THE STUDY OF VOLUME DENSITY OF TRACHEAL GANGLIONS *IN VITRO* IN NEW BORN BABIES WITH RESPIRATORY DISTRESS SYNDROME

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Abstract

Volume density of respiratory organs was studied *in vitro* in newborn babies at different age of gestation (abort, immature, premature and mature) using stereometric method. The total of 23 cases was subject to this study. The respiratory organs (trachea, lungs) were taken from autopsies of newborn babies exited from different causes. For this purpose the tissues were fixed in formalin (10%) solution, cut serially in 7μ and 10μ slabs. Volume density of the respiratory system was assessed stereometricaly using Universal testing system Weibel M 42. We observed that volume density of epithelia, musculature and glands were proportionally present in the tracheal tissue. Cellular interstitial tissue is consistently increasing and corresponds to the developmental stages of the newborn babies.

The density of tracheal ganglions is greater in premature ages of immature and premature newborns (p<0,05). Decreased number of ganglion cells is observed in mature ages (p<0,05). This is caused by intensive ramification of ganglions from serosa to deeper layers of trachea right to epithelium. Medium diameter of tracheal ganglions is greater in mature newborn babies and corresponds to developmental ages of babies.

KEY WORDS: trachea, ganglion, stereometry.

Introduction

The structure of ganglions found in the lungs is very complex; these structures are studied while making intracellular measurements in human material (1). Neurophysiological studies of ganglions show at least 3-4 groups of neurons. These neurons are inhibitory, excitatory, sensory and interneurons. In some cases even more specialized neurons are found in propria layer of the respiratory airways; initially thought to be sensory neurons, but functional definition of this neurons is still lacking. Data suggest that intrapulmonary ganglions may receive local sensory impulses as well as impulses from CNS. The presence of local sensory impulses in ganglions enables action of the reflex activity directly without central part activation. Ganglions integrate sensory impulses for local control of smooth musculature. Postganglionic fibers spread to smooth musculature of trachea and glands (2,3). The studies conducted up to date were focused on morphology and physiology of tracheal and bronchial ganglions in different species. e.g. mice (4,5), guinea pig (6), weasel (7), ship (8), human (9). Studies in humans have shown that intramural plexus of trachea and bronchia is formed by the end of fetal period. In 14 days old fetus, plexuses in external membrane of trachea and bronchia consist of a dense network of fibers and nerve cells (mono or bipolar). After that, multipolar cells appear in intramural plexus (9). Parasympathetic bronchial innervation in developed phase, shows that paraganglionar vagus nerves, which innervate smooth musculature of respiratory airways, are involved in breathing control before birth (8). Tracheal ganglions are parasympathetic structures, but also contain sympathetic sensory fibers. These problems are explained by several immunohystochemical methods (10). Noradrenergic nerve fibers are found around blood vessels in mucosa and smooth musculature of trachea, but are never observed near ganglion neurons (11).

However numerous studies have not reached clear conclusion for tracheal ganglions in cats. Two plexus in dorsal wall of trachea are found in mouse. The first one has delicate structure and is located in cervical part while the second one (80% of all ganglions with positive AChE and nerve fibers in thoracic parte) is more dense than those in cervical part. The same situation is observed in weasel (13). Ganglion localization in trachea and bronchia is mainly on the lower surface of these organs and is associated with the activity of smooth musculature (1) and the presence of mucosal glands (14). In recent years there were studies of volume and numeric density of different tissues in human and animal objects, and of their role in certain pathologic conditions of central nervous system and respiratory system. The volume density is a relative variable, which shows how larger overall space occupies the observed space in volume units, while numeric density shows how many particles (neurons) are contained in volume unit (15,16).

The aim of this was paper is analyze the *in vitro* development of ganglion cells in respiratory airways, their morphology and pathogenesis in the respiratory distress syndrome (RDS) responsible for the high mortality in newborn babies, as well as numeric density of normal and pathologic neurons in the tracheobronchial system in newborn babies in different weeks of gestations.

Material and Methods

Elaborate was performed in cooperation with the Gynaecology Obstetrics Clinic, Pathology Institute and Experimental Unit at Medical Faculty in Prishtina. For the purpose of this study we used material from autopsy of dead newborns at different weeks of gestation. The cases were grouped according to the level of development using criteria in Table 1.

Group		Weight (g)	Weeks of gestations
I	Abort	< 500 g	<22
II	Immature	500-1100 g	23-29
III	Premature	1100-2500 g	30-37
IV	Mature	>2500	>38

TABLE 1. Criteria for classification of analyzed cases into groups according to the development (n=33).

Analyzed material was collected from trachea over bifurcation level. A portion of material was used fresh for histoenzymatic analysis. The remaining tissue was fixed in 10% solution of buffered formalin for histochemical methods. The following methods have been applied:

Histoenzymatic and histochemical research methods

Endoxyl method was used for detecting esterases. The preparations have been cut with kryotom Leicca CM 1990 in 7 and 10μm thick slices. The samples fixed in 10 % buffered formalin were set in paraffin blocs. The serial slices are made in 5 and 7 μm. The slices have been stained using histochemical methods: Haematoxylin-Eozin, Sevier – Munger modification for nerve termini, Grimelius staining for argirophile granules, Luxol fast blue MBS method for myelinized fibers.

Stereometric method

Universal testing system Weibel M 42 was used for stereometric analysis. Test-system is a system of straight lines and points. This should be superimposed on morphological image for stereological count (17). In this study, we used the test-system M42 by Weibel et al. (18,19), that has 42 test-points, the test-line measures 21 d and the test-area measures 36.36 d 2 (Figure 1). M42 test-system was mounted onto x10 WH Olympus Bx50 (Japan). This system has 21 short lines with known length (d) and two test-points in each extremity (Pp, 42 test-points in total). The test-area is $36.36d^2$. The analysis have been done in five testing points (Pt) and in serial tissue slices in 7 and 10 μm (Figure 2).

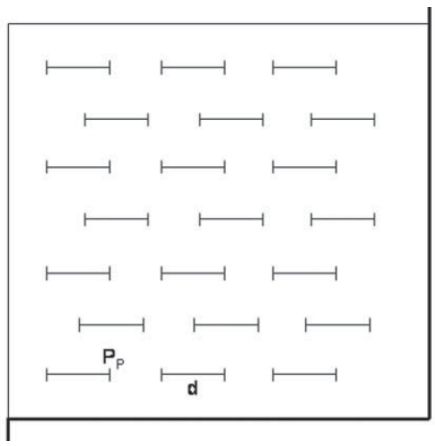

FIGURE 1. M42 test-system.

Determination of volume density

Volume density is relative variable, which shows how much overall space is occupied by the observed space in volume units. We have used universal testing system Weibel M 42, the counting have been done in five testing points (Pt). The calculation of the results is done using formula:

Vvf = Pf : Pt

where Vvf= volume density of the structure in observation phase (mm^0); Pf= number of points of system in the observation phase.

Pt= overall number of points of the testing system

The determination of numeric density (of neurons)

Numeric density is relative stereological variable, which shows how many particles (neurons) are contained in volume unit.

The calculation is made according to Abercrrombie formula:

Nv=Na:t+D

Where Nv= numeric density, Na= number of neuron section, t= thickness of serial slices, D= medium diameter of neurons

Na= Nf:At

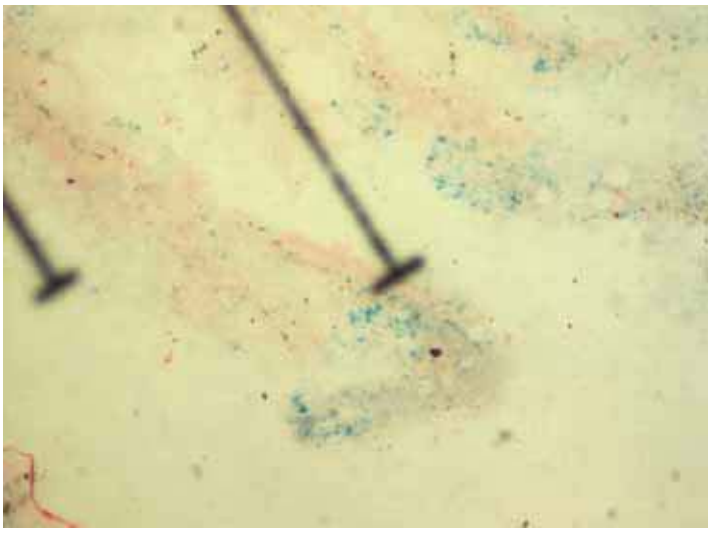

FIGURE 2. The observed testing point phase of tracheal ganglions with testing system M 42, the histoenzymatic staining in fresh tissues (indoxyl method for esterase 400 x) (3).

	Epithelium	Musculature	Gland	Cartilage
Abort	0,121±0,026	0,163±0,105	0,136±0,041	0,161±0,213
Immature	0,104±0,028	0,154±0,018	0,115±0,025	0,0±0,0
Premature	0,106±0,020	0,132±0,029	0,191±0,224	0,0±0,0
Mature	0,122±0,078	0,156±0,043	0,121±0,050	0,0±0,0

TABLE 2. The tracheal volume density in the analyzed groups of fetuses (X±SEM)

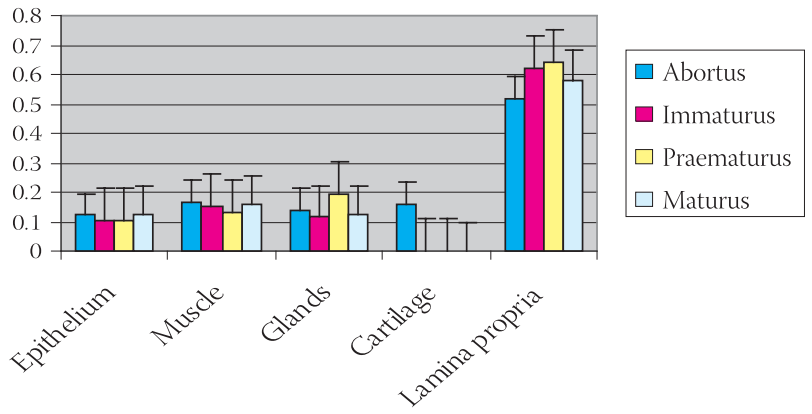

FIGURE 3. Tracheal volume density in the analyzed fetuses (X±SEM)

Nf= the number of random points in the observed phase
At= 36.36 x d²

The obtained results were statistically processed in computer program GraphPad Instant 3, with t- comparing test of columns, unpaired t- test, paired t- test, Mann-Whitney test and Wilcox test

Results

For this research we used material from autopsies of live borne babies deceased after birth and fetuses exited in different weeks of gestations. Morphological study of tracheal ganglions at different stages of fetal development, was made using histochemical and histoenzymatic methods for choline acetyl-esterase and acetylcholine – esterase. See Figure 5 (a, b, c, d, e, f,). Stereometric study has been made in serial sections of tracheal tissue, where presented density and medium diameter of ganglions, epithelium, glands, smooth musculature, cartilage and interstitial was given in volume units. After statistical processing of the results obtained from the analysis of tracheal tissues of analyzed fetuses in different ages of gestations, starting from the aborted, immature, premature and mature, we found that: interstitial in tracheal tissue has more dense presentation in volume tracheal unit, starting from early ages of newborn babies: the aborted, immature, premature and mature. In Table 2 and Figure 3 the data on density of epithelium, smooth musculature, glands and cartilages in different ages of maturity of fetuses is presented. Medium diameter of ganglions in the analyzed fetuses (immature, premature and mature) is given in Table 3. Numeric density of tracheal ganglions in the analyzed fetuses is presented in Figure 4.

	Immature	Premature	Mature
Mean (X)	14,58	17,25	22,91
SD	±3,608	±3,00	±3,508

TABLE 3. Medium diameter of ganglions in the analyzed fetuses (X±SEM)

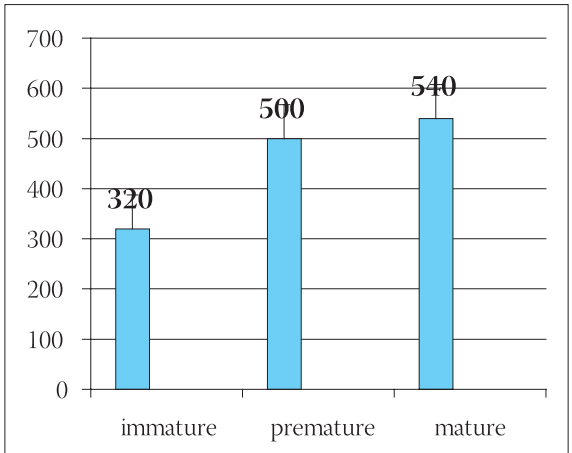

FIGURE 4. Numeric density of tracheal ganglions in the analyzed fetuses (X±SEM)

Discussion

The study shows that sympathetic nerve fibers of the trachea stem from superior cervical ganglion and stellate ganglion. Sympathetic fibers supply the trachea through tracheal laryngeal nerve from anastomosis of sympathetic ribbons and vagus nerve (10). In

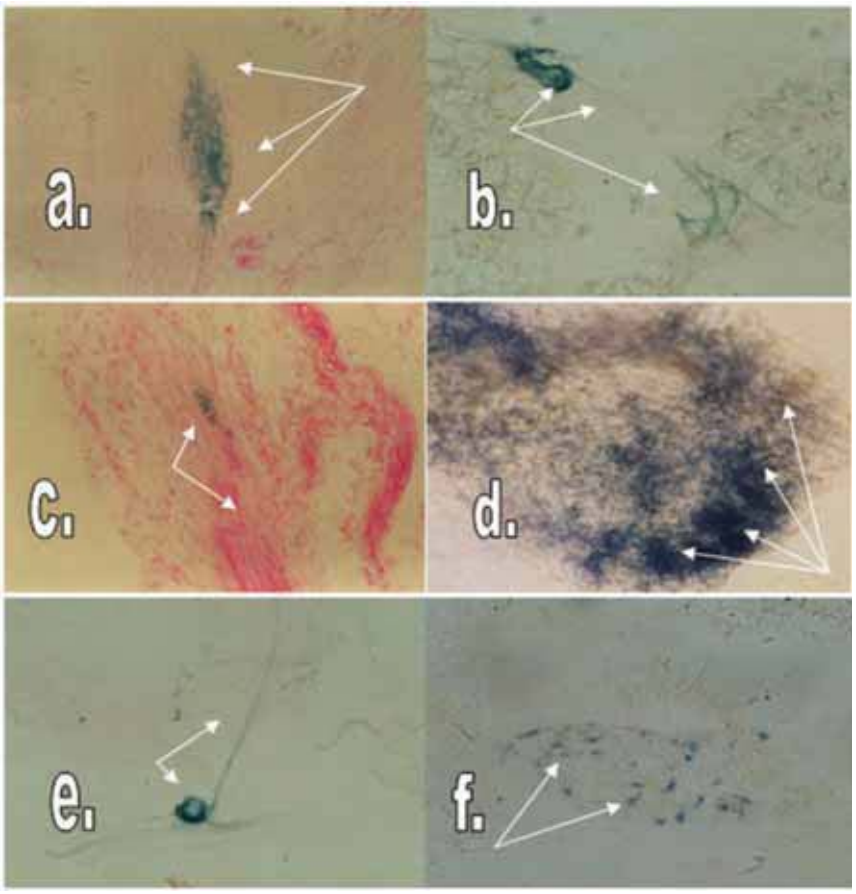

FIGURE 5. Distributions of ganglion cells in the structure of tracheal tissue in live borne babies deceased after birth and fetuses exited in different weeks of gestations:
a. Cholinereactivity well expressed in perichondrial mature ganglion containing 21 ganglion cells in mature babies (Indoxyl method for esterase; 650x).
b. Ganglion cells in lamina propria of tracheal tissue with expressed choline reactivity, with dendritic and axonal extensions around tracheal glands in prematurely born babies (Indoxyl method for esterase; 400 x).
c. Choline reactivity in smooth musculature tracheal ganglion in prematurely born babies, which contains 4 cells with oval appearance (Indoxyl method for esterase; 400 x).
d. Tracheal ganglion cells arranged in the crown form with expressed choline reactivity in mature born babies (Indoxyl method for esterase; 1600 x).
e. Ganglion cells in lamina propria of tracheal tissue with axonal extension (Indoxyl method for esterase; 400 x).
f. Group of ganglion cells with perivascular arrangement in tracheal tissue in prematurely born babies (Indoxyl method for esterase; 650x).

our observation we have noted direct connections between some tracheal ganglions and branches of cervical ganglion cells (supra, medial and inferior). The supply with sensitive innervations of neurons in trachea and bronchi has been found in nodal ganglion and vagus jugular ganglion and in ganglions of posterior upper tracheal tracts. The morphology of neurons and tracheal ganglions was examined in guinea pig and rats (1, 11). Most neurons are multipolar, with short extension (flagellate). The numbers of tracheal ganglions in the examined species are different, e.g. in guinea pig 166-327 (x -222), and rats 63-78 (70 on average). In cats, 95-210 ganglions were observed. These results suggest that there is a correlation between the number of nerve cells and the size of the respiratory system. In stereometric research of tracheal volume density of epithelial component, glands, musculature, cartilage and cell interstitial in our sample shows uniform presentation of epithelium, musculature and glands. The tracheal cell interstitial occupies dominant volume density. This shows that tracheobronchial airways of the analyzed fetuses, the aborted, immature, premature and mature, are in the phase of intense postnatal pulmonary maturity, with possible influence of external environmental factors. The obtained results of stereometric study of volume density of the respiratory airways including terminal bronchi, respiratory bronchi, alveolar ducts and alveolar sacs, present continual increase of volume density of the cellular interstitial depending on the maturity of fetuses. The main pulmonary volume representation, in all ages of the lungs development, is cell interstitial. Other constituent parts of the lungs are mainly uniformly represented. This information shows

intense cell maturity of a pulmonary tissue after birth. Sympathetic fibers from the cervical ganglions supply innervations to blood vessels, smooth musculature and mucosa, but were not found around or near the tracheal ganglions (11). Some research is concerned with the presence of VIP in parasympathetic postganglion fibers where they control cholinergic neurotransmitters with prominence of postganglion fibers. This has additional impact on ganglion transmission (20). At terminals of preganglionar nerve the presence of SP is detected (10). The examination of tracheal innervation in guinea pig, shows presence of non-adrenergic and non-cholinergic neurons. They are located in myenteric esophageal plexuses and their axons supply the trachea (6). By electrophysiological methods two types of neurons were found in tracheal ganglions. These are present only in ganglions to which neurons send sympathetic impulses (90%) and are connected with recurrent nerve steam and neurons with fast excitatory synaptic potential, located very close to smooth musculature (21). The analysis of tracheal ganglions in guinea pig by electronic microscopy, shows similarities with ganglion cells and with other spinal and parasympathetic nerve cells (1). The entire surface of the ganglions is covered with one wrapping satellite. Blood vessels, myelinated and nonmyelinated nerve fibers were found there. In morphological histoenzymatic study of respiratory airways an increase in the enzymatic activity of choline acetyltransferase (HAT) and acetylcholine esterase (ACHE) was observed. Lower the weight of fetus (aborts – immature), the more pronounced is enzymatic reactivity in tracheal smooth musculature ganglions, serose glands and tracheobronchial epithelial cells. Earlier research provides data for enzymatic reactivity of HAT and ACHE in mature ages (22). Enzymatic activity of HAT and ACHE is the most present in tracheal ganglion glands, at less developed stages, while enzymatic reactivity decreases along with age Enzymatic activity is more expressed in serosa glands, in musculature and epithelium (the neuroepithelial bodies). In terminal bronchi in epithelium and subepithelium small and large granular vesicles in direct contact with nerve endings are noticed. Tracheal cartilage is not recorded in immature, premature and mature because of limitations of stereometric network during observation phase in vitro. Denser terminals are noticed in the fetuses, which play chemoreceptive role for adaptation of musculature tonus, regulation and adaptation of blood circulation during birth (22). The nerve terminals in fern form which end in bronchial layer are sensorial and are stimulated by prolonging the bronchial wall. In bronchial smooth musculature cells the nerve endings are in claw form and are stimulated by changes in length of musculature fiber (3).

The results show that ganglion cells are noticed initially in large number in tracheal serosa, with perivascular localization in early immature stages of fetuses. Penetration of ganglion cells into deeper tracheal layers is focal. In the mature, mature postganglionar tracheobronchial fibers, which innervate more serosa and mucosal glands are noticed, while the musculature and epithelium are partially innervated. The collected data from other authors are contradictory (2, 5). A characteristic of nervous system is that neurons from different parts migrate into higher areas of nervous system. Neurons migrate radially and tangentially. It is considered that one of the migration mechanisms of young neurons is made through radial fibers of glia. In the early development, the nerve cells migrate close to 1 mm per 24 hours, whereas in the later stages of neurogenesis, for migration of cortical neurons nearly two weeks are needed (15). In our studies, we observed neuronal perivascular migration in the population of respiratory neurons.

Conclusion

On the basis of data obtained from morphological examination of trachea and pulmonary tissue *in vitro* at various stages of gestation development we found the following:

- Numeric density of tracheal ganglions is the largest at premature ages of immaturely and prematurely born babies ($p<0,05$). At mature (measured) ages there is a decrease in the number of ganglion cells ($p<0,05$).
  This is explained by the given phase of intense ramification of ganglions from serosa of deeper tracheal layers into epithelium. Average diameter of tracheal ganglions is greater in the mature age of newborn babies (measured), the value which corresponds to the developmental age of fetus.
- Enzymatic activity of HAT and ACHE is the most present in tracheal ganglion glands, at less developed stages, while enzymatic reactivity decreases with age. Enzymatic activity is more expressed in serous glands, in smooth musculature and in epithelium (neuroepithelial bodies). In terminal bronchiole small and large granular vesicles (VGM) that are in direct contact with nerve terminals are noticed in epithelium and subepithelium.

References

(1) Baluk P., Fujiwara T., Matsuda S. The fine structure of the ganglia of the guinea-pig trachea. Cell Tissue Res. 1985; 239: 51-60.

(2) John W.M. Neurogenesis of patterns for neurogenesis of gasping:noeud vital or noeuds vitals. J. Appl. Physiol. 1996; 81: 1865-1877.

(3) Shabani R., Hundozi Z., Islami H. Evoluimi i dendësisë numerike neuronale në bëthamat dorsale motorike dhe solitare të nervit vagus në palcën e zgjatur te të porsalindurit. Praxix medica. 2002; 44: 10-17.

(4) Kanemoto Y., Ishibashi H., Doi A., Akaike N., Ito Y. An electrophysiological study of muscarinic and nicotinic receptors of rat paratracheal ganglion neurons and their inhibition by Z-338. Br. J. Pharmacol. 2002; 135: 1403-1414.

(5) Shibata K., Taketani K. Excitatory effect of noradrenaline on rat airway parasympathetic ganglion neurons. Fukuoka Igaku Zasshi, 2001; 92: 377-383.

(6) Moffatt J.D., Dumsday B., McLean J.R. Non-adrenergic, non-cholinergic neurons innervating the guinea-pig trachea are located in the oesophagus: evidence from retrograde neuronal tracing. Neurosci. Lett. 1998; 248:37-40.

(7) Coburn R.F., Kalia M.P. Morphological features of spiking and nonspiking cells in theparatracheal ganglion of the ferret. J. Comp. Neurol. 1986; 254: 341-351.

(8) Perez Fontan J.J., Velloff C.R. Neuroanatomic organization of the parasympathetic bronchomotor system in developing sheep. Am. J. Physiol. 1997; 273: 121-133.

(9) Dudzinska B., Wozniak W. Intramural plexuses in the trachea and bronchi of human fetuses. Folia Morphol. (Warsz). 1977; 36: 159-166.

(10) Kummer W., Fischer A., Kurowski R., Heym C. The sensory and sympathetic innervation of guinea-pig lung and trachea as studied by retrograde neuronal tracing and double-labelling immunochistochemistry. Neuroscience. 1992; 49: 715-737.

(11) Bauluk P., Gabella G. Tracheal parasympathetic neurons of rat, mouse and guinea pig: partial expression of noradrenergic phenotype and lack of inervation from noradrenergic nerve fibres. Neurosci. Lett. 1989; 102: 191-196.

(12) Kuder T., Kuchinka J., Nowak E., Szczurkowski A. The autonomic plexus in the trachea of the cat. Sup. An. Ant. 2001; 184: 238.

(13) Baker D.G., McDonald M., Basbaum C.B., Mitchell R.A. The architecture of nerves and ganglia of the ferret as revealed by acetylocholinesterase histchemistry. J. Comp. Neurol. 1986; 246: 513-526.

(14) Choi H.K., Finkbeiner W.E., Widdicombe J.H. A comparative study of mammalian tracheal mucous glands. J. Anat. 2000; 197: 361-372.

(15) Mouton P.R., et al. Stereologycal Leng Estimation Using Spherical Probes. J. Microscopy 2002; 206: 30-35.

(16) Mouton P.R. Principles and practices of unbiased stereology:an introduction for bioscientis, The Johns Hopkins University Pres: Baltimore and London, 2002.

(17) Mandarim-de-Lacerda C.A. What in the interest of normal and pathological morphological reseach to be quantitative? The example of the stereology. Braz. J.Morphol. Sci. 1999; 16:131-139.

(18) Weibel E.R.et al. Practical stereological methods for morphometric cystology. J. Cell.Biol., 1966; 30: 23-38.

(19) Weibel E.R. Stereological methods. In: Practical methods for biologicalmorphometry. London: Academic Press. 1979; vol 1.

(20) Kalubi B., Yamano M., Ohhata K., Matsunaga T., Tohyama M. Presence of VIP fibres of sensory origin in the rat trachea. Brain Res. 1990; 522: 107-111.

(21) Lees GM., Paccitti E.G., Mackenzie G.M. Morphology and electrophysiology of guinea-pig paratracheal neurons. Anat. Rec. 1997; 247: 261-270.

(22) Fenato F., Gioretti R. Opiate related death: morphometric study of neurons from the dorsal motor neucleus of the vagus nerve. Boll.Soc. Ha. Biol. Sper. 1997; 1-2: 23-30.