Original article

Effects of protein deprivation and refeeding on the subepicardial neurons of rat: subepicardial neurons and low-protein diet

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Abstract

Heart autonomic ganglia play an important role in cardiac rhythm control, protecting against certain arrhythmias due to their parasympathetic activity. Starvation during pregnancy may cause cardiac disorders and hinder optimal cardiac performance. Also, morphology of subepicardial neuron is subjected to the influence of extrinsic factors. We studied the influence of protein deprivation on subepicardic neurons in rats at early development stages and the effect of restoration of a normal diet.: Three groups of pregnant Wistar rats were submitted to different diets according to its protein content: normal (NN group) and 5% casein (DD group), until 42 days after delivery and low protein for 21 days with refeeding for a further 21 days (RN group). All animal were weighed. The number and area of neuronal profiles were measured. The neurons were stained by histochemical methods β-nicotinamide adenine dinucleotide (NADH) and β-nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) and their ultra structure were observed.Group DD and RN animals weighed less than those from group NN. The number of neurons and the cellular profile area did not show significant differences among groups for both techniques. Endoplasmatic reticulum ribosomes in neurons of undernourished animals showed decreased electron density. Protein deprivation in early stages of development produces ultra structural changes but does not alter the number and profile area of nerve cell bodies in rats.

Keywords: neurons, protein malnutrition, morphology.

1 Introduction

Afferent (sensorial), efferent (motor), and interneurons control atrial ganglia activity in rats (ARORA, ARDELL and ARMOUR, 2000; WILSON and BOLTER, 2002; GRAY, JOHNSON, ARDELL et al., 2004). The interacting sinoatrial ganglion and the posterior atrial ganglia are responsible for the peripheral autonomic control of cardiac rate (GRAY, JOHNSON, ARDELL et al., 2004). These ganglia have intrinsic activity and work as an interdependent feedback control system. Thus, loss of central stimuli does not prevent independent ganglionic activity (ARORA, ARDELL and ARMOUR, 2000).

Subepicardial ganglia play an important role in cardiac rhythm regulation (GRAY, JOHNSON, ARDELL et al., 2004; BURKHOLDER, CHAMBERS, HOTMIRE et al., 1992). Vagal activity through these ganglia can protect against certain arrhythmias, including atrial fibrillation. Furthermore, motor and sensorial inputs can be processed by subepicardial plexus, without vagal influence (SINGH, JOHNSON, LEE et al., 1996). Knowledge of normal morphology of the subepicardial ganglia and its alterations in pathological conditions is crucial for understanding mechanisms of cardiac rhythm control. NADPH-d reactive neurons have specific functions (RICHARDSON, MARKOVIC and ANDERSON, 2003). Data suggest they may act as neuromodulators (SCHOLZ, LABENIA, DE VEENTE et al., 2002), signaling and regulating cardiac vagal activity (TAKIMOTO, AOYAMA, TANAKA et al., 2002). In addition, nitric oxide (NO) causes atrial myocardial relaxation (TANAKA, TAKANAGA, 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 can influence morphology of this plexus. For example, aging can cause an increase on the profile area of the nerve cell bodies in rats (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, malnutrition can permanently affect developmental program (SINGHAL, COLE, FEWTRELL et al., 2004), including the cardiovascular system, which is particularly vulnerable to adverse nutrition programming during pregnancy (WATKINS and FLEMING, 2009). However, the effects of low-protein diet on the structure and ultrastructure of the subepicardial neurons have not been analyzed so far.

Given the importance of these neurons, the present study aims to analyze the morphoquantitative effects of pre and postnatal protein malnutrition on the subepicardial neurons and whether re-feeding is capable of reversing such changes.

2 Methods and materials

The study was conducted according to current legislation on animal experiments of the Biomedical Science Institute of the University of São Paulo (Ethical Committee for Animal Research – CEEA).

Young male and female Wistar rats (200-240 g body weight) were mated. After conception, which was assumed when vaginal plugs or sperm were found, 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, Nielsen and Fahey Junior (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, the rats received the same diet they had during pregnancy. The offspring was weaned on the 21st day after delivery and separated into three experimental groups. The first group (NN group) of rats was maintained under normal protein diet throughout pregnancy and until they were taken for examination at 42 days. The second group was protein deprived throughout pregnancy and for 42 days postnatal (DD group). The third or re-feeding group (RN group) was composed of rats submitted to a low protein diet during the first 21 days and then received the AIN93-G normal protein diet from day 22 to day 42. All rats were 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

NADH diaphorase histochemical technique demonstrates all types of neurons (GABELLA, 1987) and was employed in five animals from each group (NN, DD and RN). Animals were perfused with Krebs solution and the thoracic cavity was opened. The heart-lung blocks were isolated and 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 then immersed in Triton-X 3% solution for 10 minutes 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. Specimens were immersed 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 under a binocular microscope at X400 magnification.

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

Five animals from each group (NN, DD and RN) were studied with this technique. Neuronal NADPH-d staining was performed according to the protocol described by Santer and demonstrates only NO-positive neurons (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 the preparation twice in phosphate buffer (10 minutes at room temperature), atria were incubated and homogenized 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

Neuronal profile area and nuclear size were determined in 100 neurons from each animal, using software for image analysis and measurement. Neurons were randomly chosen in a computerized imaging acquisition method for morphometric analysis (KS 300-Zeiss).

2.3 Statistical analysis

Results of morphometric studies were analyzed by ANOVA and post hoc Tukey tests using multiple comparisons considering the different diet groups. The level of significance was set at $p < 0.05$. (ZAR, 1984). Data analyses were performed using SPSS for Windows (Version 15.0).

2.4 Ultrastructure of intracardiac neurons

Neuronal ultrastructure was studied using the technique previously described by Bozzola and Russel (1991). Atria and ventricles of three animals per group (NN, DD and RN) 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 reimmersed in the same solution. Fragments of 2 mm close to the pulmonary and cava veins were obtained and maintained for 2 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 fixed in a 2% osmium tetroxide solution for 2 hours at 4 °C. Samples 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 1 µm 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) and analyzed in a transmission electronic microscope (JEOL, 1010).

3 Results

3.1 Weight of animals and hearts

Group DD animals weighed significantly less than normal fed animals, the same for heart weight. Reduction was 86 and 79%, respectively. Comparing groups RN and NN, body and heart weight were also reduced in the study group: 83 and 78% (Figure 1).

3.2 Numbers of neurons

The mean number of neurons NADH-reactive was 1114 ± 79 in group NN, 1031 ± 208 in group DD and 1278 ± 121 in group RN. No significant difference among groups was demonstrated (Figure 2). The mean number of neurons reactive to NADPH-d was 240 ± 47 in group NN and 321 ± 205 in group DD e 455 ± 147 in group RN. Also, statistical analysis failed to show significance for this parameter amid groups NN, DD and RN ($p = 0.07$).

3.3 Neuronal profile

Neuronal profile area in NADH reactive neurons was 363 ± 64 μm² in group NN, 347 ± 59 μm² in group DD and 307 ± 43 μm², without significant difference among groups (Figure 3). The mean size of the neuron nuclei was 116 ± 26 μm² in group NN, 105 ± 17 μm² in group DD and 84 ± 10 μm², also without significant difference (Figure 4). The profile area of neurons reactive to NADPH-d was 210 ± 62 μm² in group NN, 195 ± 59 μm² in group DD and 264 ± 49 μm² in group RN, (Figure 5). The mean nuclear area observed were 77.89 ± 22.10 μm² and 39.10 ± 10.98 μm². The mean size of neuron nuclei was 90 ± 18 μm² in group NN, 78 ± 19 μm² in group DD and 90 ± 14 μm² (Figure 6). Statistical analysis did not show significance for this parameter among groups NN, DD and RN. The ratio between cell and nuclear area was the same in all groups.

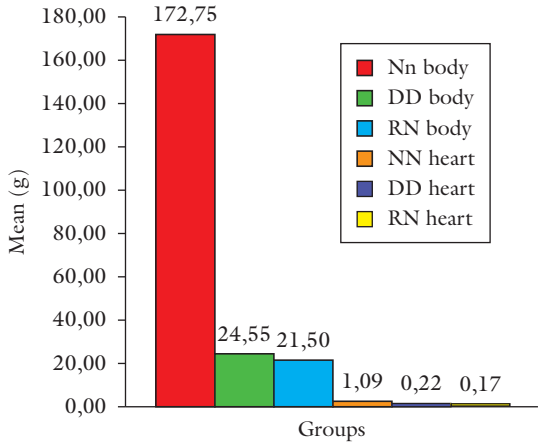

Figure 1. Distribution frequency of body and heart weight in animals from groups NN, DD and RN. For each group n = 5 and $p < 0.05$.

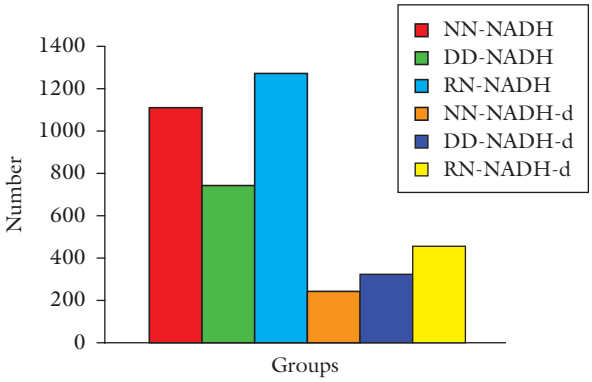

Figure 2. Distribution frequency of the number of subepicardial neurons in groups NN, DD and RN stained by NADH and NADPH-d techniques. For each group n = 5 and $p < 0.05$.

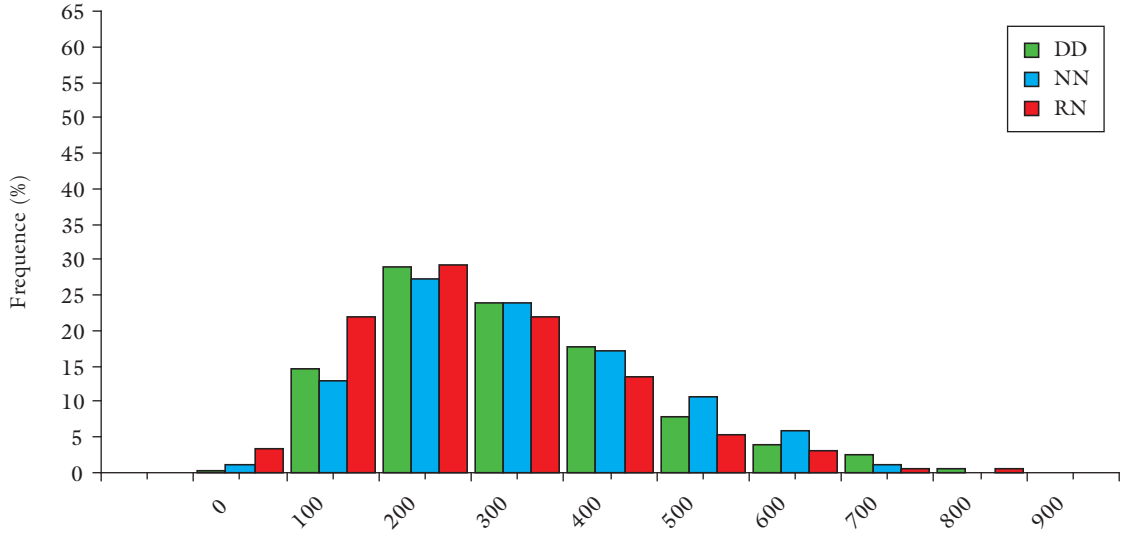

Figure 3. Distribution frequency of the area of the subepicardial neurons in animals from groups NN, DD and RN stained by NADH technique. For each group n = 5 and $p < 0.05$.

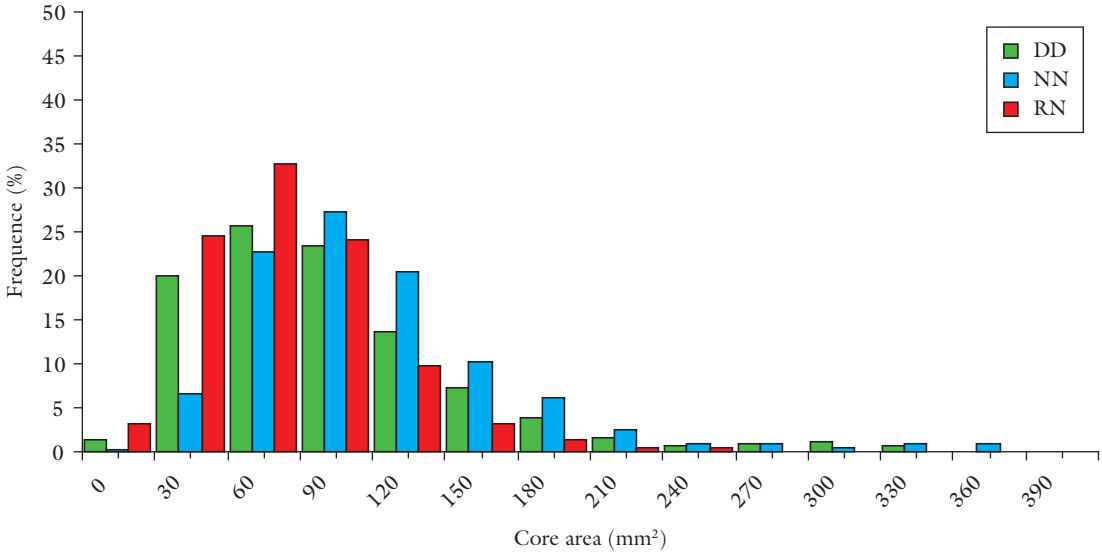

Figure 4. Distribution frequency of the area of the subepicardial nuclei in animals from groups NN, DD and RN stained by NADH technique. For each group n = 5 and p > 0.05.

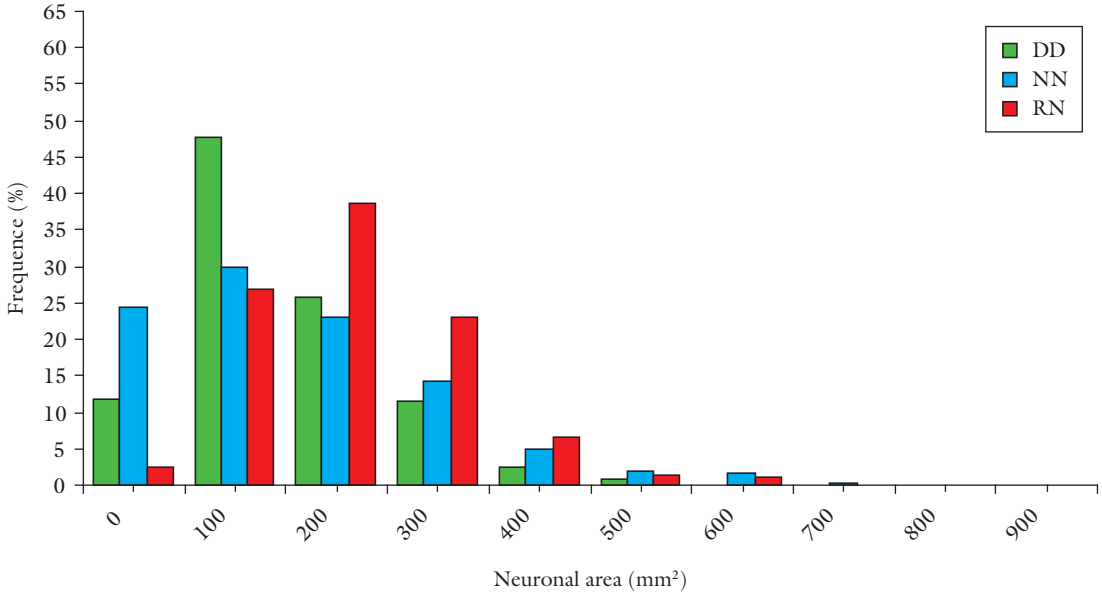

Figure 5. Distribution frequency of the area of subepicardial neurons in animals from groups NN, DD and RN stained by NADH-d technique. For each group n = 5 and p > 0.05.

3.4 Morphology and ultrastructure of intracardiac neurons

NADH diaphorase technique demonstrated subepicardial ganglia located at the subepicardic connective tissue on 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 7a-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. The endoplasmatic reticulum in group NN showed greater ribosomal density as compared to groups DD and RN. The latter showed intermediate density considering NN and DD groups (Figure 8c). Chromatin distribution in nuclei displayed sparse pattern in DD group, uniform in NN and intermediate in RN group (Figure 8f). Mitochondrial folds in groups NN and RN were transverse or oblique whereas group DD showed irregular folds.

4 Discussion

Significant reduction in body weight in experimental malnutrition was reported by many authors (CASTELLUCCI, DE SOUZA, DE ANGELIS et al., 2002; BRANDÃO,

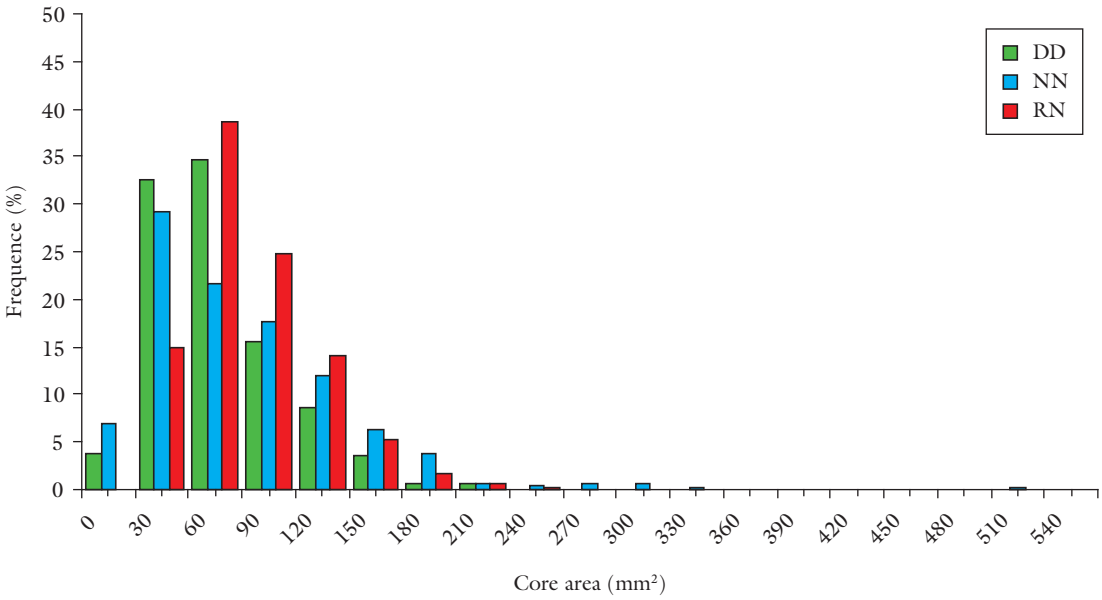

Figure 6. Distribution frequency of the area of subepicardial nuclei in animals from groups NN, DD and RN stained by NADH-d technique. For each group n = 5 and p > 0.05.

DE ANGELIS, DE SOUZA et al., 2003; GOMES, CASTELLUCCI, DE VASCONCELOS FONTES et al., 2006). It is well known that maternal undernourishment leads to offspring body weight reduction (WOODALL, JOHNSTON, BREIER et al., 1996), confirmed in our study by a mean weight reduction of 86% in animals fed with low protein diet as compared to controls. Our data also showed that re-feeding was unable to reestablish normal weight. 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. Survival depends on optimal cardiovascular performance and control of heart rate and blood pressure by subepicardical neurons may be essential.

Whole-mount preparation is an elegant 3D technique that permits neuronal visual evaluation, counting and measurements all in one preparation. Besides insufficient data provided by the small group of animals, lack of difference observed in neuron number can be due to imbalance between degeneration and maturation of precursor cells, which occurs only after immature cells reserves are exhausted[18]. The neuron cell profile area studied by NADH-d staining was not stastistically different between NN and DD groups, even though body weight in group DD was significantly lower than control animals. 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) have 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 Her, Flu, Liu et al. (2000) using NADH diaphorase staining in cardiac ganglia neuron in cell culture showed cell areas varying from 300 a 350 μm² after 40 days. In our study, the mean cell size was similar though variation was different, maybe because culture medium does not reproduce natural organ environment. Nevertheless, profile area of NADPH-d positive neurons was significantly lower in the undernourished group as compared to controls. It was described that only 2 to 4% of the ganglion neurons in rats' heart were NADPH-d reactive (KLIMASHEWSKI, KUMMER, MAYER et al., 1992), also observed in our series. Considering that full neuronal development occurs at the third week and that no changes appear after this period (HORACKOVA, SLAVIKOVA and BYCZKO 2000), neuronal size was not affected by 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. NO has a stimulatory effect by mediating vagal neurotransmission and inhibiting sympathetic neurotransmission (MARKOS, SNOW, KIDD et al., 2002). In addition, pharmacological studies in guinea pigs suggest that NO facilitates the negative chronotropic effects of vagal stimulation (SEARS, CHOATE and PATERSON, 1998; HERRING and PETERSON, 2001). Exercise training enhances cardiac vagal response and depends on neuronal nitric oxide synthethase (DANSON and PATERSON, 2003). 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 (HERRING and PETERSON, 2001).

Ultrastructural changes of the peripheral nervous system have been described by some authors. Irregular nuclei were observed with aging in spinal ganglia

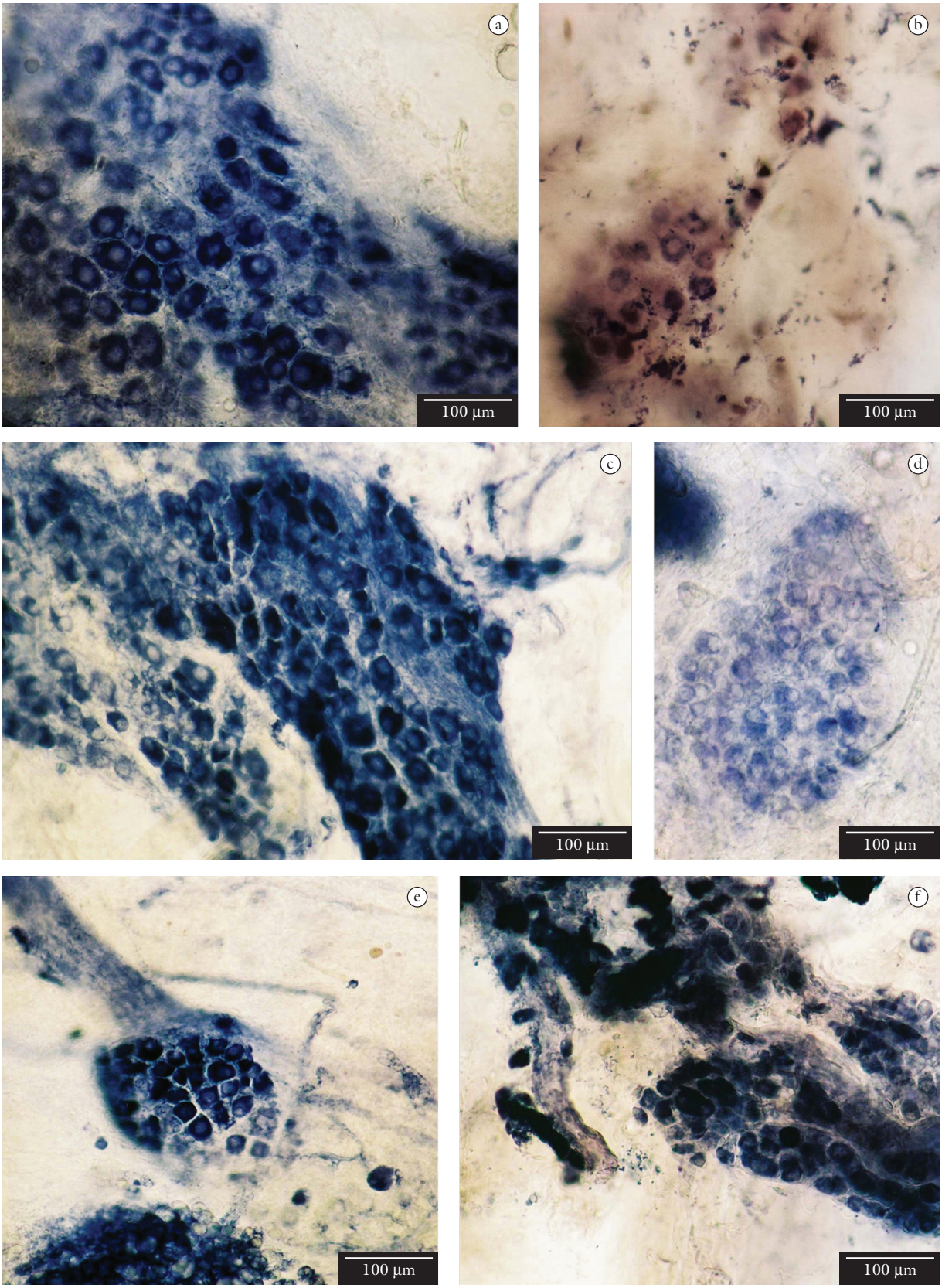

Figure 7. Subepicardial neurons of groups NN (a, b) and DD (c, d) and RN (e, f) stained by NADH (a, c, e), NADPH-d (b, d, f) histochemical techniques. No differences were noted using NADH technique in all groups. NADPH-d technique showed variable staining intensity. The few NN neurons are intensely marked (d). Many DD neurons in a round ganglion are faintly stained and RN ganglion dysplays intense staining (f). Bar = 100 µm (a, b, c, d, e and f).

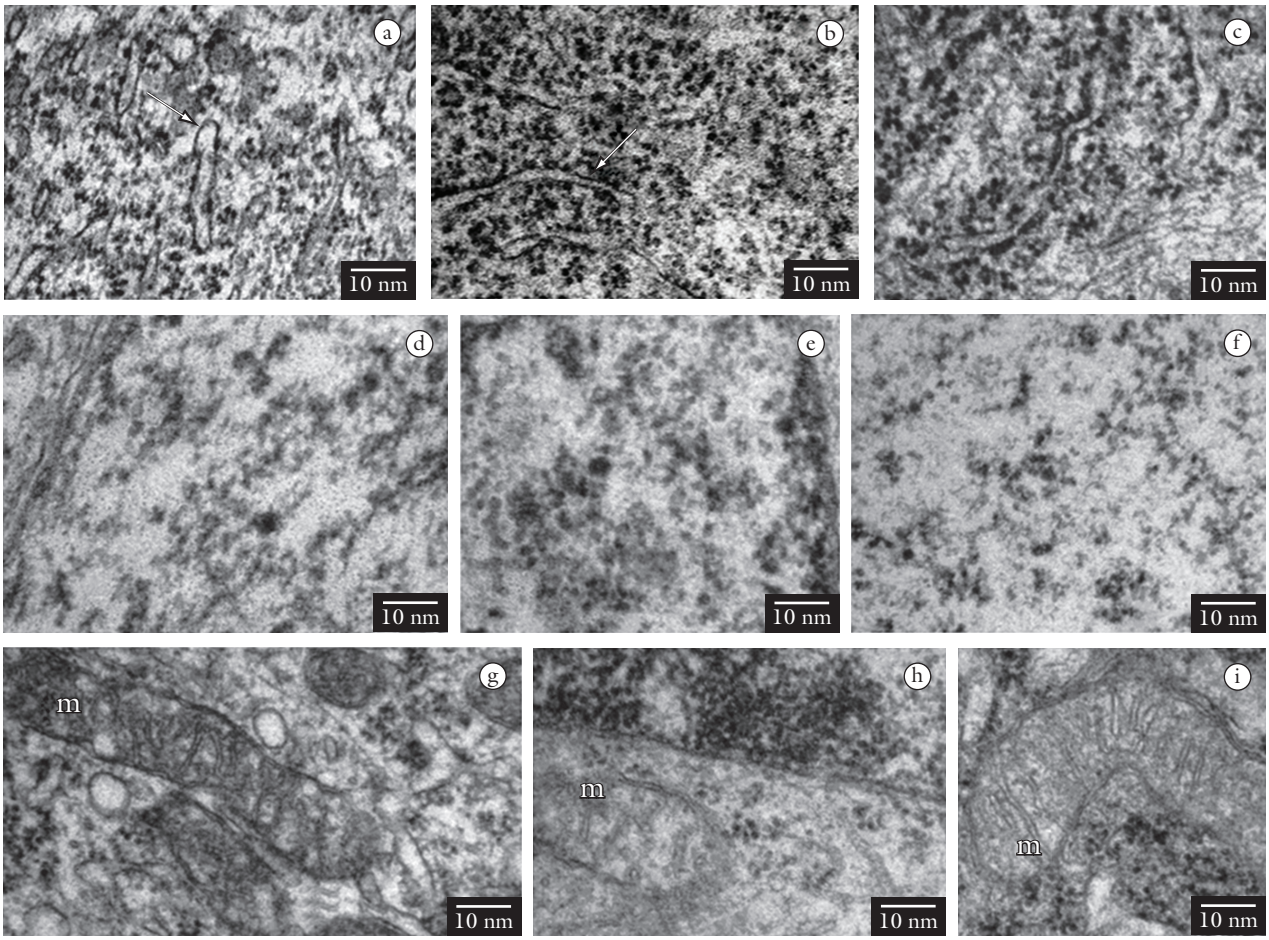

Figure 8. Electron micrographs of subepicardial neurons of group NN (a, d, g), group DD (b, e, h) and RN (c, f, i). In a, b and c, endoplasmatic reticulum (arrows) with condensed ribosomes in groups DD e RN, the later showing intermediate staining between NN e DD. In d, e and f chromatin is more uniform in group NN as compared to group DD and e RN group displays intermediate aspect considering groups NN and DD. In g, h and i, mitochondria (m) from groups NN and RN with evident transverse and oblique crests, whereas DD neurons present marked irregularity. Bars = 100 nm.

(HOPKINS, MACDONALD, MURPHY et al., 2000). Myeloid bodies, electron lucent vacuoles and degenerative changes in axon terminals were observed in the cytoplasm of subepicardial neurons in ischemic heart, suggesting that morphofunctional integrity is essential for adequate cardiac function regulation (HOPKINS, MACDONALD, MURPHY et al., 2000). We observed disarrangement of neuronal chromatin in the group submitted to a low protein diet. Some features related to malnutrition found in this study include mitochondrial edema, external membrane rupture, crest irregularity, disorganization of the endoplasmatic reticulum, and altered nuclear chromatin distribution and electrondensity of some vesicles. Similarly, Yamano, Shimada, Uamasaki et al. (1980) observed delayed neuronal maturation in embryonic cerebral cortex, including abnormal dendritic branching; yet relationship between neuronal and nuclear profile area showed no changes. Experimental starvation of pregnant animals almost universally results in high blood pressure, glucose intolerance, insulin resistance and a greater propensity to obesity (LANGLEY-EVANS, 2006). Also, protein deprivation in fetal life makes male rats more vulnerable to the effects of ischemia-reperfusion (ELMES, GARDNER and LANGLEY-EVANS, 2007). Thus, knowledge of disorders affecting these neurons may provide a wider insight into normal and abnormal rhythm control.

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