology.

KEY WORDS: Rat; Trachea; Morphology; Elastic fibers; Quantitative evaluation.

INTRODUCTION

The trachea serves as a conduit for ventilation and clearance of tracheal and bronchial secretions. Its anterolateral wall is composed of C-shaped cartilaginous rings interconnected by fibrous tissue, which is formed mainly by collagen and elastic system fibers. Its posterior wall is composed by smooth muscle and fibroelastic tissue (Minnich & Mathisen, 2007). Beneath the mucosa, the basal layer consists of an extracellular matrix rich in elastic system fibers (Minnich & Mathisen; Kamel *et al.*, 2009).

The trachea is the site of several pathologies, both acquired and congenital, all affecting the extracellular matrix (Callan *et al.*, 1988; Shivaram *et al.*, 1990). Benign tracheal stenosis following intubation and tracheostomy, "saber-sheath trachea" in chronic obstructive pulmonary disease or

the elderly, tracheomalacia and granulomas following tracheostomy are diseases commonly seen in medical practice (Callan *et al.*; Shivaram *et al.*). There are also systemic diseases with the potential to produce tracheal extracellular matrix alterations, such as amyloidosis and Wegener's granulomatosis, among others (Falk *et al.*, 1997; Daum *et al.*, 1995). These pathologic entities have a process of prominent extracellular matrix remodeling, which is not completely understood.

Few studies have focused on the tracheal morphology, especially regarding its extracellular matrix and elastic system fibers (Kamel *et al.*). While considerable attention has been focused on the elastic properties of the lung and chest wall, there is comparatively little research on the elastic

network in the major airways (Palecek & Jezová, 1988), which is surprising given its potential importance in health and disease.

An understanding of the elastic system fibers of the rat trachea would improve the basic scientific knowledge regarding composition and organization of the extracellular matrix of this organ, which has long been used as a model for human respiratory tract diseases. In this study, we characterized and quantified the elastic system fibers of the trachea in healthy rats at the age of 45 days. Our quantitative analyses were focused on volumetric density (V_v) by stereological methods (Weibel *et al.*, 1966; Mandarim-de-Lacerda, 2003; Zapata *et al.*, 2009).

MATERIAL AND METHOD

The study design and experimental protocols were approved by the Animal Care and Use Committee of the Fluminense Federal University, which based its analysis on the Guide for the Care and Use of Laboratory Animals (Bayne, 1996). The experiments described here were performed according to guidelines of the Brazilian College for Animal Experimentation (COBEA) (Schneider, 2008).

Wistar rats obtained from Experimental Morphology Laboratory (LAMEX), at Fluminense Federal University, were housed at $25 \pm 1^\circ\text{C}$ and on a 12h light–dark cycle (lights on from 07.00 hours to 19.00 hours) throughout the experiment, e.g. from birth to 45 days, and feed with stan-

dard diet *ad libitum* consisting of 23% protein, 68% carbohydrate, 5% lipid, 4% salts and 0.4% vitamins. At the age of 45 days, six rats were anesthetized with thiopental anesthesia (0.1 mL/100 g body weight) and perfused through the left ventricle with buffered saline followed by formalin solution. After perfusion, the tracheas were dissected from adjacent structures, excised and fixed in 4% formalin in 0.1 mol/L phosphate buffer (pH 7.4) by immersion for 24h at room temperature, processed according to routine histological methods and embedded in paraffin. From the paraffin-embedded samples, 5 μm -thick sections were initially stained with hematoxylin and eosin to confirm sample adequacy. Weigert's resorcin-fuchsin technique, after previous oxidation with oxone, was used for demonstration and evaluation of elastic system fibers that were stained in violet, producing a sharp contrast.

From each specimen, 5 tissue sections were analyzed, and from each section, 5 random fields were evaluated; therefore, we analyzed 25 test areas for each individual. The analyzed fields were digitized with a final magnification of $\times 400$ by a Sony CCD video camera (DXC 151-A model) coupled with a light Olympus microscope (BX52). The quantification was obtained by using an M-42 test grid system on the digitized fields on the screen of a color monitor. The volume density of the histological components was calculated according to the formula $V_v = P_p/P_t$, where V_v is the volume density, p is the tissue component to be taken into consideration, P_p is the number of test points associated with p , and P_t is the number of points of the test system. The stereological methods have been described in detail elsewhere (Weibel *et al.*; Mandarim-de-Lacerda).

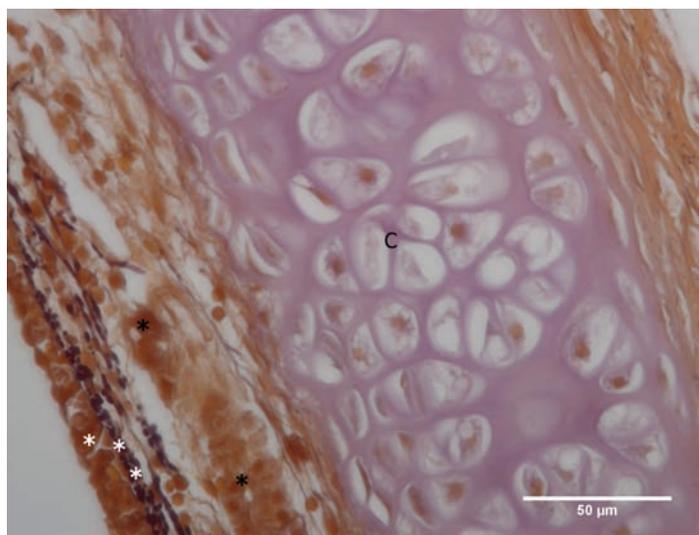


Fig. 1. Tracheal section showing the epithelium (white asterisk) and the basal layer (double white asterisk). Beneath the latter, there is a longitudinal distribution (black asterisk) of elastic system fibers with some oblique bundles. C. cartilage, X600.

RESULTS

After undergoing careful examination of the slides stained by Weigert's resorcin-fuchsin with previous oxidation, it was possible to observe the distribution of the elastic system fibers present in the trachea of the rat (Figs. 1-3).

The elastic system fibers were abundant beneath the tracheal mucosa, where they showed mainly a longitudinal distribution. There were also some bundles of oblique fibers in this region, forming an irregular network of elastic tissue (Fig. 1). In close contact to the inner surface of the cartilaginous ring, a well defined and organized circular layer was present, surrounding the whole extension of the cartilage (Fig. 2). Outside the cartilaginous rings, the elastic system fibers were exclusively arranged in a

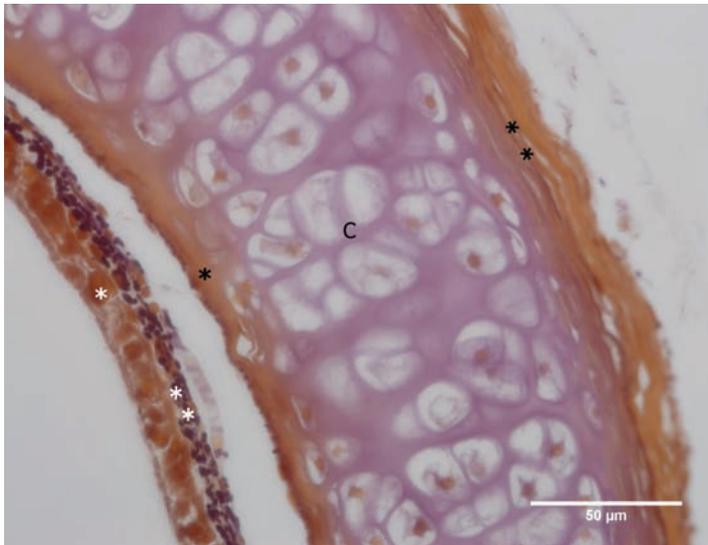


Fig. 2. Epithelium (white asterisk) and basal layer (double white asterisk). Circular layer of elastic system fibers in close contact to the inner surface of the cartilaginous ring (black asterisk). Outside the cartilage there is an exclusive circular layer of elastic system fibers (double black asterisk). C: cartilage. X600.



Fig. 3. Epithelium (white asterisk) and basal layer (double white asterisk). The circular layer of elastic system fibers is in close contact to the cartilage on its inner (black asterisk) and outer (double black asterisk) surface. C: cartilage. X600.

circular layer, following the circular cartilaginous rings (Fig. 3). At this topography, there was no longitudinal or oblique arrangement of the elastic system fibers.

The V_v of the elastic system fibers in the trachea of the rat was $2.46 \pm 0.99\%$, with a confidence interval (CI) of 2.05 – 2.087 ($p < 0.02$).

DISCUSSION

Most of the studies attempting to quantify linear structures use area density since the advent of computer-aided image analysis programs (Flotte *et al.*, 1989). These programs use an element of color properties (pixels) of the image to determine a threshold level for inclusion. In rather thin linear structures, such as elastic system fibers under analysis, the contrast between the fibers and the background is low; therefore the error introduced using color intensity as the method of measurement is high (Babinski *et al.*, 2005).

The point counting method (stereology) (Weibel *et al.*; Zapata) used in our study proved to be quite effective in avoiding the bias that frequently occurs with computerized image analyses. These methods have been used to quantify and particularly to determine the amount (expressed in percentage of volumetric density) of the elastic system fibers in a tissue (Babinski *et al.*), with particular interest on the extracellular matrix elements (Mandarim-de-Lacerda).

The aim of our study was to analyze the elastic system fibers in the trachea of Wistar rat. This animal is by far used as a model for human respiratory tract and major airway diseases. Particular emphasis was placed on Weigert's resorcin-fuchsin stain after oxidation with oxone, because it highlights all elastic components in the tissues. We performed a qualitative analysis of the distribution and organization of the fibers, and also a quantitative evaluation, providing stereological data on the concentration of elastic system fibers in the trachea of the rat.

The elastic system fibers are characterized by major extension qualities and elastic recoil (Kielty *et al.*, 2002). The location and arrangement of the fibers are related to their different functionality, which reflects local tissue mechanical properties (Kielty *et al.*). The trachea is known to change its shape and conformation during physiological processes, like normal and forced breathing, coughing and neck movements (Harris, 1959; Kamel *et al.*). The importance of normal tracheal anatomy is highlighted by congenital and acquired tracheal deformities, such as tracheobronchomegaly, tracheomalacia and benign tracheal stenosis. The extracellular matrix,

especially the elastic system fibers and collagen, is thought to play a pivotal role in these remodeling processes (Shivaram *et al.*).

In young human adults, tracheal length increases by about 20% in deep inspiration, and also lengthens with neck movements (Harris). Linear extensibility and elastic recoil of the human trachea is remarkably age-dependent, decreasing from a maximum of 50% in children to about 15-20% at 70 years of age (Kamel *et al.*).

The effect of elastin degeneration with advancing age seems to be of relevance in the respiratory system. With an estimated half-life of 70 years, elastin is the most stable and persistent protein in the human body (Vieth *et al.*, 2007), but it does degenerate in the elderly. Previous studies (Robert *et al.*, 2008) have highlighted the striking parallel between elastin degradation with aging and the declining of respiratory function, with particular emphasis on the loss of compliance of the lungs and chest wall; but researches in the elastic tissue of the trachea are lacking (Palecek & Jezová; Kamel *et al.*).

Some studies suggest that the quantity of histologically observed elastin may not have a straight correlation with properties of the tissue, such as distensibility and elastic recoil (Arteaga-Solis *et al.*, 2000; Hammond *et al.*, 1998). In Marfan's syndrome, for example, there is a molecular defect in fibrillar proteins associated with elastin, but not in elastin itself (Godfrey *et al.*, 1990). A well-known example comes from the elastic arteries with aging. Elasticity of arteries decreases with age because of extensive cross-linking and enhanced calcium binding activity; however, no histological change in staining of the elastic system fibers is apparent (Arteaga-Solis *et al.*).

Experiments with tracheal replacement in pigs have been recently attempted (Makris *et al.*, 2010). The authors used aortic allografts, and observed the replacement of the normal aorta by a dense inflammatory tissue containing islands of elastic tissue, previous to the development of a new tracheal mucosa (Makris *et al.*). The extracellular matrix, with especial regard to collagen and elastic system fibers, seems to play an important role in the process of tissue regeneration and cellular in-growth of recipient progenitor cells (Makris *et al.*; Macchiarini *et al.*, 2008). Tracheal replacement might be an option in the future, when radical surgery for primary tracheal tumors with resection of more than one-half of the tracheal length is concerned (Macchiarini *et al.*).

The elastic tissue of the human trachea is abundant and organized in discrete longitudinal bundles of elastic

system fibers in the submucosa, with a layer of circular fibers in close contact to the cartilage (Kamel *et al.*). This organization resembles that of the rat in our study, what support the use of this model for human major airway morphology.

Our results show elastic system fibers concentration and distribution in the trachea of the Wistar rat. In spite of being essentially quantitative, this study provides support for research regarding biomechanical and morphological modifications in tracheal elastic tissue. Despite elastic system fibers are well known components of tracheal extracellular matrix, no previously reported study has assessed the stereological quantification of elastic system fibers, neither in human nor in the model rat trachea. Therefore, we present original data concerning the rat trachea, which might be used as a model for studies of human major airway morphology. In the present study we described the morphological disposition of the of the elastic system fibers in the rat trachea, what reach a volumetric density of $2.46 \pm 0.99\%$.

In conclusion, elastic system fibers are one of the extracellular matrix's components concerning the trachea. These fibers seem to give the trachea the capacity to increase in length and diameter, and also are essential to its elastic recoil (Harris; Hammond *et al.*). Furthermore, this analysis of the elastic system fibers of the rat trachea provides original data on normal components of this organ, with special regard to using it as a model for studies in human trachea.

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RESUMEN: La matriz extracelular es un elemento clave en la morfología y fisiología de la tráquea, tanto en estados normales como patológicos. Sin embargo, poco se sabe acerca de su composición y organización. Se llevó a cabo un estudio detallado de la organización morfológica y la densidad volumétrica de fibras del sistema elástico en la tráquea de ratas Wistar. Se utilizaron seis ratas Wistar edad de 45 días de vida. La tráquea fue extraída después de anestesia con tiopental y la perfusión a través del ventrículo izquierdo con solución salina tamponada seguido de solución de formalina. Las muestras fueron fijadas en formalina e incluidas en parafina y se procesaron histológicamente. Las fibras del sistema elástico fueron evaluadas en microscopía de luz utilizando la técnica de Weigert, resorcina-fucsina después de la oxidación con Oxone. Los estudios morfométricos fueron realizados por el método de conteo de puntos. Las cantidades se expresaron (% media) como la densidad volumétrica y se determinaron en 25 campos al azar para cada animal. Las fibras elásticas del sistema fueron identificadas por bajo la mucosa traqueal que muestra principalmente un perfil longitudinal. También se encontraron algunos

haces de fibras oblicuas en esta región, formando una red irregular de tejido elástico. Cerca de la superficie interna del anillo cartilaginoso, una capa bien definida circular está presente. La densidad volumétrica de las fibras del sistema elástico en la tráquea de ratas Wistar fue de $2,46 \pm 0,99\%$. Los datos del presente estudio proporcionan información inicial sobre las fibras del sistema elástico de la tráquea de rata, que puede ser utilizado como un modelo para el estudio de la morfología de las vías respiratorias humanas. Los resultados reportados en este documento constituyen la base de investigaciones continuas en la tráquea de la matriz extracelular por estereología.

PALABRAS CLAVE: Rata; Tráquea; Morfología; Fibras elásticas; Evaluación cuantitativa.

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