VIA MEDICA

ORIGINAL ARTICLE

Folia Morphol.
Vol. 72, No. 4, pp. 322–327
DOI: 10.5603/FM.2013.0054

ISSN 0015–5659
www.fm.viamedica.pl

Cholinergic innervation of human mesenteric lymphatic vessels

V. D'Andrea[1], E. Bianchi[2], S. Taurone[2], F. Mignini[3], C. Cavallotti[4], M. Artico[2]

[1]*Department of Surgical Sciences, Sapienza University of Rome, Rome, Italy*
[2]*Department of Sensory Organs, Sapienza University of Rome, Rome, Italy*
[3]*School of Drug and Health Products Sciences, University of Camerino, Camerino, Italy*
[4]*Department of Anatomical, Histological, Medico-legal and Locomotor System Sciences, Sapienza University of Rome, Rome, Italy*

[Received 20 March 2013; Accepted 14 April 2013]

Background: *The cholinergic neurotransmission within the human mesenteric lymphatic vessels has been poorly studied. Therefore, our aim is to analyse the cholinergic nerve fibres of lymphatic vessels using the traditional enzymatic techniques of staining, plus the biochemical modifications of acetylcholinesterase (AChE) activity.*
Materials and methods: *Specimens obtained from human mesenteric lymphatic vessels were subjected to the following experimental procedures: 1) drawing, cutting and staining of tissues; 2) staining of total nerve fibres; 3) enzymatic staining of cholinergic nerve fibres; 4) homogenisation of tissues; 5) biochemical amount of proteins; 6) biochemical amount of AChE activity; 6) quantitative analysis of images; 7) statistical analysis of data.*
Results: *The mesenteric lymphatic vessels show many AChE positive nerve fibres around their wall with an almost plexiform distribution. The incubation time was performed at 1 h (partial activity) and 6 h (total activity). Moreover, biochemical dosage of the same enzymatic activity confirms the results obtained with morphological methods.*
Conclusions: *The homogenates of the studied tissues contain strong AChE activity. In our study, the lymphatic vessels appeared to contain few cholinergic nerve fibres. Therefore, it is expected that perivascular nerve stimulation stimulates cholinergic nerves innervating the mesenteric arteries to release the neurotransmitter AChE, which activates muscarinic or nicotinic receptors to modulate adrenergic neurotransmission. These results strongly suggest, that perivascular cholinergic nerves have little or no effect on the adrenergic nerve function in mesenteric arteries. The cholinergic nerves innervating mesenteric arteries do not mediate direct vascular responses.* (Folia Morphol 2013; 72, 4: 322–327)

Key words: cholinergic nerve fibres, neurotransmitter, acetylcholinesterase, mesenteric vessels

INTRODUCTION

The lymphatic vessels have been studied in different organs from morphological [1, 22], as well as functional point of view [27, 29].

Some morphological and probably functional interactions between nervous fibres and structures of the lymphatic system have been described in different organs, such as the monkey bladder [12], human lymph nodes [18], thoracic duct of the dog [11], rat skin [28], Peyer's patches of the cat's ileum [10], intestine of the guinea pig [5], mesenteric lymphatic vessels of ox [16, 20], of sheep [8], of guinea pig [7],

Address for correspondence: Dott.ssa S. Taurone, Department of Sensory Organs, V.le del Policlinico 155, 00161 Rome, Italy, tel: +39 0649918038, e-mail: t.samanta@yahoo.it

femoral lymphatic vessels [9], lymph nodes [14] and thoracic duct of rat.

Published papers are still not exhaustive regarding the role of the cholinergic innervation within the lymphatic vessels. Knowledge of the physiological or pathological mechanisms regulating the functions of the lymphatic system is incomplete, therefore, some diseases that probably have a morphological basis, may be considered as functional disorders.

In recent years, there has been a growing interest in the morpho-functional links between cholinergic nervous fibres, lymphatic vessels and lymphatic organs [2].

Nevertheless, deeper knowledge of the cholinergic nervous control of the lymphatic system is becoming increasingly important in order to clarify the structural basis of diseases affecting the lymphatic circulation.

The authors' objective is to analyse the cholinergic innervation of lymphatic vessels using both traditional enzymatic techniques for the staining of acetylcholinesterase (AChE)-positive nerve fibres, plus biochemical dosage of AChE activity.

MATERIALS AND METHODS

In this study, the following experimental procedures were performed: 1) drawing of mesenteric tissues containing lymphatic vessels; 2) cutting of tissue; 3) staining of tissue; 4) staining of nerve fibres; 5) enzymatic staining of AChE; 6) proteins amount; 7) biochemical amount of AChE activity; 8) quantitative analysis of images; 9) statistical analysis of data.

1) Drawing of the samples: fragments of human mesenteric tissues, containing lymphatic vessels, were harvested during autopsies with written informed consent from the relatives of the dead donors, and used for the experimental procedures described later on. After pre-fixation and cut in serial sections (20–40 μm) on a cryostat, all sections were dried "in vacuo" under P_2O_5 atmosphere for 60 min.

2) Staining of tissues: the tissues were stained by means of classical staining with eosin-orange. If a deeper shade of red is desired in staining, add 0.5 mL of glacial acetic acid to each 100 mL of standard solution, as reported by Townsend [26].

3) Staining of nerve fibres: the nervous structures were stained using the method of Bodian [3]. It can be used to verify the percentage stain of the nerve fibres. In fact, this method stains nerve fibres and neurofibrils in black.

4) Enzymatic staining of AChE: in order to demonstrate cholinergic structures, the sections were processed according to the direct-colouring thiocholine method [13]. The substrate used was acetylcholine iodide (0.5 mM). Control sections were incubated to demonstrate non-specific cholinesterases using butyrilthiocholine iodide (Sigma, USA) and BW 284c51 (Wellcome, G.B.) as substrate and inhibitor respectively. The incubation was performed for 1 h and 6 h at room temperature. The sections were washed, counterstained in eosin and mounted with Entellan. Iso-octamethyl-pyrophosphoramide (iso-OMPA) was used at a concentration of 1×10^{-6} M as an inhibitor of non-specific cholinesterase activity, because it inhibits peripheral aspecific cholinesterase without pronounced effects on the specific AChE [6]. Control slides were incubated in buffer without either substrate or non-specific cholinesterase inhibitor. After the histoenzymatic staining, the sections were examined and photographed under a Zeiss Photomicroscope.

The techniques presently available for an accurate enzymatic staining of cholinergic nerve fibres consist of the visualisation of the enzymatic activities correlated with acetylcholine synthesis and/or catabolism. These techniques are not very reliable and, therefore, do not allow a satisfactory quantitative assay of this enzyme [19]. Consequently, our enzymatic results are only significant from a comparative point of view between samples from each homogeneous group.

5) Estimation of protein content: In all the experiments, autopsy samples were cut into small pieces, the lymphatic vessels were identified, drawn by scissors, placed on an ice-cold homogenisation buffer, homogenised in a Potter-Heim apparatus and ultra-centrifuged for collecting 2 phases: supernatant and pellet. Tissue protein concentrations were determined by the method described by Lowry et al. [17], using bovine serum albumin as a standard.

6) Biochemical amount of AChE: the AChE activity was measured both in supernatant and pellet by a colorimetric method. This method is designed for determination of tissue AChE activity and is based on the main principle that thiocholine formed during hydrolysis of substrate rapidly reacts with DTNB (5-5'-dithiobis-2-nitrobenzoic acid) and releases a coloured 5-thio-2-nitrobenzoate anion with maximum absorption at 416 nm. The AChE activity is expressed in International Units [IU] (nanomoles of substrate hydrolysed per minute per mg of protein).

Now, there are more sophisticated techniques for the identification and quantitative evaluation of many enzymatic activities, including AChE activity (immuno-

histochemistry, immune-electron microscopy, PCR techniques, immunoblotting, ELISA, HPLC). Nevertheless, many of these techniques can only be applied on fresh homogenates and not on small fragments coming from autopsies at least 1 day after death. Moreover, many tissues require a pre-fixation in Bouin's liquid that makes it impossible to apply these new techniques.

7) Quantitative analysis of images (QAI): in order to evaluate the amount of AChE, quantitative analyses of the intensity of the histochemical staining were performed on slides using a Quantimet Analyzer (Leica®). The values of control sections, incubated without substrates, were considered as 'zero'. Examinations were performed for each slide separately, evaluating the standard error of the mean (= SEM). QAI may be subjected to experimental bias. To ensure accuracy, a double blind technique using multiple replicates and obtained from different evaluations must be used. The values reported represent the intensity of staining for each sample and are expressed in conventional units [CU] ± SEM. The conventional units are arbitrary units furnished and printed directly by the Quantimet system.

8) Statistical analysis of data: the statistical methods used throughout this study must be interpreted as an accurate description of the data rather than a statistical inference of such data. The preliminary studies of each value were performed using basic sample statistics. Mean values, maximum and minimum limits, variations, standard deviation (SD), SEM, and correlation coefficients were performed according to Serio [21]. A correlative analysis of the morphological and biochemical data was performed by comparing the significant differences for each group of the results with the corresponding values of the other homogeneous groups. Correlation coefficients denote a significant level less than 0.001 ($p < < 0.001$), while the correlation coefficient is not significant when $p > 0.05$ (NS). This correlation coefficient was calculated according to Castino and Roletto [4].

RESULTS

Our results are reported in Figures 1–4 and summarised in Tables 1 and 2. Figure 1 is a light microscope image of many mesenteric lymphatic vessels in longitudinal section. Nuclei of endothelium are well stained by orange-eosine. They are prominent in the vessel lumen. The wall of these vessels appears at high magnification in Figure 2 stained by means of the Bodian's method. A dense network of nerve fibres with swellings and crossings along their course can be observed on the wall of the mesenteric lymphatic vessels. The relevant density of cholinergic nerve fibres is clearly visible.

Figures 3 and 4 show the AChE staining of the nerve fibres running along on the wall of the mesenteric lymphatic vessels. Numerous varicosities and swellings are visible along the course of the cholinergic nerve fibres thus suggesting an accumulation of the neurotransmitter within the fibres innervating the mesenteric vessels. Figure 3 was performed after an incubation time of 1 h (partial staining of a few nerve fibres), while Figure 4 was obtained after an incubation time of 6 h (total staining of many nerve fibres).

Table 1 contains the results of the AChE staining. Samples stained after 1 h of incubation and after 6 h of incubation were assessed by means of QAI, which was performed for a great number of images ($n = 50$, 10 fields for every image). We can see that after a short time of incubation the values are 26.8%, while after a long time of incubation the values reach 58.3%, when 0 is the black and 100 is the maximum value of the samples stained with the Bodian's method.

Table 2 shows the amount of the biochemical assay of AChE activity in pellet and in supernatant of arrangements of the lymphatic vessels after 1 and 6 h of incubation. We can observe that within the wall of the mesenteric lymphatic vessels (supernatant) the amount of the AChE activity is 32.6% after 1 h of incubation and 71.5% after 6 h of incubation. Therefore, the biochemical results confirm the data of morphological results.

DISCUSSION

Pathological and clinical findings

The lymphatic vessels have been studied in different organs from morphological and clinical point of view. However, the function of cholinergic innervation remains unknown. The present study investigated cholinergic innervation.

It is generally believed that the vessel wall of lymphatic vessels is less innervated than that of blood vessels. In man and in most animals, nerve fibres are confined to the adventitia, where they run longitudinally to the major axis of the vessel. Some morphological and probably functional interactions between nervous fibres and structures of the lymphatic system have been found in different animal organs. To date, published papers are not exhaustive as far as the distribution of nerve fibres within human lymphatic vessels is concerned. Knowledge of the physiological or pathological mechanisms regulating the functions of the lymphatic

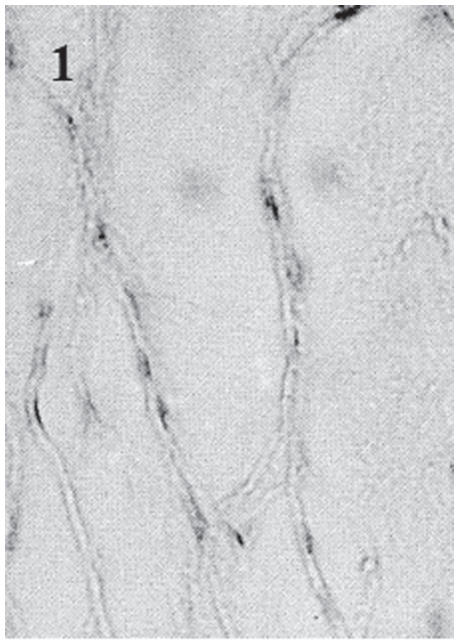

Figure 1. Light microscopy images of the human mesenteric lymphatic vessels. Longitudinal section of same lymphatic vessels, the nuclea of endothelium are prominent in lumen (magnification 4 ×).

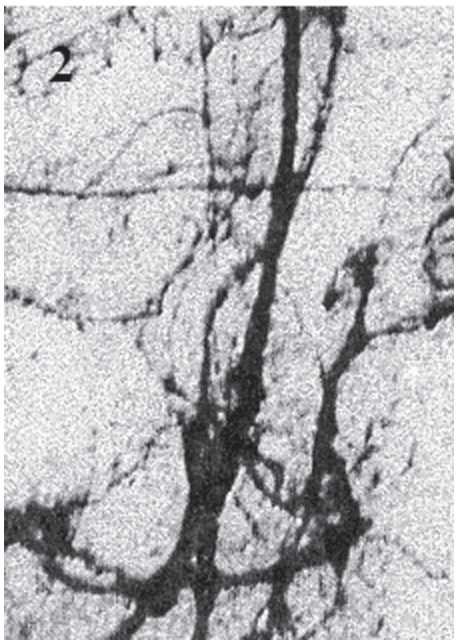

Figure 2. Bodian staining of nerve fibres. Along the wall of vessels we can observe numerous nerve fibres with thick bands, swellings and crossings on their course (magnification 400 ×).

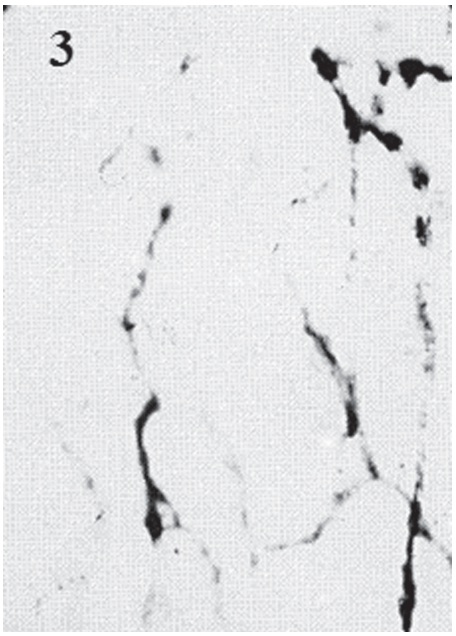

Figure 3. Acetylcholinesterase (AChE) staining of nerve fibres. After 1 h incubation we can see many AChE-positive nerve fibres along the wall of the lymphatic vessels (magnification 400 ×).

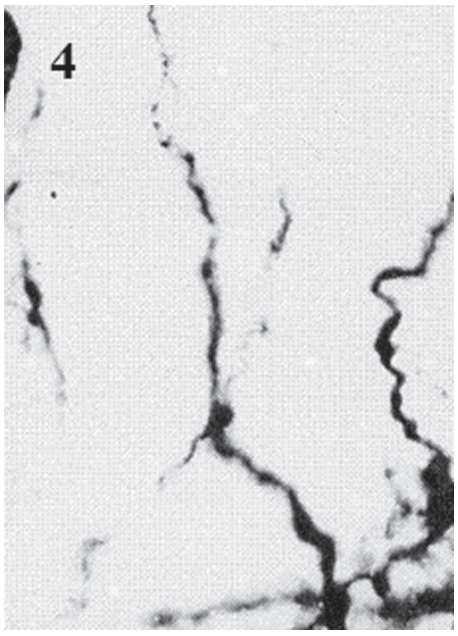

Figure 4. Acetylcholinesterase (AChE) staining of nerve fibres. After 6 h of incubation we can see the total staining of many AChE nerve fibres (magnification 400 ×).

system is incomplete, meaning that some diseases that probably have a morphological basis, may currently be erroneously considered as functional disorders.

Our results show that AChE positive nerve fibres are localised in the wall of the large human mesenteric lymphatic vessels.

Immunoreactivity for vasoactive intestinal peptide, a neuropeptide present in many cholinergic parasympathetic nerve fibres, was found to be sparse in human lymphatic vessels [9]. These results seen to suggest a parasympathetic role on the function of the lymphatic vessels. Our results show that AChE nerve

Table 1. Quantitative analysis of images of the acetylcholinesterase staining in the wall of the human mesenteric lymphatic vessels after 1 h and 6 h of incubation time

	1 h of incubation	6 h of incubation
Bodian [%]	26.8 ± 3.4	58.3 ± 4.1

Table 2. Biochemical assay of acetylcholinesterase activity in the wall of the human mesenteric lymphatic vessels after 1 h and 6 h of incubation time

	1 h of incubation	6 h of incubation
Pellet	8.1 ± 3.0	19.6 ± 2.1
Supernatant	32.6 ± 1.2	71.5 ± 2.5

fibres are sparse in mesenteric lymphatic vessels. Lymphatic capillaries are composed of a single layer of endothelial cells resting on a basement membrane. Their water channels are permeable to all interstitial fluid components, including protein. Interstitial fluid enters in these capillaries by bulk and fluid flows through lymph nodes and ends in 2 lymphatic ducts that drain into subclavian veins in the lower neck. Lymphatic vessels carry interstitial fluid back to the cardiovascular system and compensate for net filtration out of blood capillaries. Additionally, the lymphatic system provides a pathway across which the fat absorbed in the bowel reaches the blood. Infections causing blockage of the lymphatic system lead to accumulation of interstitial fluid, called oedema.

We may hypothesise that lymph flow is a passive phenomenon without the role of actively contracting the lymphatic vessels. On the other hand, a possible active contraction of the smooth muscle cells near the wall of the lymphatic vessel may be controlled by sympathetic nerve fibres. In the same conditions, what is the role of the AChE positive nerve fibres localised in the wall of lymphatic vessels? In our study the lymphatic vessels appear to contain few cholinergic nerve fibres. Therefore, perivascular nerve stimulation is expected to stimulate the cholinergic nerves innervating the mesenteric arteries to release the neurotransmitter AChE, which activates muscarinic or nicotinic receptors to modulate adrenergic neurotransmission. These results strongly suggest that perivascular cholinergic nerves have little or no effect on the adrenergic nerve function in mesenteric arteries. Previous studies [25] have shown that AChE induces the endothelium-independent vasodilation of denuded mesenteric vascular beds, which is mediated by endogenous calcitonin gene-related peptide (CGRP) released from CGRPergic nerves via direct stimulation of muscarinic AChE receptors located on CGRPergic nerves [23, 24]. A high concentration of AChE induces adrenergic and CGRPergic-dependent vasodilation via nicotinic AChE receptors by the same mechanisms as nicotine-induced vasodilation [23]. Substance P is a vasodilatator in blood vessels and increases the frequency of contraction in lymphatic vessels. CGRP has been demonstrated to potentiate substance P-induced effects via inhibition of neuropeptide degradation. Therefore, an increase in intraluminal pressure or appropriate thermal or chemical stimuli would cause the local release of substance P and CGRP, inducing lymphatic vessel wall contraction and propulsion of lymph. It is generally believed that the contraction of the lymphatic vascular wall is exclusively myogenic, the role of innervation being limited to modulation of the intrinsic contractile activity. The cholinergic nerves innervating mesenteric arteries do not mediate direct vascular responses. As in blood vessels, the haemodynamic regulation of both vasoconstriction and dilatation plays an important role in the blood circulation. Similarly, we may hypothesise that the lymph flow is not only a passive event, but that sympathetic and parasympathetic neurotrasmitters might play a certain role in supporting lymphatic circulation.

AChE is a rather resistant enzyme. In fact, the sections showing the histochemistry of this enzyme are maintained even after the fixation in formalin, and can be kept in a freezer at low temperatures for some months, without reducing the enzymatic activity. Moreover, AChE is resistant enough to the autolytic post-mortem phenomena (even organs harvested during autopsy are used to demonstrate AChE histochemistry). However, AChE is not thermoresistant (a temperature higher than 50°C may disactivate AChE). Techniques for cholinesterase localisation, using the specific inhibitors, are at the moment the only techniques able to show cholinergic nervous fibres at optic microscopy [15].

CONCLUSIONS

In conclusion, our findings confirmed the hypothesis that lymph flow is under actively controlled by parasympathetic AChE-positive nerve fibres too. In fact, the presence of a dense network of cholinergic fibres with evident varicosities and swellings within the wall of mesenteric vessels may lead us to hypothe-

sise an active role of these fibres in the regulation of the caliber and the functionality of the mesenteric lymphatic vessels, thus suggesting a more important and active contribution of the cholinergic innervation in the human mesenteric compartment.

Nevertheless, further morphological, biochemical and functional studies are needed to better define the role of the AChE-positive nerve fibres in human lymphatic mesenteric vessels.

REFERENCES

1. Azzali G (1990) Bases morphologiques et ultrastructurales du sysrem vasculaire lymphatique. Acta Biomedica Ateneo Parmense, 61: 1–40.
2. Azzali G (1992) Morpho-functional features in the absorbing peripheral lymphatic vessels of the diaphragm. Cong European Group of Lymphology, Prague 15–16.
3. Bodian D (1936) A new method for staining nerve fibers and nerve ending in mounted paraffin sections. Anat Rec, 65: 89–97.
4. Castino M, Roletto E (1992) Statistica applicata. Piccin, Padua.
5. Cooke HJ, Wang YZ (1994) H3 receptors: modulation of histamine-stimulated neural pathways influencing electrogenic ion transport in the guinea pig colon. J Auton Nerv Syst, 50: 201–207.
6. Du Bois KP, Dull J, Coon JM (1950) Studies on the toxicity and pharmacological action of octamethyl Pyrophosphoramide (OMPA, Pestox III). J Pharmacol Experim Therapie, 99: 376–393.
7. Guarna M. Pucci AM, Alessandrini C, Volpi N, Fruschelli M, D'Antona D, Fruschelli C (1991) Peptidergic innervation of mesenteric lymphatics in guinea pigs: an immunocytochemical and pharmacological study. Lymphology, 24: 161–167.
8. Hart HR, Thornbury KD, McHale NG (1993) Neurotransmission in isolated sheep mesenteric lymphatics. Microvasc Res, 46: 310–319.
9. Hukkanen M, Konttinen YT, Terenghi G, Polak JM (1992) Peptide-containing innervation of rat femoral lymphatic vessels. Microvasc Res, 43: 7–19.
10. Ichikawa S, Sreedharan SP, Goetz EJ, Owen RL (1994) Immunohistochemical localization of peptidergic nerve fibers and neuropeptide receptors in Peyer's patches of the cat ileum. Regul Pept, 54: 385–395.
11. Igarashi T, Ikomi F, Ohhashi T (1998) Electrical stimulation--induced alpha1-and alpha2-adrenoceptors-mediated contraction in isolated dog thoracic ducts. J Auton Nerv Syst, 71: 18–24.
12. Ji RC, Kato S (2000) Intrinsic Interrelation of lymphatic endothelia with nerve elements in the monkey urinary bladder. Anat Rec, 259: 86–96.
13. Karnowsky MJ, Roots L (1964) A direct colouring thiocholine method for cholinesterase. J Histochem Cytochem, 12: 219–221.
14. Kim M, Lee H, Lee N, Choi M, Kim J, Chang D, Choi M, Yoon J (2011) Ultrasound-guided mesenteric lymph node iohexol injection for thoracic duct computed tomographic lymphography in cats. Vet Radiol Ultrasound, 52: 302–305.
15. Koelle GB, Volle RL, Holmstedt B, Karczmar AG, O'Brien RD (1963) Anticholinesterase agents. Science, 141: 63–65.
16. Lobov GI, Pan'Kova MN (2012) Effect of histamine on contractile activity of smooth muscles in bovine mesenteric lymph nodes. Bull Exp Biol Med, 152: 406–408.
17. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem, 193: 265–275.
18. Panuncio AL, De La Pena S, Gualco G, Reissenweber N (1999) Adrenergic innervation in reactive human lymph nodes. J Anat, 194 (Part 1): 143–146.
19. Patrick WLA, Besley GTN, Smith IJ (1980) Histochemical diagnosis of Hirschprung's disease and a comparison of the histochemical and biochemical activity of acetylcholinesterase in rectal mucosal biopsies. J Clin Pathol, 33: 336–343.
20. Sacchi G, Weber E, Agliano M, Comparini L (1994) Subendothelial nerve fibers in bovine mesenteric lymphatics: an ultrastructural and immunohistochemical study. Lymphology, 27: 90–96.
21. Serio A (1986) Appunti delle lezioni di statistica sanitaria. Kappa, Rome.
22. Shimoda H, Isogai S (2012) Immunohistochemical demonstration of lymphatic vessels in adult zebrafish. Acta Histochem Cytochem, 45: 335–341.
23. Shiraki H, Kawasaki H, Tezuka S, Nakatsuma A, Nawa H, Araki H, Gomita Y, Kurosaki Y (2001) Adrenergic nerves mediate acetylcholine-induced endothelium-indipendent vasodilation in the rat mesenteric resistence artery. Eur J Pharmacol, 11: 231–242.
24. Takenaga M, Kawasaki H, Wada A, Eto T (1995) Calcitonin gene-related peptide mediates acetylcholine-induced endothelium-independent vasodilation in mesenteric resistance blood vessels of the rat. Circ Res,76: 935–941.
25. Tangsucharit P, Takatori S, Sun P, Zamami Y, Goda M, Pakdeechote P, Takayama F, Kawasaki H (2012) Do cholinrgic nerves innervating rat mesenteric arteries regulate vascular tone? Am J Physiol Regular Integr Comp Physiol, 303: R1147–R1156.
26. Townsed FM (1960) Manual of histologic and special staining techniques. Mc Graw-Hill, New York.
27. Weid PY (2013) Lymphatic myogenic constriction: how lymphatic vessels pump lymph uphill, J Physiol, 591 (Part 2): 391–392.
28. Yamada K, Hoshino T (1996) An examination of the close relationship between lymphatic vessels and nerve fibres containing calcitonin gene-related peptide and substance P in rat skin. Nogoya J Med Sci, 59: 143–150.
29. Zgraggen S, Ochsenbein AM, Detmar M (2013) An important role of blood and lymphatic vessels in inflammation and allergy. J Allergy (Cairo), 2013: 672381.