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Human coronary vessels: Distribution of cholinergic nerve fibres and age-related changes

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Abstract. *Background.* Cholinergic nerve fibres were studied in the human coronary vascular tree by means of acetylcholinesterase staining and choline acetyltransferase activities on autopsy samples of coronary vessels (arteries, veins, and micro-vessels). *Methods*. Samples of human coronary vessels were harvested in younger and older subjects. Samples were exposed to the enzymatic and or immune staining for acetylcholinesterase and choline acetyltransferase, two enzymes linked to the metabolism of acetylcholine. The morphological data were subjected to the quantitative analysis of images and to the statistical analysis of data. *Results*. Both acetylcholinesterase and choline acetyltransferase activities are localised in the human coronary vessels. Structures resembling cholinergic nerve fibres are located in the extra-parenchymal and intra-parenchymal branches of these vessels. *Discussion and Conclusions*. The quantitative analysis of images and statistical analysis of data demonstrate that the cholinergic innervation of coronary vessels (especially the extra-parenchymal branches) is well represented. Moreover, in older subjects both the enzymes are strongly decreased. The extra- and intraparenchymal branches of the human coronary arteries and veins are provided with cholinergic nerve fibres, which could control the efferent sensitive pathways and the autonomic nerve fibres of the coronary vascular tree.

Keywords: coronary vessels, Cholinergic Nerve Fibres (CNF), acetylcholinesterase (AChE), choline acetyltransferase (ChAT), histochemistry, age-related changes.

INTRODUCTION

Heart failure (HF) is one of the most common chronic pathologies in the elderly population and is characterised by a decrease in left ventricular function and biohumoural changes such as hyperactivation of the sympathetic system, the renin-angiotensin-aldosterone system and the inflammatory system (Hunt et al., 2009). The prevalence of HF in the general population has been estimated to be between 0.4 and 2%. In Italy about 2 million patients are affected by HF and more than a third of these do require an average annual hospitalisation involving a significant cost for the national health system (Rego et al., 2004).

The hyperactivity of the Sympathetic Nervous System (SNS) observed in heart failure initially represents an adaptation process aimed at compensating for the reduction in cardiac performance; however, the chronic increase in plasma CA is associated with a marked dysregulation of cardiac β-adrenergic receptors both at the receptor and post-receptor levels, responsible for a structural damage characterised by: ventricular hypertrophy, focal necrosis, inflammation and increased collagen deposition resulting in myocardial interstitial fibrosis. Furthermore, chronic HF is associated with a progressive loss of both adrenergic and cholinergic cardiac nerve fibres.

Cardiac tissue is extensively innervated by the autonomic nervous system, which is characterised by the presence of sympathetic and parasympathetic fibres. Sympathetic neurons release norepinephrine, which acts post-synaptically on the β1-AR and β2-AR receptors, whereas parasympathetic fibres release acetylcholine which acts on muscarinic receptors. The integration of sympathetic and parasympathetic fibres and their respective neurotransmitters is capable of modulating heart rate (chronotropic function), electrical impulse conduction speed (dromotropic function), myocardial relaxation (lusitropic function) and myocardial contraction (inotropic function) (Kapa et al., 2016).

Studies in the literature report that the density of cardiac nerve fibres decreases by up to 50% with age and is found to be altered in numerous pathological conditions (e.g. myocardial infarction) (Hopkins et al., 2000), in which cell proliferation occurs following Wallerian degeneration and disorganised axonal regeneration with consequent repercussions on cardiac function.

Despite numerous studies, cholinergic innervation in the human coronary vascular district has not been completely clarified (Hunt et al., 2009). Different animal species, in fact, have been showing different answers to acetylcholine (AChE). In particular, this enzyme is able to induce vasodilatation and vasoconstriction (Wang et al., 2006) in isolate preparations of coronary arteries, with the key role of endothelial cells (Rengo et al., 2004), through stimulation of muscarinic receptors (Kapa et al., 2016) and smooth muscle cells fibres. Moreover, an intra-coronary injection of acetylcholine in living humans produces an increase of the coronary circulation rate, hence a strong vasodilatation.

To explain these apparent contradictions, acetylcholine was supposed to induce vasodilatation in small coronary branches, while vasoconstriction of the large calibre extra-parenchymal branches (Bakovic et al., 2013; Hirsch & Kaiser, 1971, Staoyanou et al., 2021).

Subsequent studies on the nervous regulation of coronary circulation and on the cholinergic neuromodulation of the coronary vessels have failed to agree with one another (Ludmer et al., 1986; Armour, 2011).

Therefore, it was opted to carry out the present study to assess the presence in the human coronary vessels of acetylcholinesterase (AChE) and/or choline acetyltransferase (ChAT) positive nervous fibres, and their distribution in younger and older individuals.

MATERIALS AND METHODS

All procedures were in accordance with the ethical standards of the Declaration of Helsinki (1964) of the World Medical Association (https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/). All the photographs were made before 1979, when written informed consent was not available. Samples of coronary arteries, veins, and heart parenchyma (for the studies of the coronary microcirculation) were harvested, during autopsies performed 24-36 hours after death in 24 individuals of both sexes, whose ages had an average of 26 years in n= 8 subjects and of 76 in n= 16 subjects.

It has been previously demonstrated by More and Fatty (Moore & Fatty, 1958) that cholinesterase activity remains stable in tissues for as long as 24-48 hours after death, so that an autopsy series can be used also for immunochemical staining. The whole diameter of each vessel was measured with a calibrated gauge and all vessels were collected with the following calibre class (vd. Rhodin, 1967): 1) extra-parenchymal branches of large capacitance with diameter > 500 μm; 2) intra-parenchymal arteries of large calibre with diameter >150 μm; 3) arteries of middle calibre with diameter between 50-150 μm; 4) arterioles of small calibre with diameter between 35-50 μm; 5) micro-vessels with a diameter between 10-35 μm 6) capillaries with a diameter < 10 μm; 7,8

and 9) veins of small, middle and large calibre (with the same diameter as the related class of arteries).

Capillaries are made by only the endothelial layer. The vessels of middle calibre possess endothelial and adventitial layer, while the larger vessels possess three layers (endothelial, middle smooth muscle and adventitial). After harvesting, the samples were washed for 30-60 minutes at 4°C in an isotonic Na Sulphate solution and subsequently submitted to the following procedures : 1) either frozen and serially cut under a refrigerated microtome in order to obtain 8-10mm thin slices; 2) or paraffin embedded and serially cut in slices of 4-5µm, in transversal thin sections for the entire length of the vessel. These samples are considered as whole-mount and include all the thickness of the wall of the vessel; 3) other samples were dissociated under an operative microscope with the aid of a micro-scissor, into thin sheets, corresponding with the three normal components of the vascular wall (intima, media and/or adventitia). The samples 2 and/or 3 were made transparent by adding a drop of glycerol and mounted on glass slides for light microscopy. The sample 1 was vacuum-dried and dehydrated for 60 minutes in a P_2O_5 gaseous atmosphere. Then all the samples were submitted to: 1) Bodian's morphological method for the staining of all the types of nerve fibres; 2) enzymatic method for the staining of acetyl-cholinesterase (AChE) (this method stains AChE-positive nerve fibres); 3) immuno-histochemical method for choline acetyltransferase (ChAT) (this method stains specifically cholinergic nerve fibres); 4) quantitative analysis of images; 5) statistical analysis of the data.

1) Staining of all nerve fibres. All the nerve fibres were stained using the Bodian's method (Bodian, 1936). This method can be used to verify that a stained structure is nervous in nature. Indeed, it stains all nerve fibres and neuro-fibrils. After fixation, the sections were treated with: i. 1% Protargol solution (colloidal silver), ii. reducing solution (Hydroquinone + sodium sulphite), iii. 1% Gold chloride solution, iv. 2% oxalic acid solution and counterstained with 0.03% aniline blue. The nerve fibres and neuro-fibrils are stained in black.

2) AChE staining: The slices were incubated in a buffer phosphate solution containing 0.5 mM acetylcholine iodide (Koelle, 1963) in the presence of cholinesterase specific inhibitor iso-OMPA 10-6 mole/litre (Du Bois et al., 1950). The reaction lasted 30-60 min (short time). The controls were performed either avoiding the substrate or adding the specific inhibitor (Karnowsky & Root, 1964)

The addiction of eserine (specific inhibitor) to incubation medium or the lack of iodide acetylcholine substrate caused the negativity of the reaction expressed by not coloured CNF. The sections were washed in a cold phosphate buffer, stained for haematoxylin-eosin as contrast, mounted in Entellanâ (Merck) and observed under light microscopy.

3) ChAT staining: Transverse cryostat sections of our samples (20-40 m) were mounted on glass slides and allowed to dry at room temperature. The sections were then incubated at room temperature with anti-ChAT monoclonal antibodies Lyophilised Monoclonal (NCL-ChAT) Clone 38B12 (Novocastra) was used diluted 1:50 as indicated by the producer. Choline acetyltransferase (ChAT) is a 68kD enzyme which catalyses the synthesis of acetycholine (ACh) from choline and acetyl coenzyme A. The human ChAT gene encodes two proteins, the 68kD ChAT enzyme and a 27kD protein immunologically related and co-expressed with ChAT in cholinergic neurons of the central and peripheral nervous system. The smaller proteins may play a role in the regulation of ACh synthesis. NCL-ChAT specifically labels central and peripheral cholinergic neurons. NCL-ChAT does not label some central axons in the insular cortex or in the internal capsule, non-cholinergic structures, endothelial cells and microglia (Novocastra Catalog 2003). ChAT is the key enzyme for the turnover of acetylcholine (Perse, 1972). Slides were then rinsed and incubated each with 1 ml, diluted 1:200 in phosphate buffer, of a biotinylated goat anti-rabbit secondary antibodies. Sections were then incubated with an avidin-biotin-peroxidase complex, diluted 1:200 in phosphate buffer, (K3468, Dako Corporation, Carpentera, CA, USA) for 20 min, developed in acetate-imidazole buffer (containing 0.25 % nickel sulphate, 0.04 % diamino-benzidine, and 0.005 % hydrogen peroxide) counterstained with haematoxylin – eosin, dehydrated and mounted. Negative controls were performed with iso-type matched irrelevant antibodies, while positive controls were made with specific antisera raised against peripheral ChAT. All samples, without any selection, were stained, observed, photographed, counted by quantitative analysis of images, and submitted to statistical analysis. On the contrary, the images were selected in a limited number and are representative of the tissue sample in general. Only these images have been described in our morphological results, while the general results are comprehensive also of the findings emerging from all the observed images. Observations and photographs were performed with a photo-microscope Carl Zeiss (Jena Germany) PMQ II.

4) Quantitative analysis of images: A quantitative analysis of the intensity of the staining was performed by means of a Quantimet Analyser (Leicaä), provided with specific software including internal controls. The values coming from samples incubated without substrate were considered as 'zero'. The values reported in our experiments represented the intensity of staining for each type of vessel and are expressed as Conventional Units (C.U.) ± standard error of the mean; further detailson QAI and on definition of CU are reported in the Manual of the Quantimet Leica 2000 image analyser (Manual of methods: Quantimet Leica 2000).

5) Statistical analysis of data: To ascertain the significance of QAI, it is mandatory to perform a statistical analysis including basic statistical methods such as: mean values, maximum and minimum limits, variations, Standard Deviation (SD), Standard Error of Mean (SEM), probability index (p) and Student's t-test. The majority of these data were calculated, but not tabled (only SEM and p are tabled). All these statistical results demonstrate a high significance of our morphometrical data (Castino & Roletto, 1992).

RESULTS

In stretched flat on slides or in the serial sections of the left and right coronary arteries as well as of anterior and posterior interventricular branches we can observe AChE positive structures that had been identified as cholinergic nervous fibres (CNF). CNF have been observed in all three layers of the coronary arteries (adventitia, media, and intima) and in both times of incubation used (short and long times). The addiction of eserine (specific inhibitor) to incubation medium or the lack of iodide acetylcholine substrate caused the negativity of the reaction expressed by not coloured CNF. Comparing the figures 1 and 2 stained for AChE with short and long times of incubation we can see that CNF were organized in a plexus localized in outer adventitia zone. We can observe a strong increase of colouring with long times of incubation. Figures 3 and 4 are stained for ChAT with short and long times of incubation.

Table 1. Variations of the cholinergic nerve fibres in the human coronary vessels.

Coronary vessels	Subjects (n=12)
Large calibre coronary extraparenchymal branches*	19.3±1.6
Small calibre coronary extraparenchymal branches*	4.2±1.4**
Coronary veins*	1.5±0.16**

* All results are expressed in conventional unit (=CU) ± SEM.

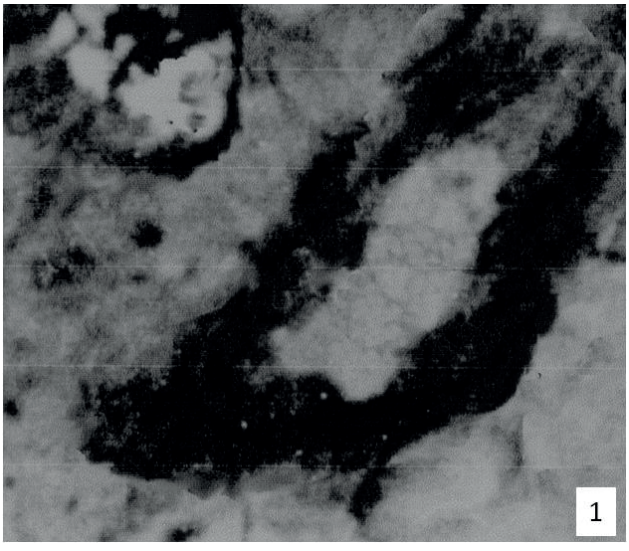

Figure 1. Full-thickness preparation of adventitia of a large intraparenchymal branch of the right coronary artery of a 57-year-old male individual. AChE activity after a short incubation time. Note the presence of staining in the adventitia of the vessel. (Original magnification: 450 x; calibration bar 10 mm). Image made in 1978.

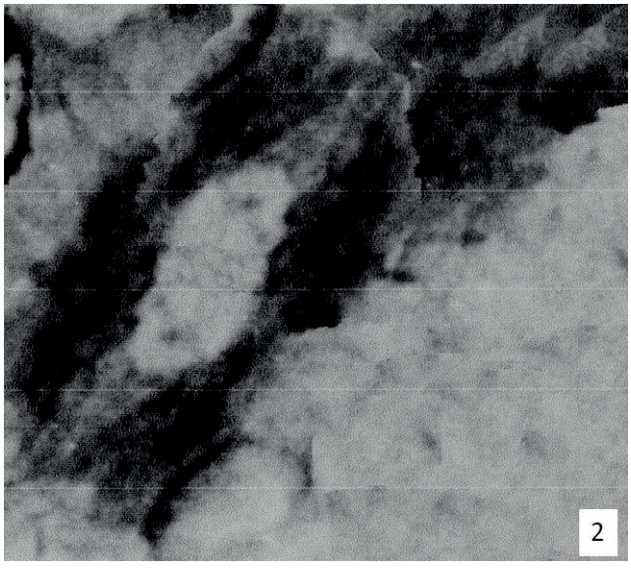

Figure 2. Full-thickness preparation of adventitia of a large intraparenchymal branch of the right coronary artery of the same subject as in Fig. 1 (serial section). AChE activity after a long time of incubation. We can observe a strong increase of the staining in the adventitia of the vessel. (Original magnification: 450 x; calibration bar 10 mm). Image made in 1978.

Table 2. Regional variations of the AChE-positive nerve fibres in the human coronary vessels after 1h or 6h of incubation.

Human coronary vessels	Incubation 1h	Incubation 6h
Left coronary artery (adventitia)*	29.3 ± 1.6	70.1 ± 0.9**
Posterior intra-ventricular branch*	25.2 ± 1.4	67.3 ± 0.6**
Microcirculation (arterioles and capillaries)	0	0
Small intraparenchymal branch (whole thickness transversal section)*	31.5 ± 1.6	82.4 ± 0.6**

* All results are expressed in conventional unit (=CU) ± SEM** P = 0.001 incubation 6h vs 1h.

Table 3. Age-related changes of the AChE-positive nerve fibres in the human coronary vessels.

Human coronary vessels	Young (n=8)	Old (n=16)
Large extra-parenchymal branches*	19.3 ± 1.6	10.1 ± 0.9**
Small intra-parenchymal branches*	14.2 ± 1.4	7.3 ± 0.6**
Microcirculation (arterioles and capillaries)	0	0
Large and Small veins*	11.5 ± 1.6	5.4 ± 0.6**

* All results are expressed in conventional unit (=CU) ± SEM.
** P = 0.001 older vs younger.

The results confirm the same observation as in figures 1 and 2. Great arterial branches appeared more diffusely innervated than smaller branches. In the larger arteries the periadventitial plexus is formed by thick CNF (from 20 to 30 μm in diameter) and by a few thin CNF (Fig. 1,2). In the smaller arteries thin CNF prevail on the thick ones. The coronary capillaries are not provided with CNF (Table 2), while the contiguous portions of myocardial tissue appears to be provided with CNF and others nervous structures resembling nerve endings. The coronary veins were provided with few CNF (Table 1). In the great arterial branches, the presence of some elbow shaped masses of AChE positive material very close to the CNF could be observed (Fig. 3,4). QAI performed on a great number of preparations yielded the opportunity to observe relevant regional variations and to highlight the stain's modifications in large and small calibre coronary branches (Table 1).

Therefore, in stretched flat on slides of the external layer (adventitia) or in the serial sections of the left and right coronary arteries as well as of anterior and posterior intra-parenchymal small branches we can observe both AChE and ChAT positive structures that had been identified as cholinergic nerve fibres (CNF) (Tables 3,4). CNF were organised in a plexus localised in outer adventitia or periadventitia. Large arterial branches

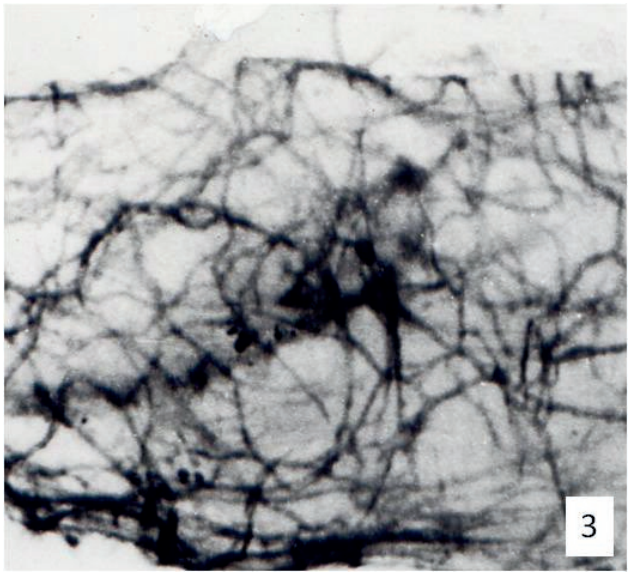

Figure 3. Left coronary artery in a 61-year-old male individual; full-thickness preparation of adventitia. Chat activity after a short time of incubation, periadventitial plexus. Two Chat-positive glomerular structures are located close to cholinergic fibres (Original magnification 450 x calibration bar 10 mm).

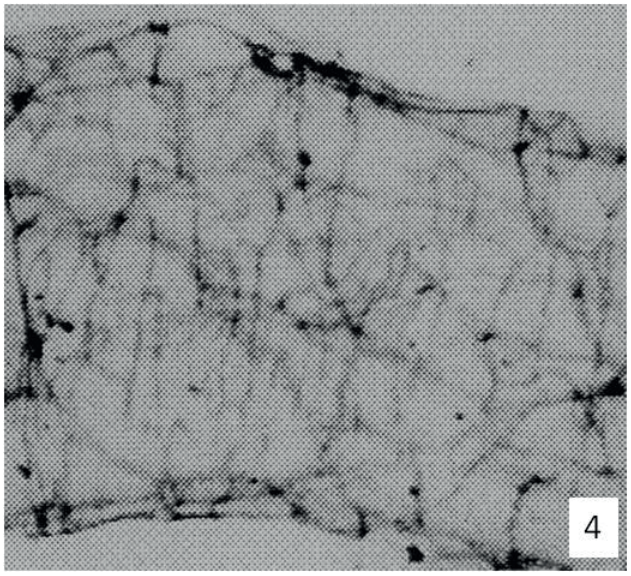

Figure 4. Left coronary artery in the same subject as in Fig. 3 (serial section); full-thickness preparation of adventitia. AChE and ChAT activity, periadventitial plexus. We can observe a strong increase of the staining of AChE and ChAT activity localized in the cholinergic nerve fibres similar as them in surrounding tissues (Original magnification 450 x calibration bar 10 mm).

Table 4. Age-related changes of the ChAT-positive nerve fibres in the human coronary vessels.

Human coronary vessels	Young (n=8)	Old (n=16)
Large extra-parenchymal branches*	29.3 ± 1.8	11.1 ± 1.3**
Small intra-parenchymal branches*	24.2 ± 1.6	7.3 ± 1.6**
Microcirculation (arterioles and capillaries)	0	0
Large and Small veins*	16.8 ± 1.3	5.8 ± 0.7**

* All results are expressed in conventional unit (=CU) ± SEM.
** P = 0.001 older vs younger.

appeared more diffusely innervated than smaller ones. In the larger arteries the periadventitial plexus is formed by thick CNF (from 20 to 30 μm of diameter) and by a few thin CNF (Tables 1,2). In the smaller arteries thin CNF prevail over the thick ones. In the large arterial branches, we observed the presence of some elbow shaped masses of AChE positive material very close to the CNF. QAI on a great number of preparations gave the opportunity to observe important regional variations and to highlight the modifications in old age. About changes related to age it came out that during old age there is a strong reduction of CNF (Table 3).

DISCUSSION

The control by the autonomic nervous system of correct cardiovascular function is influenced by various pathological and physiological factors such as: contractile activity of the myocardium, blood pressure, psycho-physical stress, metabolic diseases; in order to better understand the mechanism through which these factors participate in the onset and progression of cardiovascular diseases, it is essential to know the anatomical characteristics regarding the innervation of human cardiac tissue and above all the role played by aging on cardiac innervations (Mandsager et al. 2015; Millet et al., 2022; Harris et al., 2004).

The heart is innervated by the cardiac plexus, whose formation includes parasympathetic fibres coming from the vagus nerves and orthosympathetic fibres derived from ganglia and trunks of the cervical and thoracic tract of the orthosympathetic chain (Brack et al., 2015; Pather et al., 2003).

Nerve filaments originate from the cardiac plexus which, accompanying the right and left coronary arteries and their branches, are distributed to the heart; some fibres go to the sinoatrial node and the atrioventricular node, others to the atrial and ventricular myocardium and to the wall of the great vessels (Pather et al., 2003).

Our results show that CNF are present in extraparenchymal branches of coronary arteries, while on the contrary CNF are not present in the coronary microcirculation.

Sherf and co-workers (Sherf et al., 1977), studying the ultrastructure of human coronary vessels, described the presence of fibres and nervous endings even in small calibre arteriole and in microcirculation.

In our study, coronary capillaries and microcirculation appear devoid of CNF.

Nevertheless, in coronary blood flow the constriction and the dilatation of microcirculation and/or of the pre-capillary sphincters play an important role. So, the absence of CNF seems to indicate that cholinergic system does not play a role in the coronary microcirculation (Houghton et al., 1998).

More likely, the stained CNF may represent afferent nerve fibres providing information to nervous central system (Shigei et al., 2010).

This hypothesis seems to be supported by the presence of AChE positive glomerular formations in CNF plexus. These formations are similar to cholinergic afferent terminations located in rich baroreceptors areas as aortic arch and the carotid sinus (Norcliffe-Kaufmann et al., 2019). CNF have also been considered as sensory or afferent fibres. These receptors are localized around the large branches of coronary arteries. Brown suggested that these receptors were probably located in the wall or near the large calibre coronary arteries (Norcliffe-Kaufmann et al., 2019).

Acetylcholine is the chemical mediator of postganglionic parasympathetic endings. Since there are at the moment, no techniques able to histochemically demonstrate acetylcholine, cholinergic nervous fibres in peripheral nervous system are pointed out thanks to the stain of enzyme that catabolizing acetylcholine: (acetylcholinesterase-AChE). In origin, the technique used to localize AChE in tissues was proposed in 1953 by Gerebtzoff (Gerebrzoff, 1953). After that were brought many modifications to the original technique to reduce the artefacts (coming from the enzyme diffusion) and to increase the specificity and the intensity of the reaction. Karnowsky and Root (Karnowsky & Root, 1964) introduced the "cholinesterase direct pointing out technique" using thiocholine. This method represents, at the present time, one of the most used techniques to point out AChE at electron microscopy. Nevertheless, histochemical techniques, above mentioned, show and AChE, the 'true cholinesterase', and not specific cholinesterase. To eliminate non-specific cholinesterase, sections must be treated with phosphoric acid esters (DFP, Mipafox, Iso OMPA, etc.); these substances inhibit non-specific cholinesterase without alter the 'true AChE'.

AChE is a rather resistant enzyme. In fact, the sections used to show the histochemistry of this enzyme stand even the fixation in formalin and can be kept in freezer at low temperatures for some months, without reducing the enzymatic activity. Moreover, AChE is resistant enough to the autolytic *post-mortem* phenomenons. However, AChE is not thermoresistant (a temperature higher than 50°C may inactivate AChE). Techniques for cholinesterase localisation using the specific inhibitors, are, at that present, the only techniques able to show cholinergic nervous fibres at optics microscope (Koelle, 1963). In relation to the strong presence of parasympathetic innervation in heart many studies were performed to assess the cholinergic innervation pattern. These studies have highlighted the cholinergic nervous fibres distribution in the heart, in the coronary and pulmonary vessels and in the aorta and caval veins.

It has been demonstrated that CNF are distributed in coronary vessels but in small laboratory animals, coronary vessels have a limited cholinergic innervation. Our findings seem to agree with all these data. Nevertheless, further morphological and functional studies are needed to define the role and function of CNF in the coronary circulation.

In our study, a reduction in CNF was highlighted in elderly subjects compared to young subjects. In the heart of elderly people, changes are found especially at the level of the myocardial tissue and coronary arteries; these changes almost always depend on various pathological factors that occur at an advanced age and which, associated with physiological aging, have repercussions on the correct functioning of the cardiovascular system. Several studies confirm that changes related to aging are caused by the prolongation of the duration of contraction, with a consequent decrease in inotropic responses to catecholamines and cardiac glycosides and an increase in mechanical refractoriness; furthermore, alterations in myocardial relaxation are also highlighted which can be correlated with the prolongation of contractile activity (Bruzzone et al., 2003; Docherty, 2002). In light of these data, it appears clear that the vascular insufficiencies that occur during aging can be a consequence of alterations in cardiac electrical activity, specifically alterations in the duration of the action potential and alterations at the level of the sarcoplasmic reticulum. With age, changes in electrical activity are also evident at the level of the sinoatrial and atrioventricular nodes as well as in the bundle of His and Purkinje fibres. These changes appear to be caused by both anatomical and physiological modifications of the main arteries. Furthermore, aging also appears to be related to a change in the effect and above all in the effectiveness of the drugs used for the treatment of vascular insufficiencies (Bruzzone et al., 2003; Docherty et al., 2002; Njemanze et al., 2016). Therefore, during aging both anatomical and physiological cardiac remodelling occurs with a consequent slowdown of some functions relating to the electrical activity of the heart. It is clear that this process can play a fundamental role in the development and progression of cardiac and vascular insufficiency that develops at an advanced age. The autonomic nervous system also regulates cardiovascular homeostasis through regulation of heart rate, myocardial contraction, and vasoconstriction, contributing largely to such age-related changes (Bruzzone et al., 2003; Docherty, 2002; Njemanze et al., 2016; Francis Stuart et al., 2018).

AUTHORS' CONTRIBUTIONS

ST, AC and VP designed the study. FF, IV, LC, FMG, FC and VS consulted literature and collected data, ST, AC and FF wrote the paper. FMG reviewed and edited the manuscript. All authors read and approved the manuscript.

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