Original article

Low protein diet during perinatal period decreases the number of subepicardial neurons in rats

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,
Department of Surgery, School of Medicine, University of São Paulo – USP

[2]São Judas Tadeu University

*E-mail: flaea@usp.br

Abstract

Subepicardial plexus of the atria is an interdependent heart control system and its structure and function may be influenced by external factors. We studied the influence of protein deprivation on subepicardial neurons at early development stages by subjecting rats to a low protein diet (5% of casein) during pre and post natal period. Atrial neurons were identified by histochemical methods β-nicotinamide adenine dinucleotide (NADH) reaction, β-nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) reaction and Acetylcholinesterase (AChE) reaction and counted in 21 day-old rats. Besides a significant reduction in body and heart weight, the total number of neurons decreased 46% in the undernourished group as compared to control animals. Our data suggest that deficient nutrition during early developmental period may produce irreversible deleterious changes later in life.

Keywords: subepicardial neurons, morphometry, protein undernutrition.

1 Introduction

The subepicardial ganglia have an important role in cardiac rhythm regulation (BURKHOLDER, CHAMBERS, HOTMIRE et al., 1992; CALARESU and StLOUIS, 1967; PARDINI, PATEL, SHMID et al., 1987). Vagal activity of these ganglia protects against certain arrhythmias, including supraventricular fibrillation. Moreover, motor and sensory inputs can be independently processed inside the subepicardial plexus, without preganglionar influence (SINGH, JOHNSON and LEE, 1996). Knowledge of the morphology of the involved nervous structures, specifically of the subepicardial nervous plexuses, is essential for understanding mechanisms involved in heart control (EDWARDS, HIRST, KLEMN et al., 1995; KENNEDY, HARAKALL and LYNCH, 1998; STEELE, GIBBINS, MORRIS et al., 1994). Neurons subjected to natural factors as aging or extrinsic pathological factors can undergo changes in their morphology. For example, aging can cause a decrease in the neuron number of the subepicardial plexus of up to 40% in humans (AMORIM and OLSEN, 1990) and 70% in rats (AKAMATSU, De SOUZA, LIBERTI et al., 1999).

More importantly, low weight and size at conception, secondary to malnutrition in different gestational ages, are related to cardiovascular diseases in adulthood (BARKER, GLUCKMAN, GODFREY et al., 1993; SINGHAL, COLE and FEWTRELL). 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 the effect of malnutrition on cardiac performance (DROTT and LUNDHOLM, 1992) and cardiac muscle fibers (CHAUHAN, NAYAK and RAMALINGASWAMI, 1965). Despite the demonstration of those cardiac functional disorders due to pre and post natal protein malnutrition, this is the first report to describe the structural changes of the subepicardial neurons under those circumstances.

Thus, we consider that both quantitative and qualitative studies of the subepicardial neurons represent a major contribution to the understanding of deleterious consequences caused by malnutrition to the heart.

2 Material and methods

2.1 Diet groups

Animal experiments were performed in accordance to Ethical Principles in Animal Research (COBEA) adopted by the Brazilian College of Animal Experimentation and approved by the Ethical Committee for Animal Research (CEEA) of the Biomedical Sciences Institute of the University of São Paulo. *Rattus norvegicus* (*Wistar*) male and female rats weighing 200 to 240 g were housed together to couple during a period of seven to ten days, during which a normal diet (N) to the control group and a low protein diet (D) to the experimental group were offered. The diets, with *ad libitum* water supply were respectively AIN-93G purified and supplemented with 20% casein and the AIN-93G with 5% casein, according to the protocol described by Reeves, Nielsen and Fahey Jr. (1993). Rats were maintained under standard conditions at 21 °C, with a 12 hours light-dark cycle. After this period, females were separated and housed in individual cages and assigned to groups D or N according to their diets. They were kept with the respective diets till their nestlings reached 21 days of extra uterine life, time determined to wean. The mean number of rats per brood established was eight, being discarded broods whose mothers ate the

nestlings and the surplus of broods with eight nestlings. At the 21st day of life, rats were identified according to their diet group as N or D. Animals of group N (n = 5) and D (n = 5) were weighed and sacrificed with a single dose of intraperitoneal pentobarbital (HYPNOL®-Fontoveter) 30 to 40 mg.kg^{-1} (WAYNFORTH and FLECKNELL, 1992). The thoracic wall was opened and their heart and basal vessels were removed.

2.2 Histochemical study

2.2.1 β-nicotinamide adenine dinucleotide (NADH) reaction

Five animals from each group (N and D) were used for this technique (GABELLA, 1971). Following removal, hearts were immediately washed and immersed in Krebs solution. Atria were then removed and kept in the same solution for a period of 2 hours. After removal from Krebs solution, atria were dissected under magnification, to remove superficial adipose tissue. Atria were then immersed in Triton-X 3% solution for 10 minutes, to facilitate staining medium penetration, washed in Krebs solution and kept for a period of 45 to 60 minutes in a Nitro-Blue Tetrazolium (NBT-Sigma) 0.5 mg.mL^{-1} solution in distilled water; 25 parts of sodium phosphate buffer (0.1 mol.L^{-1}; pH 7.3) and 50 parts of water; 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), in which they were kept 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.

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

Five animals from each group (N and D) were used for this technique (SANTER, 1994). Following removal, atria were removed and dissected as reported above. They were then immersed in a fixative solution of paraformaldehyde 4% in phosphate buffer (pH 7.2) at 4 °C for 30 minutes. After washing in phosphate buffer (2 times during 10 minutes, at room temperature), atria were incubated and continuously shaken for 60 minutes at 37 °C in the specific medium for NADPH demonstration: β-NADPH (0.1 mg.mL^{-1}) in reduced form (Sigma) and a Nitro-Blue Tetrazolium (NBT-Sigma) solution (0.5 mg.mL^{-1} in phosphate buffer containing 0.2% Triton X-100). Atria were placed on slides as whole mount preparations in glycerin.

2.2.3 Acetylcholinesterase (AChE) reaction

Five animals from each group (N and D) were used for this technique (BALUK and GABELLA, 1989) modified in the Autonomic Nervous System Laboratory of the University of São Paulo. Following removal and dissection, atria were immersed in a fixative solution of paraformaldehyde 4% in phosphate buffer (pH 7.2) for one hour at 4 °C and then washed overnight in a Krebs solution with hyaluronidase 2.000 U.T.R (ASPEN) and iso-OMPA (tetra-isopropyl pyrophospharamide - SIGMA) at 4 °C. Specimens were then incubated for a period of 24 hours shaking at 4 °C, in a medium containing 0.01 g of acetylcholine, 13 mL phosphate buffer 0.1 mol.L^{-1} (pH 6.0), 1.0 mL of sodium citrate, 2 mL of copper sulfate (30 nmol.L^{-1}), 2 mL of distilled water and 2 mL potassium ferrocyianide (5 nmol.L^{-1}) and triton 0.3% (1% total volume) (KARNOVSKY and ROOTS, 1964). After this period, atria were dehydrated through a graded series of alcohol (from 70 °GL to absolute) for 5 minutes each passage, made translucent in three 5 minutes passages in xylol before mounting on slides with synthetic resin.

2.3 Morphometry of subepicardial neurons

Neuronal counting was performed on the whole mount preparations (groups N and D) stained by NADH and NADPH methods under a binocular light microscope.

2.4 Statistical analysis

All data (neuronal counts, animals' weight, and weight of the hearts) 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 with SPSS for Windows (Version 15.0).

3 Results

3.1 Quantitative analysis

3.1.1 Body weight and heart weight

The body and heart weight of animals from the experimental group were respectively 71 and 68% lower ($p < 0.05$) than the control group. The heart weight was proportional to the animal body weight (Table 1; Figure 1).

3.1.2 Numbers of neurons

The mean number of neurons reactive to NADH was 1463 ± 135 in group N and 784 ± 105 in group D. There was a 46% decrease in relation to group N which was statistically significant ($p < 0.05$) (Table 2; Figure 2). The mean number of neurons reactive to NADPH-d was 558 ± 241 in group N and 435 ± 158 in group D. Statistical analysis did not show

Table 1. Body weight and heart weight in groups N and D (g).

Number animals	Groups	Body weight ± SD	Heart weight ± SD	Heart weight/ body weight × 100
11	N	*48.44 ± 18.39	0.44 ± 0.19	0.90 ± 0.19
9	D	*14.19 ± 2.94	0.14 ± 0.04	1.01 ± 0.30

SD = Standard deviation; * = $p < 0.05$; N = normal diet (control group); and D = low protein diet (experimental group).

significance for this parameter between groups N and D, (p = 0.07) (Table 2, Figure 2).

3.2 Qualitative analysis

3.2.1 Morphology of intracardiac neurons

In both groups, the subepicardial ganglia revealed with the NADH (Figure 3 a-b) diaphorase technique were located in the subepicardial connective tissue on the external surface of both atria musculature. Ganglia displayed elongated, starry, polygonal, or round patterns with variation in the staining intensity, mainly in the NADPH reaction (Figure 3 c-d). In group D, intensely stained isolated neurons were observed. Neurons presented different sizes (small, medium and large), with oval, spindle, and pear like forms. The nuclei had spherical forms and were predominantly located in the periphery of the cytoplasm. With the AChE technique, neurons presented superimposed in both groups, without significant qualitati ve difference among them (Figure 3 e-f).

4 Conclusion

Different methods have been used to induce experimental malnutrition (LEPRI, BRUSCHI and MOURA, 1994; ALIPI, META, OLIVERA et al., 2002; CASTELUCCI, De SOUZA, De ANGELIS et al., 2002; BRANDÃO, De ANGELIS, De SOUZA et al., 2003; GOMES, CASTELLUCCI, De VASCONCELOS FONTES et al., 2006) that results in a significant decrease in the body weight of the studied specimens. In this study, we observed a decrease in the body and heart weight of 71 and 68%, respectively in undernourished animals as compared to controls. This data reinforce the proportion between body and heart weight. Protein malnutrition decreased the number of neurons in rat subepicardial ganglia. The total number of subepicardial neurons increases with development in rats and reach maturation within 21 days (HORACKOVA, SLAVIKOVA and BYCZKO, 2000). We showed that protein malnutrition causes significant decrease of the neuronal population in relation to the control group. This finding can be of great importance for the heart physiology after weaning, when the animal should be physically independent and presumably needs stronger cardiovascular response for survival, considering that subepicardial neurons act in heart rate control. Decrease in neuronal counting secondary to malnutrition has been described by several authors, not only affecting the heart but other organs as well (SALAS, DIAZ and NIETO, 1974; DEO, K., BIJALINI and DEO, MG., 1978; PAULA-BARBOSA, ANDRADE and CASTEDO, 1989; SANTER and CONBOY, 1990). The observed neuronal loss is similar to that observed during aging process, despite the fact that aging is a natural phenomenon (BARKER, GLUCKMAN, GODFREY et al., 1993). Interestingly, NADPH-d neurons did not show the same behavior under those conditions. Maybe there is a quantitative preservation of those neurons during malnutrition states.

Our findings of 38% of NADPH-d positive neurons of the total neurons of the subepicardial plexus were similar to those found in dogs (ARMOUR, SMITH, LOSIER et al., 1995) and in rats for the nodose ganglion (KOIKE, HISA, UNO et al., 1998), which differs from the 10% of the NADH stained heart neurons in mice (MAIFRINO, LIBERTI, CASTELUCCI et al., 2006). As NADPH-d positive neurons

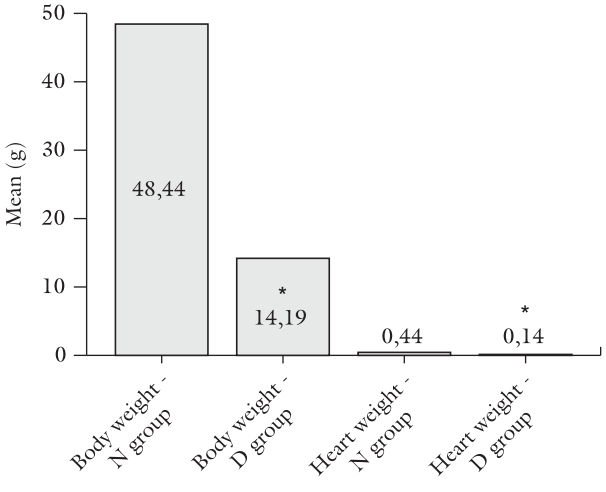

Figure 1. Distribution frequency of body weight and heart weight in animals from groups N, D. For each group n = 5* p < 0.05.

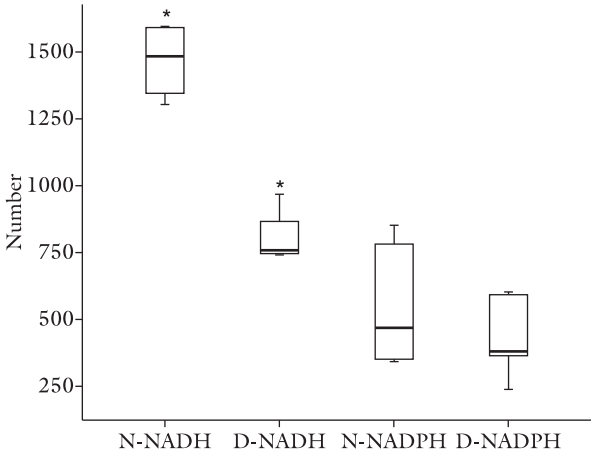

Figure 2. Distribution frequency of the number of subepicardial neurons in groups N and D stained by NADH and NADPH techniques N group n = 5; D group n = 5. * p < 0.05.

Table 2. Total number of subepicardic neurons in the hearts of rats from groups N and D stained by the NADH and NADPH techniques.

Reaction	NADH N	NADH D	NADPH-d N	NADPH-d D
Mean	1463*	784*	558	435
SD	135	105	241	158

SD = Standard deviation; * = p < 0.05; N = normal diet (control group); and D = low protein diet (experimental group) n = 5.

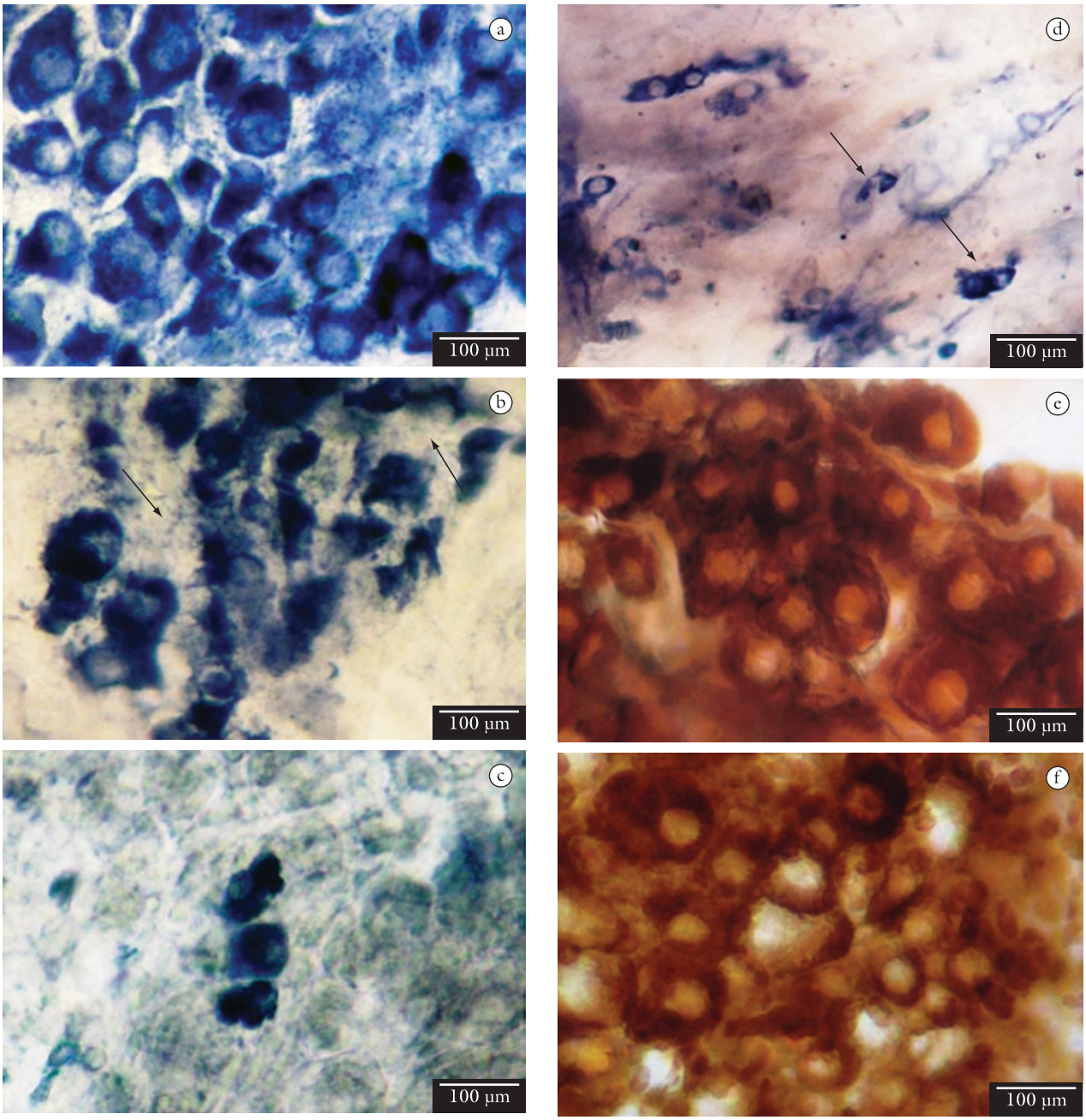

Figure 3. Subepicardial neurons of groups N (a, c, e) and D (b, d, f) stained by a, b) NADH; c, d) NADPH; and e, f) AChE histochemical techniques. In group D isolated neurons can be seen (arrows) (d). In C, ganglion with only three NADPH reactive neurons and in E and F cholinergic neurons intensely stained in groups N (e) and D (f).

in the cardiac plexus of the atria have distinct morphology, it is possible they also have different functions.

Our data matches Klimaschewski, Kummer and Mayer (1992), finding only 2 to 4% NADPH-d reactive neurons in rats' heart ganglia. The fact that there was no statistical difference in the number of NADPH reactive neurons between the two groups studied suggests that malnutrition does not influence this class of neurons. Maybe this class of neurons was not affected due to a lack of sensitivity to malnutrition at this developmental period of life.

In general, nutritional state 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.

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