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PRE AND POST NATAL UNDERNUTRITION INFLUENCES THE DEVELOPMENT OF THE SUBEPICARDIC GANGLION CAPSULE

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ABSTRACT

The subepicardic ganglion capsule of rat is constituted by connective tissue, type I collagen and rare fibers of the elastic system. Undernutrition is associated to defects of the connective tissue as lesions and altered growth. As collagen is the most prevalent protein in all tissues, its presence was evaluated in a proteic undernutrition model (5% casein) in the subepicardic plexus ganglions of pre and post natal rats with 21 and 42 days and after post natal restart of refeeding. Collagen fibers were identified by picrosirius staining with polarization microscopy and transmission electronic microscopy. Elauninic and oxytalanic elastic fibers were not identified by their staining characteristics and fine structural morphology for the coloration of Verhöeff and Resorcina Fuccina with and without previous passage for the oxona. It was not observed, by optical microscopy, differences between the collagen and elastic fibers in the subepicardic ganglion capsule of the studied groups. However morphometric analysis by transmission electronic microscopy showed that the undernourished groups of 21 and 42 days presented difference in the outline area of the collagen fibers in transversal sections. There was an increase in collagen fiber area of group D (21 days) suggesting an acceleration in the development, once the values obtained in that group were similar to the group NN (nurtured of 42 days). With undernutrition up to 42 days there was a decrease in the collagen fiber area in transversal sections. It was not observed recovery in collagen area after refeeding from 21 to 42 days.

Key words: subepicardic ganglia - collagen fibers - capsule - elastic system fibers

INTRODUCTION

Several studies in different animal species, including human, has been demonstrating that the nervous cells of the cardiac atrium of mammals possess a capsule of connective tissue [1, 5, 6, 10-12, 14, 21]. The subepicardic capsule sends septa to the interior of the ganglion which constitutes a net support for the nervous plexus [1, 4, 10] and maintains the arrangement and the interaction between nerve cells and the glial cells [2]. However till now effects of proteic undernutrition (5% casein) over the subepicardic ganglionar capsule of rat was not addressed. The present work focused the identification of collagen and other elastic system fibers of rats submitted to proteic undernutrition from gestation to 21 and 42 days, and after refeeding, through histological techniques and transmission electronic microscopy.

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MATERIALS AND METHODS

Animals and feeding regimens

We performed animal experiments in accordance with Ethical Principles in animal research (COBEA) adoptes by Brazilian College of Animal Experimentation and was approved by the Biomedical Sciences Institute/ USP – Ethical Committee for animal research (CEEA) (in 23/04/02 meeting).

Rattus norvegicus (variety Wistar) from the biotery of ICB1 of the University of São Paulo were used. Young, males and females rats coupled during a period from seven to ten days, during which a proteic diet for the control group, denominated nurtured (N) and a diet hypoproteic for the experimental group, or undernourished (D) was offered without restrictions. Diets with water supply without restrictions were, respectively, purified AIN-93G1 complemented with 20% of casein and AIN-93G1 with 5% of casein, according to established protocol [15]. The diet AIN-93G was used by its properties of giving support to the growth, gestation and nursing phases of the rodent.

After that period, females were separate in individual cages and divided in groups N and D, according to the diet regimen used. Animals were maintained with the respective diets until the nestlings reached 21 days of extrauterine life, time for weaning. The biotery atmosphere was kept calm and animal keepers were always the same due to the increased sensibility of the undernourished animals and for not losing the brood due to the stress that can induce the mother's cannibalism and weakness of the nestlings taking them to death. The ambient temperature was monitored, once undernourished animals have a decrease of corporal temperature that can take them to hypothermia and consequent death. It was settled down four as minimum brood number and eight as maximum, being despised the broods with less than 4 nestlings, broods whose mothers ate the nestlings and the surplus of broods with 8 nestlings. At the 21st day of life the nestlings, according to the diets, were identified as groups N and D. Rats of groups N and D maintained with the respective diets until they completed 42 days formed the groups NN and DD respectively. The experimental group in which diet was reintroduced (RN), was composed by rats from group D that were kept from the 22nd day with proteic diet until they completed 42 days of life.

All animals (groups N, D, NN, DD and RN) were weighted and identified through tail numbering, being two of each brood chosen randomly in order to be sacrificed and submitted to analysis.

Euthanasia and heart preparation

After euthanasia, accomplished with the injection of Hypnol to 3% (Fontoveter), skin was dissected and a medline sternostomy performed exposing the heart which was removed with the proximal basal vessels.

Staining for collagen and elastic system fibers of the subepicardic plexus

The hearts of 3 animals per group (N, D, NN, DD and RN) were washed with phosphate buffer and immersed in 10% formalin solution, for a period of 1 hour. After fixation, the atria were isolated and dissected under magnification and microsurgical technique, in order to take off the connective and fatty tissue. The tissue was kept in the same fixative for 24 hours. Following, the atria were washed for 2 to 4 hours in running water, dehydrated in a series of alcohols (from 70% to absolute), and then in three xilol series before being embedded in paraffin. Adjacent serial 5μm thick sections were obtained in a microtome and stained for collagen fibers with Picro-sirius according to Junqueira et al. (1979). Sections were analyzed under polarized light.

Fibers of the elastic system (elastic, elauninic and oxitalanic), were stained with ferric hematoxilin [21] and resorcin-fucsin [19] with and without previous oxone passage, according to techniques previously described [7, 8, 13, 17].

Ultra-structure of subepicardic plexus neurons

Transcardiac perfusion was performed in three animals per group (N, D, NN, DD and RN) with a syringe, using a 2% glutaraldehyde solution in sodium phosphate buffer (0,1M, pH 7.3). Atria and fragments of 2mm close to the pulmonar and cava veins were dissected as previously described and kept in the same fixative for 2 hours at room temperature. Specimens were then washed in sodium phosphate buffer solution (0,1M, pH 7.3) and fixated in 2% osmium tetroxide for two hours at 4ºC. The pieces were washed with saline and kept in an 0,5% uranil acetate aqueous solution overnight in the dark at room temperature for a period of 12 hours. Following, dehydration was performed in a growing series of alcohols (from 70% to absolute), two baths of propylene oxide for 15 minutes, and embedding in

oxipropylene resin (1:1) from 8 to 12 hours and finally inclusion in another resin (ARALDITE) [3].

Adjacent serial ultrathin sections were obtained by means of an ultramicrotome and stained with toluidine blue. Sections were then stained with a saturated alchoholic uranil acetate solution and lead citrate, according to previous reports [16]. Specimens were then examined by transmission electronic microscopy at the histology department of ICB/USP.

Morphometric image analysis of subepicardic ganglion capsule collagen fibers

The estimated total outline areas of collagen fibers in transversal sections were analysed in a mean fiber number of 266 per group (N, D, NN, DD, RN) through a computerized image analyzer (AxioVision, Zeiss).

Statistical treatment

Statistical analysis was performed by comparing separately N and D groups from groups NN, DD and RN. Data were submitted to a variance analysis for multiple comparisons (Tukey's test) [20] according to the diet group factor (N, D, NN, DD, RN), and the statistical program SPSS 13.0 was used.

RESULTS

Qualitative aspects

Collagen and elastic system fibers associated to the subepicardic plexus

All the histological sections of the ganglions stained for collagen fibers showed a capsule of variable thickness that was not related to the diet group. Very defined septa were found forming "loci" among themselves separating the neurons. Under polarized light, it was noticed that in the constitution of the capsule as of the septa, collagen type I red, orange and yellow fibers prevailed (figure 1).

In respect to the elastic fibers, with the different used stainings, it was not observed the presence of them in the ganglions nor in the septa (figures 2 and 3).

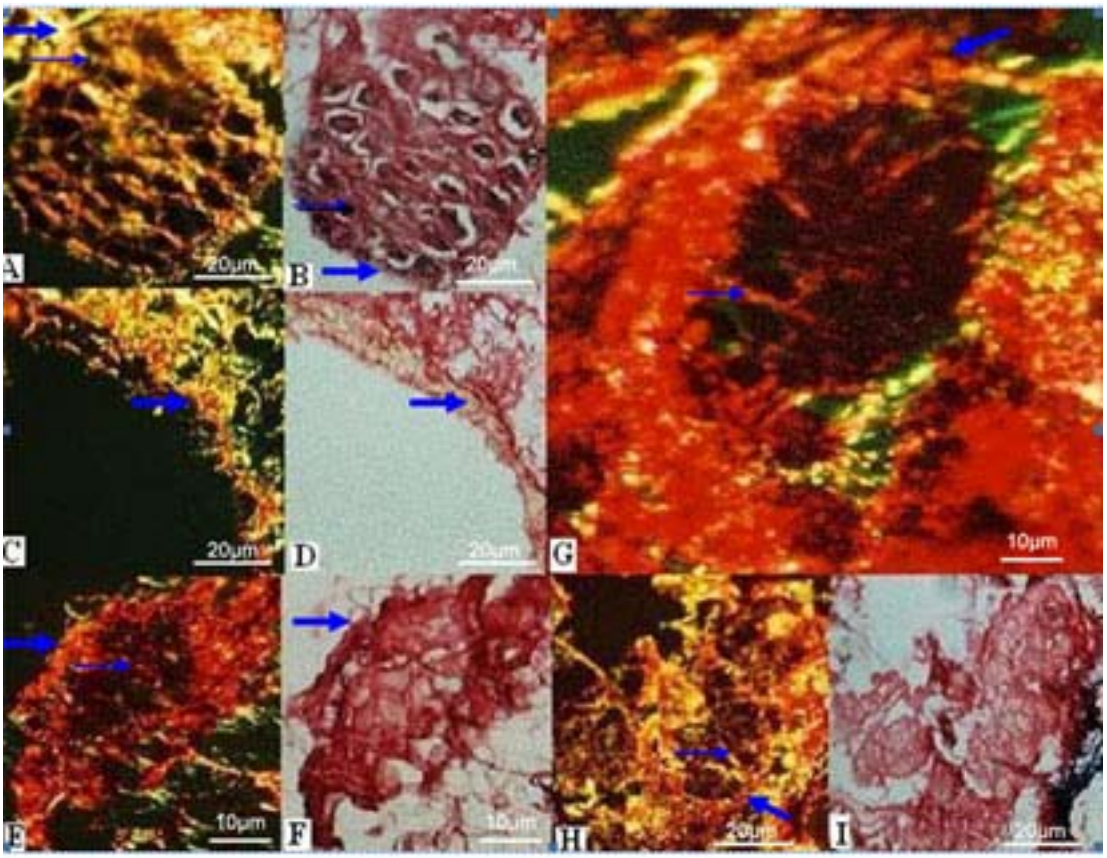

Figure 1 - Sections of subepicardic ganglions of rat. Groups RN (A-B); DD (C-D);NN (E-F); D (G) and N (H-I) - stained for Picrosirius technique. Note the capsule (large arrow) and the septa (small arrow) of connective tissue involving neurons. Under polarized light (A, C, E, G, H) the prevalence of collagen fibers type I is verified (red, orange and yellow).

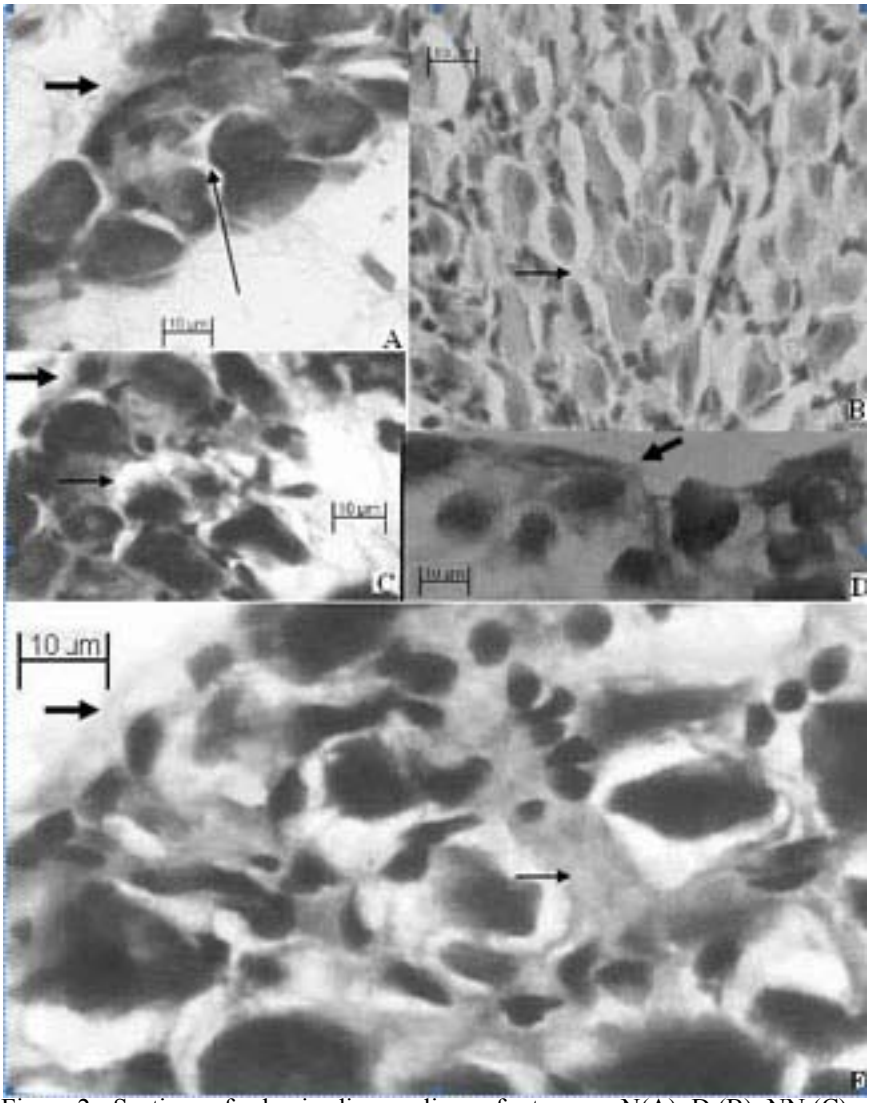

Figure 2 - Sections of subepicadic ganglions of rat, group N(A); D (B); NN (C); DD (D); RN (E) stained by Verhöeff technique. Ganglions do not present elastic fibers in the capsule (large arrow) nor in the septa (thin arrow).

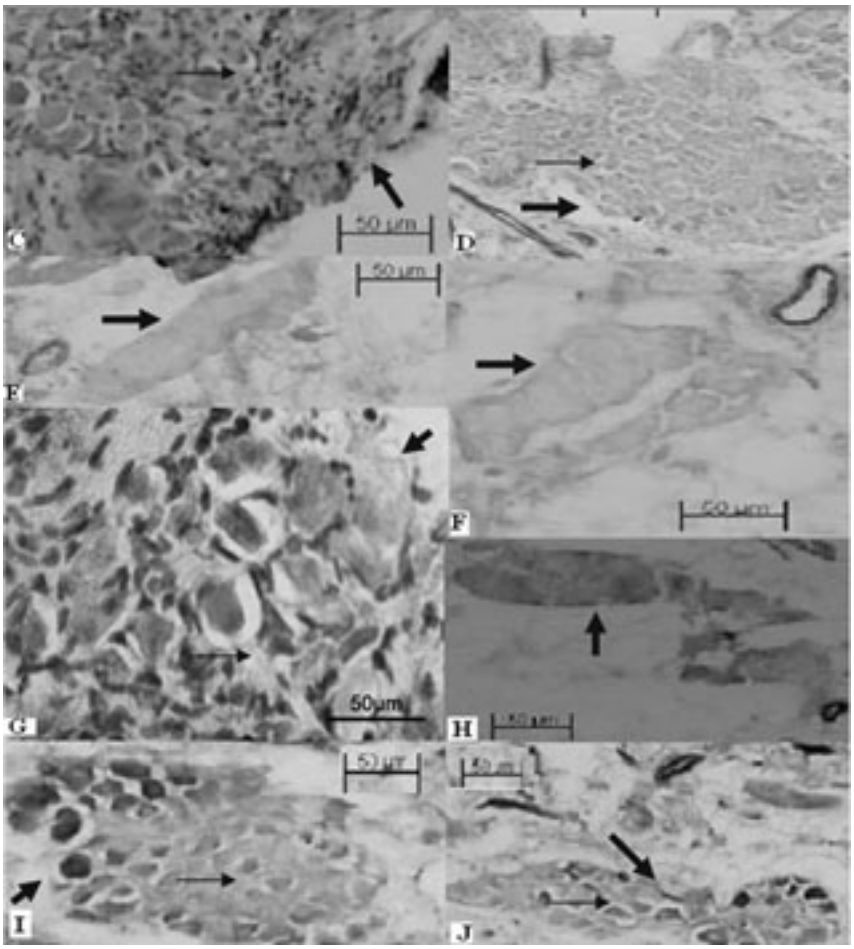

Figure 3 - Sections of subepicardic ganglions of rat, group N(A-B); D (C-D); NN (E-F); DD (G-H); RN (I-J) stained by Weigert - Resorcin Fucsin technique with (A-C-E-G-I) and without (B-D-F-H-J) oxone. Note that ganglions do not present oxitalanic and elaulinic fibers in the capsule (large arrow) nor in the septa (thin arrow).

Ultra-structure

The collagen fibers involve the ganglion and its satellite cells being disposed in different orientations forming nets in all studied groups. It was not observed neurons without collagen fibers capsule (Fig 4).

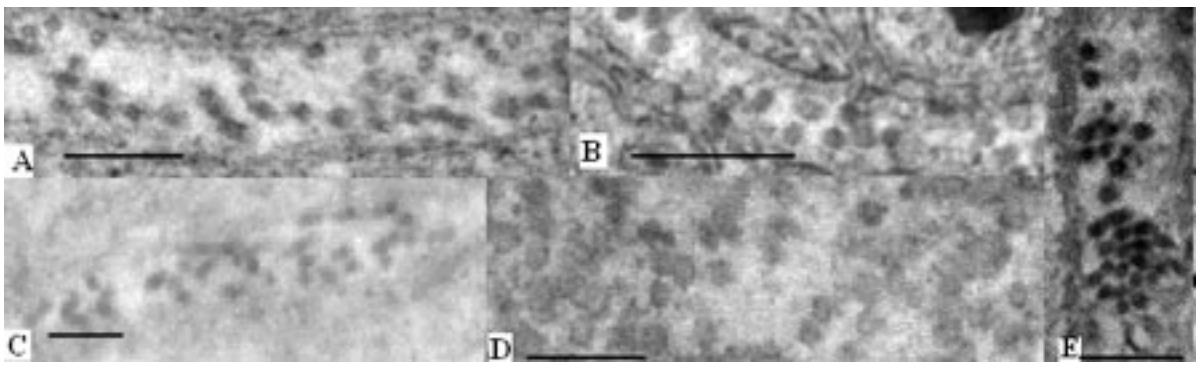

Figure 4 - Electronmicrography of collagen fibers in the capsule of subepicardic ganglions of rat, group D (A); N (B); DD (C); NN (D); RN (E). Bars=500nm

Quantitative aspects

The mean outline area value of collagen fiber in tranversal sections was 1375,24 ± 59,83nm2 for group N and 1888,10 ± 83,07nm2 for group D ($p < 0,001$), being observed statistical difference. The mean outline area value of collagen fibers in tranversal section for groups NN, DD and RN were respectively 1919,30 ± 101,31nm2, 1167,01 ± 46,63nm2 and 1522,50 ± 88,48nm2. Significant reduction was observed in the group DD and RN in relation to the group NN ($p < 0,001$) and in the group DD in relation to the group RN ($p < 0,05$) Table 1 and figure 5.

Table 1 - Mean outline area values (inm²) of collagen fiber in transversal sections

Group / n	Mean	Standard Deviation
N_collagen (272)	1375,24	59,83
D_collagen (301)	1888,10	83,07
NN_collagen (120)	1919,30	101,31
DD_collagen (587)	1167,02	46,63
RN_collagen (51)	1522,50	88,47

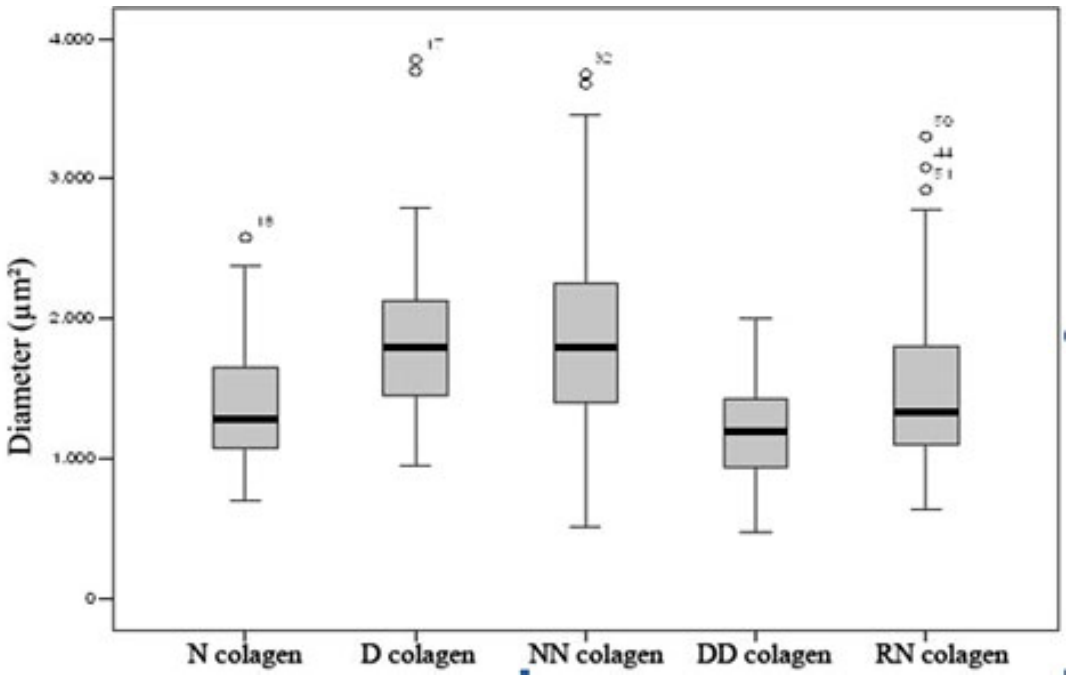

Figure 5 - Graphic shows the distribution frequency of the outline area of collagen fibers in transversal sections of subepicardic ganglion plexus in groups N, D, NN, DD and RN.

DISCUSSION

Fibrils of the elastic system were not found among the collagen as it is commonly encountered in the ganglionar capsule of the esophagus mioentheric plexus [4].

Optical microscopy showed ganglions with and without capsule in all studied groups, however that was not confirmed in ultra-structure, once all ganglions are involved by conjunctive tissue. Probably the thickness of the capsule influences its appearance in optical microscopy, being this thin enough to give the impres-

sion of not existing to the optical microscope. It is referred in human heart ganglions the presence or not of the ganglionar capsule at optical microscopy [12] fact that was not discussed by the It is referred in human heart ganglions the presence or not of the ganglionar capsule at optical microscopy [12] fact that was not discussed by the author in ultra-structure. The neurons of the ganglion are separated from each other by collagen fibers that involve the nervous cell, as confirmed by the picrosirius technique (Sirius Red) and polarization. In this technique, type III collagen was barely observed in comparison to type I [9]. These findings suggest that the difference in the increase of the outline collagen fiber area occurs in the collagen type I along the growth and development up to 21 days as observed with the aging, where the capsule of the heart ganglions presents an increased amount of collagen fibers type I, besides there are no elastic fibers in young nor in old animals [1]. Fibers of the elastic system were not seen in the ganglionar capsule at optical microscopy and ultra-structural analysis. The mechanical stress is supported by collagen fibers which are responsible for maintain the neuronal organization during the heart diastole in the ganglion. Collagen absorbs mechanical stress and offers support for neuronal organization. It was observed difference in the size of collagen fiber area in transverse section with the undernutrition up to 42 days, and despite refeeding, fibers areas were not recovered. At this phase, differently from the aging process where collagen fibers increase, there is a decrease in the collagen fiber area, suggesting a deterioration process as a consequence of proteic undernutrition. So it can be suggested that the collagen fibers of the ganglionar capsule of the subepicardic plexus follows the protein regimen (5% casein), resulting in ultra-structural changes observed in histology and morphometric analysis. The present study also suggests that larger periods of undernutrition could lead to greater changes in the ganglionar capsule, since collagen is a support protein in the organism. It seems that up to 21 days of proteic undernutrition there is an acceleration in the development of the collagen fiber area, fact that is not sustained up to 42 days of undernutrition, probably because a deterioration process of the collagen fibers at this phase. Finally it was not observed recovery of the collagen fiber area after restarting normal food intake from the 21st to the 42nd days. In conclusion proteic undernutrition alters the collagen fibers outline area in tranversal sections of the ganglionar capsule of the subepicardic plexus, and may influence the organization, development and maturing of nervous cells.

REFERENCES

1. AKAMATSU, F.E.; DE SOUZA, R. R.; LIBERTI, E.A. (1999) Fall in the number of intracardiac neurons in aging rats. Mech. Aging. Dev. **109**. 153-161.
2. BERGSTEINSDOTTIR, K.; HASHIMOTO, Y.; GRENNAN, A.; MIRSKY, R.J.K.R. (1993) The effect of the three dimensional collagen type I preparation on the structural organization of guinea pig enteric ganglia in culture. Exp. Cell. Res. **207**. 67-75, 1993.
3. BOZZOLA, J.J.; RUSSEL, L.D.(1991) Electron microscopy principles and techniques for biologist: Jonen and Bartlett publisher. Boston.
4. DE SOUZA, R.R.; CARVALHO, C.A.F.; WATANABE, I. (1988) Identification of collagen, elastic, elaunin and oxytalan fibers in ganglia of the myenteric plexus of the human oesophagus. Anat. Embryol. **179.** 97-102.
5. DE SOUZA, R.R.; GAMA, E.F.; CARVALHO, C.A.F.; LIBERTI, E.A. (1996) Quantitative study and architecture of nerves and ganglia of the rat heart. Acta Anat. **156**. 53-60.
6. ELLISON, J.P.; HIBBS, R.G.(1976) An ultrastructural study of mammalian cardiac ganglia. J. Mol. Cell. Cardiol., **8**. 89-101
7. FULLMER, H.M.; LILLIE, R.D. (1958) The oxytalan fiber: a previous underscribed connective tissue fiber. J. Histchem. Cytochem., **6**. 425-430.
8. GREENSPAN, F.P. (1946) The convenient preparation of peracids. Am. Chem. Soc. **68**. 907.
9. JUNQUEIRA, L.C.U.; BIGNOLAS, G.; BRETANI, R. (1979) Picro sirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. Histochem. J. **11**. 447-455.
10. KING, T.; COAKLEY, J.B. (1958) The intrinsic nerve cells of the cardiac atria of mammals and man. J. Anat. **92**. 353-376.
11. PARDINI, B.J.; PATEL, K.P.; SHMID, P.B.; LUND, D.D. (1987) Localization, distribution and projection of intracardiac ganglion cells in the rat. J. Auton. Nerv. Syst. **20**. 90-101.
12. PAUZIENE, N.; PAUZA, D.H. (2003) Electron microscopic study of intrinsic cardiac ganglia in the adult human. Ann. Anat. **185**. 135-148.
13. PEARSE, A.G.E. (1968) Histochemistry. Theoreti-

cal and applied. London: J and A Churchill, 3rd.
14. RANDALL, W.C.; ARDELL, J.; CADERWOOD, D.; MILOSAVLJEVIC, M.; GOYAL, S.C. (1996) Parasympathetic ganglia innervating the canine atrioventricular nodal region. J. Auton. Nerv. Syst. **6**. 311-323.
15. REEVES, P.B.; NIELSEN, F.H.; FAHEY JR., G.C. (1993) - AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J. Nutr. **123**. 1939-1931.
16. REINOLDS, E.S. (1963) The use of lead citrate at high Ph as an electron opaque stain in electron microscopy. J. Cell. Biol. **17**. 208-212.
17. SAMPAIO, S.B.; COTTA-PEREIRA, B. (1971) Distribuição das fibras elásticas, elaunínicas e oxitalânicas na derme superior em pele humana. A. Bras. Dermatol. **46**. 333-347.
18. WIEGERT, C. (1989) Ùber eine Methode zur Färbung elastisher Fasern. Zbl. All. Path. Pathol. Anat. **9**. 289-292.
19. SPANHEIMER,R.; SLATEV, T.; UMPEIRREZ, B.; DIGIROLAMO, M. (1991) Collagen production in fasted and food-restricted rats response to duration and severity of food deprivation. J. Nutr. **12**. 518-524.
20. ZAR, J.H. (1984) Biostatistical analyses: Prentice Hall, Englewoods Cliffs, Second ed. New Jersey.
21. YUAN, B.X.; ARDELL, J.L.; HOPKINS, D.A.; LOSIER, A.M.; ARMOUR, A. (1994) Gross and microscope anatomy of the canine intrin sic cardiac nervous system. Anat. Rec. **239**. 75-87.
22. VERHÖEFF, F.H. (1908) Some new staining methods of wide applicability including a rapid differential stain for elastic tissue. J. Am. Med. Ass. **50**. 876-877.