Original article

Effect of prenatal low-protein diet in subepicardial neuron of rat: a morphological study

Akamatsu, FE.[1]*, Gama, EF.[2], Andrade, M.[1], De Souza, RR.[2] and Jacomo, AL.[1]

[1]Human Structural Topography, Laboratory of Medical Research (LIM 02),
Department of Surgery, School of Medicine, University of São Paulo – USP,
Rua Capitão Otávio Machado, 817, CEP 04718-001,
Chácara Santo Antonio, Santo Amaro, São Paulo, SP, Brazil

[2]Laboratory of Human Movement, São Judas Tadeu University – USJT,
Rua Taquari, 546, CEP 03166-000, Mooca, São Paulo, SP, Brazil

*E-mail: flaea@usp.br

Abstract

Heart autonomic ganglia are known to play an important role in cardiac rhythm control, protecting against certain arrhythmias due to their parasympathetic activity. Cardiac disorders may arise following starving states during pregnancy; cardiac performance and cardiac fibers have been shown to suffer deleterious effects under starvation. Morphology of these plexuses may suffer interference of extrinsic factors, but data is still lacking about the effects of low protein diet during pregnancy and early postnatal period on subepicardial neuron structure. Two groups of pregnant Wistar rats were submitted to different diets according to its protein content, normal and 5% casein, until 21 days after delivery. The offspring was divided in two groups, D and N, according to their mother´s diet, low and normal protein respectively, and then sacrificed. The atrial neurons were identified by β-nicotinamide adenine dinucleotide (NADH) and adenine dinucleotide phosphate diaphorase (NADPH-d) staining. Profile areas of the nerve cell bodies were measured. NADH staining did not show significant differences between groups but NADPH- d profile areas of nerve cell bodies from group D were smaller than in control group. Ultrastructural changes were observed in group D rats: agglomerated ribosomes, increase in nucleoli density and irregular chromatin.Low-protein diet in rats at early developmental stages interferes in size, and ultrastructure of subepicardial neurons. Even though underfeeding during perinatal period did not produce neuronal death, neuron development is delayed and permanent changes can supervene in long term.

Keywords: low-protein diet, morphometry, subepicardial neurons, ultrastructure, undernutrition.

1 Introduction

Afferent (sensorial), efferent (motor), and interneurons control atrial ganglia activity in rats (CHENG, POWLEY, SCHWABER et al., 1997; ARORA, ARDELL and ARMOUR, 2000; WILSON and BOLTER, 2002). Autonomic heart ganglia have intrinsic activity and work as an interdependent feedback control system. Thus, loss of central stimuli does not prevent independent ganglia activity (ARORA, ARDELL and ARMOUR, 2000). Subepicardial ganglia play an important role in cardiac rhythm regulation (CALARESU and STLOUIS, 1967; PARDINI, PATELl, SHMID et al., 1987; BURKHOLDER, CHAMBERS, HOTMIRE et al., 1992). Knowledge of normal morphology of the subepicardial nervous plexuses and its alterations in pathological conditions is crucial for understanding mechanisms of cardiac rhythm control (STEELE, GIBBINS, MORRIS et al., 1994; EDWARDS, HIRST, KLEMN et al., 1995; KENNEDY, HARAKALL and LYNCH, 1998). A subpopulation of NADPH-d reactive neurons displays a specific function of cardiac neurons (RICHARDSON, GRKOVIC and ANDERSON, 2003). Despite the unclear role of nitrous oxide (NO) in neuronal function, data suggest it may act as a neuromodulator (SCHOLZ, LABENIA, DE VENTE et al., 2002), signaling and regulating cardiac vagal activity (CONLON AND KIDD, 1999; TAKIMOTO, AOYAMA, TANAKA et al., 2002). In addition, NO causes atrial myocardial relaxation (TANAKA, TAKAYAMA, HAYAKAWA et al., 2001), inhibits sympathetic stimuli to the heart (SEARS, CHOATE and PATERSON, 1998) and locally controls cardiac function under normal and pathological conditions (ARMOUR, SMITH, LOSIER et al., 1995).

Extrinsic factors such as aging can influence morphology of this plexus (AKAMATSU, DE SOUZA and LIBERTI, 1999). On the other hand, it is well known that low weight and size at birth, secondary to malnutrition at different gestational ages, are related to cardiovascular diseases in adulthood (BARKER, GLUCKMAN, GODFREY, et al., 1993; SINGHAL, COLE, FEWTRELL et al., 2004). Considering the increased growth rate in the first weeks after conception, factors that can alter growth such as malnutrition, can also presumably affect developmental program permanently (SINGHAL, COLE, FEWTRELL et al., 2004). Some studies have shown on the effect of malnutrition on cardiac performance (DROTT and LUNDHOLM, 1992). However, none has analyzed the effects of low-protein diet on the structure of the subepicardial neurons. Given the importance of these neurons, the present study aims to analyze the effects of pre and postnatal protein malnutrition on the morphology of subepicardial neurons.

2 Material and methods

The study was conducted according to current legislation on animal experiments of the Biomedical Science Institute of the University of São Paulo. Young male and female Wistar rats (200-240 g body weight) were mated. After conception, which was assumed to have occurred when vaginal plugs or sperm were found, the females were placed in individual cages. The nourished mothers received an AIN-93G normal protein diet and the undernourished mothers received the AIN-93G diet with 5% casein (Rhoster Indústria e Comércio Ltda, São Paulo, Brazil), according to the protocol of Reeves (REEVES, NIELSEN and FAHEY, 1993). The rats were maintained under standard conditions at 21 °C, with a 12 hours light–dark cycle, and supplied with water ad libitum. Following delivery, adult rats and their offspring received the same diet the mother had during pregnancy. There were two experimental groups according to their diet: low (D) and normal protein diet (N). Experimental groups were kept for 21 days and then weighed and sacrificed with a single intraperitoneal dose of pentobarbital (HYPNOL® Fontoveter) 30 to 40 mg.kg^{-1} at the left inferior quadrant (WAYNFORTH and FLECKNELL, 1992). The thoracic wall was opened and their heart and basal vessels were removed for examination.

2.1 Histochemical method

2.1.1 β-nicotinamide adenine dinucleotide (NADH) reaction

Five animals from each group (N, D) were submitted to this technique. Demonstration of neurons by NADH diaphorase histochemical technique was previously described (GABELLA, 1987). Animals were perfused with Krebs solution and the thoracic cavity was opened. The heartlung blocks were isolated and, subsequently, the atria were separated from the ventricles by careful dissection with stereoscopic microscope. Subepicardical fatty tissue was removed using ophthalmologic instruments. Atria were then removed and kept in Krebs solution for 30 minutes and afterwards immersed in Triton-X 3% solution for 10 minutes, to facilitate staining medium penetration and washed in Krebs solution. Samples were kept for 45 to 60 minutes in a solution of Nitro-Blue Tetrazolium (NBT-Sigma) 0.5 mg.mL^{-1}, sodium phosphate buffer (0.1 mol.L^{-1}; pH 7.3) and 0.5 mg.mL^{-1} of β-nicotinamide adenine dinucleotide (NADH-Sigma) in reduced form. The staining reaction was interrupted by immersing the atria in a formalin fixative solution 10% in sodium phosphate buffer (0.1 mol.L^{-1}; pH 7.3) for a period of 1 to 3 days. At the end of this period atria were processed as whole mount preparations under stereoscopic magnification and assembled on slides in glycerin solution. The profile areas of the nerve cell bodies were measured by examining the whole-mount preparations under a binocular microscope at 400× magnification.

2.1.2 β-nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) reaction

Also five animals from each group (N, D) were studied with this technique. Neuronal NADPH-d staining was obtained following the protocol described by Santer (1994). Atria were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) pH 7.2 solution at 4 °C for 30 minutes. After washing in phosphate buffer (2 times during 10 minutes at room temperature), atria were incubated and homogeneized for 60 minutes at 37 °C in specific medium for NADPH-d demonstration: b-NADPH (0.1 mg.mL^{-1}) in reduced form (Sigma) and nitro-blue tetrazoliun (Sigma) solution (0.5 mg.mL^{-1}) in phosphate buffer containing 0.2% Triton X-100.

2.2 Morphometry of subepicardic neurons

The neuronal profile area was determined in 100 neurons randomly chosen and respective nuclei on each of the total membrane specimens (100 neurons of each of the five animals per group) by means of a computerized image acquisition method for morphometric analysis (KS 300-Zeiss). Neuronal profile of acquired images was automatically measured by the software.

2.3 Statistical analysis

All data (neuronal profile area) were presented as means ± SD. The results of morphometric studies were statistically analyzed using Student's t test. The level of significance was set at $p < 0.05$. Data analyses were performed using SPSS for Windows (Version 15.0).

2.4 Ultrastructure of intracardiac neurons

For this part of the study, the technique described by Bozzola and Russel (1991) was employed. Atria and ventricles of three animals per group (N, D) were perfused with a 2% glutaraldehyde fixative solution in sodium phosphate buffer (0.1 mol.L^{-1}, pH 7.3). Then, atria were removed and dissected as previously reported and immersed in the same fixative solution. Two millimeters fragments close to the basal pulmonary and cava veins, where cardiac neurons are located, were dissected and kept for two hours in the same fixative solution at room temperature. Then, specimens were washed in sodium phosphate buffer (0.1 mol.L^{-1}, pH 7.3) and post fixed in a 2% osmium tetroxide solution for two hours at 4 °C. Specimens were then washed in saline and immersed in a 0.5% aqueous solution of uranyl acetate for a period from 8 to 12 hours. Dehydration of specimens was obtained through serial baths in progressive concentrations of alcohol (from 70 °GL to absolute). After two baths of propylene oxide for 15 minutes, specimens were embedded in oxipropylene resin (1:1) from 8 to 12 hours, before inclusion in Araldite. Semi thin sections 1μm thick were obtained and stained with toluidine blue. Ultra fine sections were stained with a saturated alcoholic solution of uranyl acetate and lead citrate (REYNOLDS, 1963). Specimens were then analyzed in a transmission electronic microscope (JEOL, 1010).

3 Results

3.1 Neuronal profile

Area of the neuronal profile reactive to NADH was 212.05 ± 24.41 μm² in group N and 166.59 ± 32.79 μm² in group D, without significant difference between groups ($p > 0.05$). The mean size of the neuron nuclei was 72.82 ± 13.13 μm² in group N and 55.06 ± 9.88 μm² in group D, also without significant difference between groups ($p > 0.05$) (Figure 1). The profile area of neurons reactive to NADPH-d was 230.57 ± 68.10 μm² in group N and 100.05 ± 28.88 μm² in group D, representing a statistically

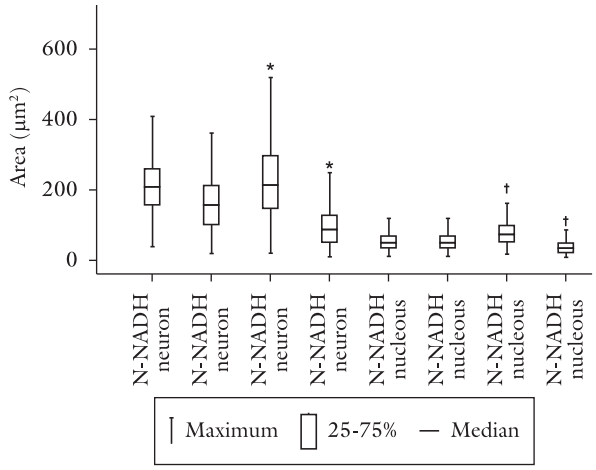

Figure 1. Distribution frequency of the area of the subepicardial neurons and their nuclei in animals from groups N, D stained by NADH and NADPH-d techniques. For each group n = 5 * $p < 0.05$ † $p < 0.05$

significant decrease of 57% (Figure 2). The mean nuclear area observed were 77.89 ± 22.10 µm² and 39.10 ± 10.98 µm², respectively in groups N and D, a significant decrease of 49%. The ratio between cell and nuclear area was the same in both groups (Figure 1).

3.2 Morphology and ultrastructure of intracardiac neurons

NADH diaphorase technique demonstrated subepicardial ganglia located at the subepicardic connective tissue of the external surface of both atria musculature. The ganglia presented elongated, starry, polygonal, or round patterns and some variations in staining intensity were observed, mainly with the NADPH reaction (Figure 2c, d). Also, neurons had different sizes (small, medium and large), with oval, spindle, and pear-like forms. The nuclei were spherical and predominantly found at the peripheral area of the cytoplasm. In all groups, ganglia presented a thin capsule of connective tissue constituted by collagen fibers irregularly arranged (Figure 3e, j). The endoplasmatic reticulum in group N

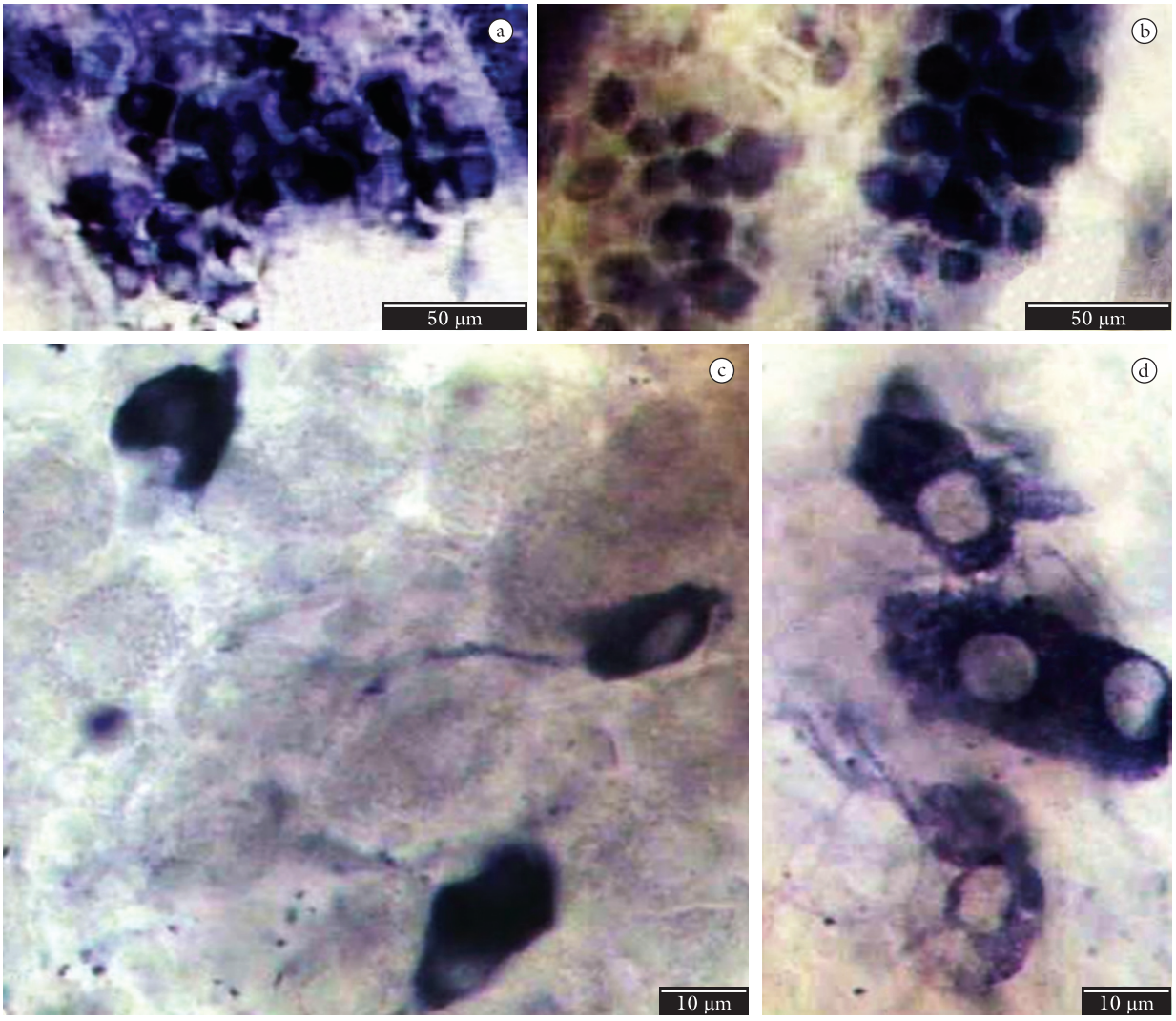

Figure 2. Subepicardial neurons of groups N (a, c) and D (b, d) stained by NADH (a, b), NADPH-d (c, d) histochemical techniques. In group N and D the area of the neuronal profile reactive to NADH (a, b) shows no difference between groups. In all groups and both reactions some variations in staining intensity were observed. In C and D only 2 to 4% of the ganglion neurons in rats' heart are NADPH-d reactive, Bars = 50 µm (a, b) and, Bars = 10 µm (c, d).

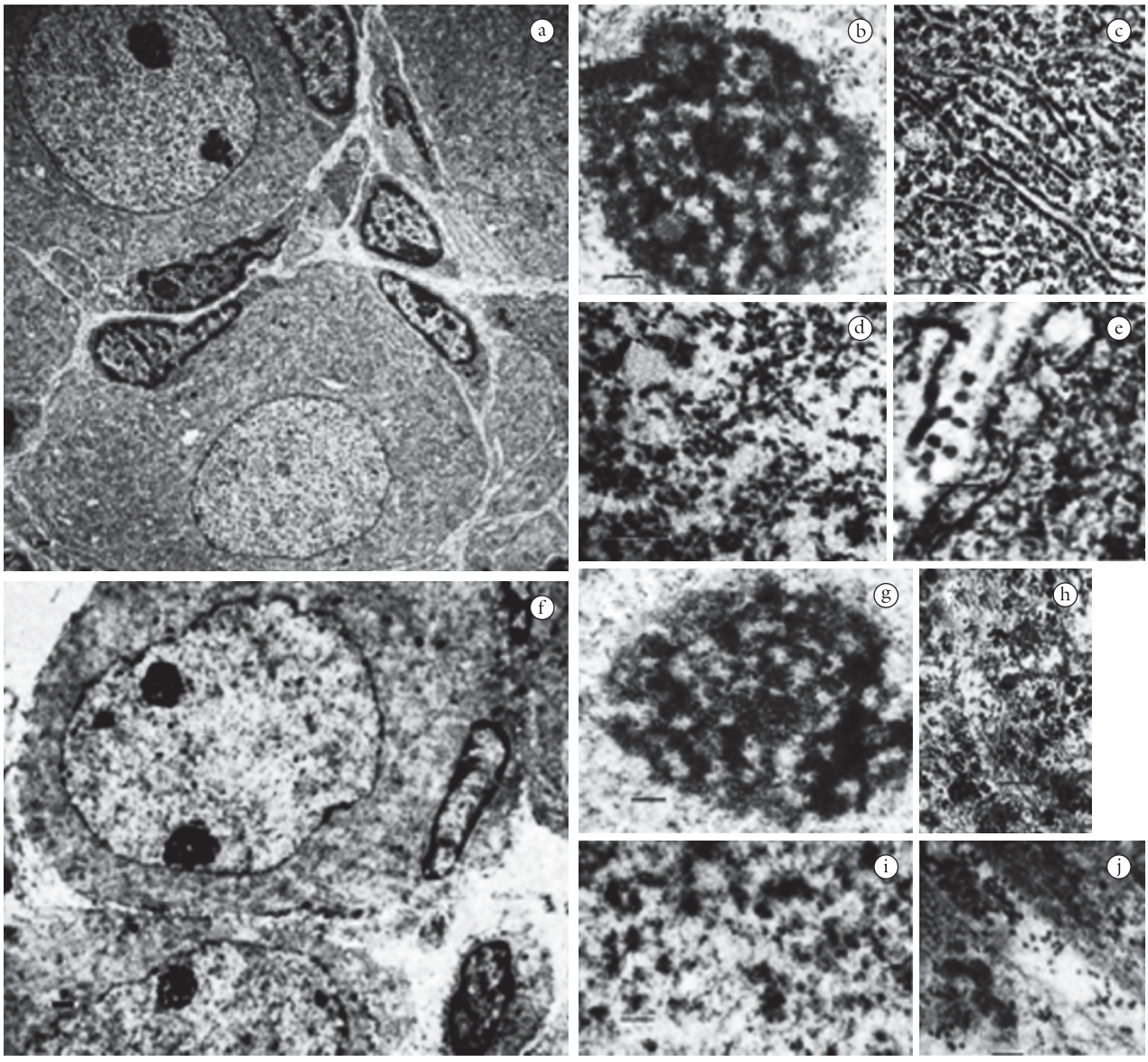

Figure 3. Electron micrographs of subepicardial neurons of group N (a, b, c, d, e) and group D (f, g, h, i, j). In H, it can be seen, a ribosomes agglomerate, and in I, chromatin disorganization. In E and J collagen fibers form the ganglion capsule. b, c, d, g, h, i (Bars = 500 nm) e, h, j (Bars = 100 nm) a, f (Bars = 100 micra).

showed a great number of ribosomes aligned at the external surface of its membranes in a regular pattern (Figure 3c). In group D, ribosomes were disposed in agglomerates with the membranes not so evident (Figure 3h) and irregular chromatin (Figure 3i). In this group, nucleoli appeared to be more electrondense (Figure 3g).

4 Discussion

Malnutrition produces severe underdevelopment in many behavioral aspects of experimental animals, such as locomotion, feeding, mounting, and prancing (MASSARO, LEVITSKY and BARNE, 1977). Motherly behavior also changes, like taking inadequate care of the brood (SMITH, SECKL, EVANS et al., 2004). This may be particularly important to physiological heart functions, especially after weaning, when the animal should be physically independent and needs larger cardiovascular request for survival, a fundamental role of subepicardical neurons that control heart beats and blood pressure.

Whole-mount preparation is a 3-dimensional technique whose advantage over sections is that one can view; make measurements of neuron somata and compare regions of the entire intracardiac plexus all in one preparation. The neuron cell profile area studied by nicotinamide adenine dinucleotide (NADH) diaphorase staining, were not stastistically different between N and D groups, even though body weight in group D animals were significantly lower than control ones. This finding differs from what is observed in aging where subepicardial neuron cell profile area in rats (AKAMATSU, DE SOUZA and LIBERTI, 1999) and cardiac neurons in dogs (PAUZA, PAUZIENE, PAKELTYTE et al., 2002) are of a bigger size. This data suggest that there

is no direct correlation between malnutrition and aging regarding morphofunctional patterns, even though both are recognized as stressing factors to nervous cell survival. Interestingly, neuron cell profile area studied by Her, Fu, Li et al. (2000) using NADH diaphorase staining in cardiac ganglia neuron in cell culture showed cell areas varying from 100 to 450 μm² after 6 days and 200 to 300 μm² after 7 days. In our study, the mean size of the cells were similar, variation were different (group N, 40 to 529 μm² and group D, 20 to 529 μm²), suggesting that culture medium does not reproduce natural organ environment. Nevertheless, profile area of NADPH-diaphorase positive neurons was significantly lower in the undernourished group if compared to controls. Klimaschewski described that only 2 to 4% of the ganglion neurons in rats' heart were NADPH-d reactive (KLIMASCHEWSKI, KUMMER and MAYER, 1992), fact that is supported by our findings of NADPH-d reactive neurons. Considering the fact that complete cardiac neuronal development occurs at the third week and that no changes appear after this period (HORACKOVA, SLAVKOVA and BYCZKO, 2000), neuronal size was affected by malnutrition, probably as a consequence of a maturation delay in neurons reactive to NADPH-d. Horackova demonstrated that hearts of newborn rats are able to mediate reflex changes in cardiac activity and that the intrinsic cardiac neurons in rats change during the first post natal month (HORACKOVA, SLAVKOVA and BYCZKO, 2000). These changes are related to the development of the extrinsic nervous control of the heart. Thus, variations in NADPH-d reactive neurons profile areas until 21 days are due to a vulnerability of the nervous system to malnutrition.

The results of several studies strongly support the hypothesis that nitrous oxide released from neuronal sources has an important facilitator action on the vagal control of the heart (CONLON, COLLINS and KIDD, 1998; CONLON and KIDD, 1999; MARKOS, SNOW, KIDD et al., 2002). Elvan, Rubart and Zipes (1997) demonstrated that NO has a stimulatory effect in mediating vagal neurotransmission and modulation of sympathetic effects and an inhibitory role in sympathetic neurotransmission. In addition, prior pharmacological studies in guinea pigs suggest that NO facilitates the negative chronotropic effects of vagal stimulation (CONLON, COLLINS and KIDD, 1998; SEARS, CHOATE and PATERSON, 1998; CHOATE and PETERSON, 1999; HERRING, GOLDING and PATERSON, 2000; HERRING and PETERSON, 2001). Danson and Paterson (2003) investigated whether enhanced cardiac vagal responsiveness elicited by exercise training was dependent on neuronal nitric oxide synthethase. It was suggested that the mechanism of action of NO might be facilitation of acetylcholine release, either at the preganglionic- postganglionic or at postganglionic-muscle synapse (CONLON and KIDD, 1999; HERRING and PETERSON, 2001).

Ultrastructural changes were observed in neurons of the peripheral nervous system by some authors. Irregular nuclei were observed with aging in the spinal ganglia (AMENTA, 1993). Myeloid bodies as well as electron lucent vacuoles and axon terminals displaying degenerating changes were observed in the cytoplasm of subepicardial neurons in ischemic heart. This is an indication of the effects on the functional integrity of this final common regulator of cardiac function in disease states (HOPKINS, MACDONALD, MURPHY et al., 2000). Our results showed a disarrangement of the chromatin and presence of agglomerated ribosomes in the cytoplasm of the neurons in the group submitted to a low protein diet. Some features related to malnutrition found in this study include the organization of the endoplasmatic reticulum, nuclear chromatin distribution and electrondensity of some vesicles. Similarly, Yamano, Shimada, Uamasaki et al. (1980) observed that embryonic cerebral cortex had delayed neuronal maturation, including dendritic branching; yet relationship between areas of neuronal and nuclear profile area showed no changes. In general, nutritional strain in human adult hearts does not lead to functional deficits (CHAUHAN, NAYAK and RAMALINGASWAMI, 1965). However, the same strain during the developmental period of life can produce, in the long term, permanent changes or even death. It is necessary to have further studies with a larger observation time and to assess the impact of renutrition in these neurons.

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