

◆ MANUSCRITO ORIGINAL

Auerbach plexus structure with NADH histochemistry in a line of obese rats: effects of dietary restriction

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Summary

This report aims to study architectural Auerbach plexus structure with NADH histochemistry (nicotinamide adenine dinucleotide, reduced form), along ages and their modifications with restricted diet in obese β line rats. Experimental groups were: 1) After weaning, male rats were fed *ad libitum* (ALD) with standard rat chow. Autopsies were done at 2, 4, 8, 12, and 18 months old. 2) After weaning, one group was fed *ad libitum*, another group of rats were maintained on a restricted diet (RD). Autopsy was performed at 8 months of age. 3) After weaning, male rats were fed *ad libitum* (ALD) with standard rat chow. At 60 days old one group was continued with standard rat chow. Another group was fed with a restricted diet (RD). Autopsy was performed at 120 days old. After autopsy, segments of small intestine, proximal and distal colon were processed for NADH histochemistry. 1) At 2 months of age some empty spaces ("neuronal ghosts") were seen between neurons. Later on partial to total disruption of reticular structures was seen along ages. 2) In RD rats of 8 months of age, a mesh-like structure similar to normal control rats was observed. In ALD rats, partial to total disruption of mesh-like structures was seen. 3) In RD rats of 4 months of age, disruption intermingled with normal mesh-like zones was seen, more severe in ALD rats. Changes in Auerbach plexus structure (disruption of mesh-like appearance) in this line of rats were quite different from normal control rats suggesting dismetabolism effects. Die-

tary restriction delayed alterations in Auerbach plexus structures in obese rats.

Key words. obesity, myenteric plexus, diabetes, small intestine, colon.

Estructura del plexo de Auerbach con técnica histoquímica del NADH en una línea de ratas obesas: efectos de la restricción dietaria

Resumen

Estudiamos las modificaciones con la edad y con una dieta restrictiva de la estructura reticulada del plexo de Auerbach en ratas β (obesas) en intestino delgado, colon proximal y distal que fueron procesados con la técnica histoquímica de NADH (nicotinamide adenine dinucleotide, reduced form). Los grupos experimentales fueron: 1) Después del destete ratas machos fueron alimentadas *ad libitum* (DAL) con dieta balanceada comercial y autopsiados a los 2, 4, 8, 12 y 18 meses de edad. A los 2 meses de edad se observaron espacios vacíos interneuronales y a edades mayores mostraron una destrucción parcial a total de la estructura reticular. 2) Después del destete un grupo fue alimentado con DAL y otro con dieta restringida (RD). Se realizó la autopsia a los 8 meses de edad. En los animales con RD de 8 meses de edad se pudo observar una estructura normal reticulada muy similar a otras ratas controles. En las ratas con DAL se observó una ruptura parcial y/o total de la estructura reticulada. 3) Después del destete, las ratas fueron mantenidos con DAL hasta los 60 días de edad en que un grupo siguió con DAL y otro con RD. La autopsia se hizo a los 120 días. Se observaron zonas con una destrucción parcial de la estructura reticulada que se entremezclaron con zonas con-

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servadas. La destrucción fue más severa en las ratas con DAL. La restricción dietaria pospone la destrucción de la arquitectura reticulada del plexo de Auerbach en las ratas obesas, diferenciándose con las imágenes que presentan los animales controles. Así en las ratas obesas se sugiere un efecto del dismetabolismo (obesidad, hipertriacilglicerolemia, normocolesterolemia) probablemente bajo influencias genéticas.

Palabras claves. *obesidad, plexo mientérico, diabetes, intestino delgado, colon.*

Diet affected small intestine on mucosa growth, morphology, and cell cytokinetics.^{1,2} In adult male Lewis rats, restricted diet fed at an amount of food equivalent to 60% of ALD animals, a reduction of body and intestinal weights were found.³ Enteric nerves responded to physical and chemical stimuli originated in the intestinal lumen by initiating reflex pathways that regulate epithelial function.⁴ The most striking characteristics of the myenteric plexus were the heterogeneity of the neuronal populations and the complexity of their organization. Histochemically, distinct types of neurons were described, which probably use different substances as transmitters.⁵ There are several histological and histochemical techniques that can be used as an approach for the understanding of the ENS (enteric nervous system) physiology, such as NADH,⁶ cuproline blue,⁷ GPG 9.5⁸ and Giemsa.⁹ Each of these techniques have their followers and their detractors.

Since no data were found about Auerbach plexus structure in obese rats, it seemed attractive to explore architectural disposition along ages and the effects of a restricted diet on Auerbach plexus in obese rats using NADH histochemical technique.

Materials and methods

Animals

Obese β line rats (II Mb/Fm β) were raised in the facilities of our School of Medicine (Department of Biology), being the original IIM inbred stock a set of several rat strains bred as parallel lines from a single out bred colony in 1948.^{10,11} These rats are hypertriacilglicerolemic, normocolesterolemic, and hyperglycemic at adult age (model of type 2 diabetes).¹² Hyperinsulinemia is present since postpubertal age.¹³

Experimental procedures, housing and feedings

- 1) After weaning (22 days old), male rats were fed *ad libitum* (ALD) with standard rat chow (Cargill, Argentina). Autopsies were done at 2, 4, 8, 12 and 18 months old (n=6, for each age group).
- 2) Ten male rats were fed *ad libitum* (ALD) with standard rat chow (Cargill, Argentina) consisted of crude proteins 32 kcal %, lipids 19 kcal % and carbohydrates 49 kcal %. Caloric content was 326.40 kcal/100g. Adult ALD rats consumed an average of 23.4 \pm 1.3 g per day each.¹³ Another group of rats (n=10) was maintained on restricting food intake to 70 % of that eaten by *ad lib-fed* rats.¹⁴ Autopsy was performed at 8 months of age.
- 3) Male rats were fed with standard rat chow (ALD) since weaning until 60 days old. Afterwards, 5 rats continued with standard chow and 4 rats were fed with a restricted diet (RD). Autopsy was performed at 120 days old. All rats were placed in a room with standard environmental conditions, under a 12 hours light/12 hours dark schedule cycle and given tap water *ad libitum*. At autopsy, rats were euthanased by ether overdose. Abdomen was cut and opened along the midline: small intestine, from pylorus to the ileocaecal junction, and colon was dissected out from ileo-cecal junction up to 2 cm above anus. According to Freeman et al,¹⁵ proximal (ascending colon) may be distinguished grossly by the presence of oblique mucosal folds and distal (descending) colon may be distinguished grossly by the presence of longitudinal folds. Gut was flushed with PBS (phosphate buffered saline) at 4 °C in order to remove contents and trim fat and mesentery. Ethical Committee from our School of Medicine approved the experimental protocols.

NADH histochemistry

At 25 cm from pylorus, a segment (3-4 cm) from small intestine was sealed at both ends with linen threads and dilated with cold PBS until the specimen started to curl. Segments of proximal and distal colons were similarly processed. NADH (nicotinamide adenine dinucleotide, reduced form) histochemistry was done complying with Gabella⁶ modified as following: specimens were incubated in 0.5% Triton X-100 for 10 minutes. After a brief washing in PBS, specimens were incubated for 60-70 minutes in 10 mg nitroblue tetrazolium (Gibco, Japan),

20 mg NADH (Gibco, Japan), 20 ml phosphate buffer, 20 ml distilled water. They were rinsed in PBS 2-3 times and then fixed in 3% paraformaldehyde (Merck, USA) in PBS for 24 h at 4 °C. Samples were cut at mesenteric border, placed in Petri dish with the serosa face down and the mucosa uppermost. Under dissecting microscope using watchmaker forceps (Brussels's type # 7), the mucosa and submucosa were carefully peeled off, resulting in strips of both circular and longitudinal muscle, with the myenteric plexus sandwiched between the muscle layers and attached with serosa. These strips were rinsed in PBS and mounted with the serosa uppermost, with a local aqueous mounting (Kero, Argentina) and formaldehyde.

Neuronal counts

Neuronal counts were done in a light microscopy (Leitz) with a graticule, bearing in mind that when neurons were crossed on the margins, only those placed in upper and right sides were considered. The neuronal counts are expressed as neurons/mm².

Analytical studies

Triacylglyceridemia,¹⁶ cholesterolemia,¹⁷ glycemía¹⁸ were measured in blood obtained from vein tail.

Results

Longitudinal studies in β rats

Since 2 months old Auerbach plexus mesh-like aspects were structurally present, but some empty spaces (neuronal "ghosts") could be observed between ganglionic neurons (Figure 1, a and b). Partial disruption of mesh-like aspects could be observed (Figure 1, d). Afterwards, advanced disruption (Figure 1, f) ended in isolated neurons was seen with total disruption of mesh-like structures, (Figure 1, h and i). Small intestine (Figure 1, i) was more affected than colon (Figure 1, h). More detailed descriptions could be seen elsewhere.¹⁹

Long term restricted diet

Morphometric data can be seen in Table 1. Body, abdominal fat pads, small intestine and colon weights were lower in RD than ALD rats. Biochemical data can be seen in Table 2. Triacylglyceridemia was lower in RD rats. Auerbach plexus from RD rats (8 months old) was quite similar to that described in other control lines of rats (Wistar,

Sprague-Dawley). In small intestine, ganglia were long and thin, their long axis being almost invariably parallel to the circumference of the gut. They were interconnected with nerve tracts (NADH-positive) showing mesh-like structures (Figure 1, e). In proximal colon ganglia were elongated along circular muscles and polygonal figures in the interconnection with nerve tracts formed polygonal mesh-like structures. In distal colon ganglia were elongated along circular muscles and nerve tracts ran longitudinally forming rectangular mesh-like structures. In the ganglia NADH-positive and NADH-negative neurons could be observed. All neurons showed a round eccentric uncoloured nucleus. NADH-positive neurons ranged from a rim of cytoplasm (small neurons) to abundant cytoplasm, round or oval shaped neurons (medium and large) and NADH-negative. As stated by Johnson et al,¹⁷ non-coloured cytoplasmic neurons could be observed. As background smooth muscles were faintly stained, resembling parallel lines (Figure 1, c, e and f). Scarce blood vessels (as no coloured tube-like structures) could be described. Modifications of these patterns could be observed in ALD fed rats: 1) hypocoloured neuronal cytoplasm; 2) non-coloured cytoplasm, leaving empty spaces (Figure 1, a); 3) normal neuronal ganglia continued by non-coloured cytoplasmic neurons, with elongated nucleus; 4) disruption of mesh-like structures (Figure 1, d and f); 5) nerve tracts with their NADH positivity lost (Figure 1, h and i); and 6) severe changes that included disruption of mesh-like structure, changes in cytoplasmic neuronal border, loss of parallel muscle disposition (muscle clotted stained blot), and vascular muscle stained with NADH (Figure 1, h). Intact zones were intermingled with the disruptions of the plexus. Neuronal count was lower in ALD rats (Table 3).

Restricted diet rats for 60 days long

Morphometrical data can be seen in table 1: rat body weights and abdominal fat pads were lower in RD rats. Plasma parameters can be seen in Table 2: triacylglycerides were lower in RD rats. Auerbach plexus in small intestine, proximal and distal colon in RD rats showed zones of mesh-like aspects of Auerbach plexus (Figure 1, c), intermingled with disrupted zones. In ALD rats more severe plexual disruption was observed (Figure 1, d). No differences in neuronal count were seen between groups (table 3).

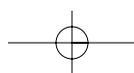
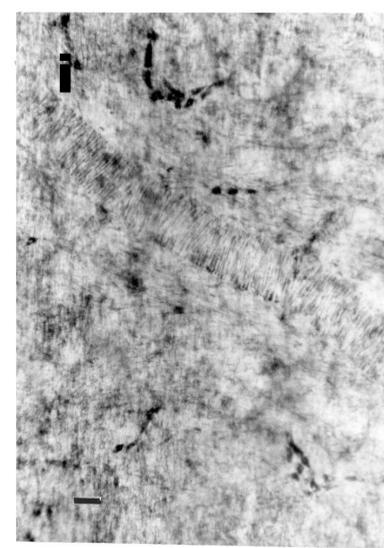
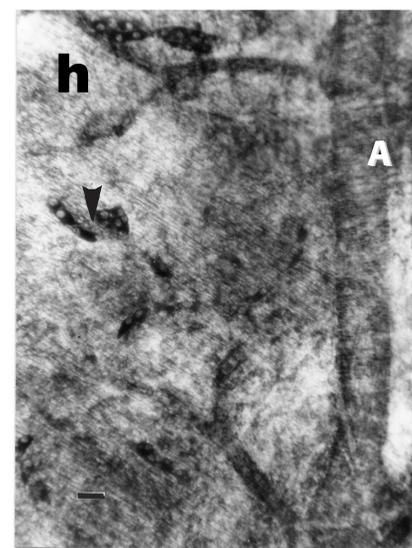
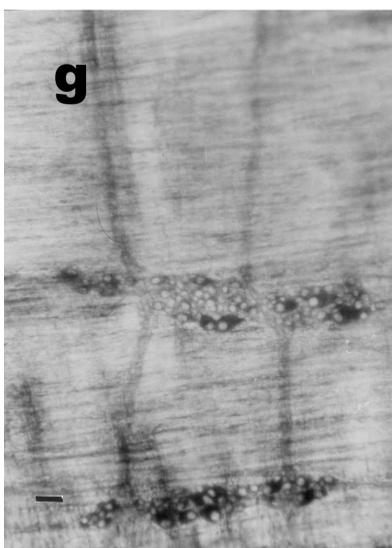
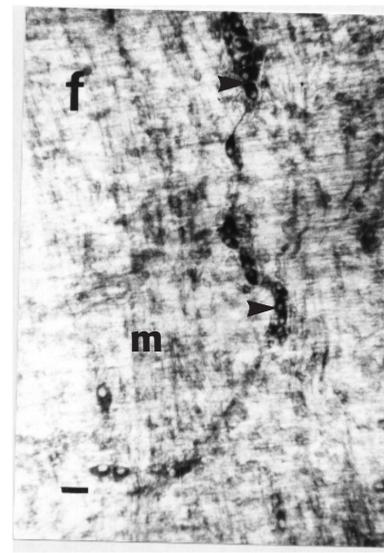
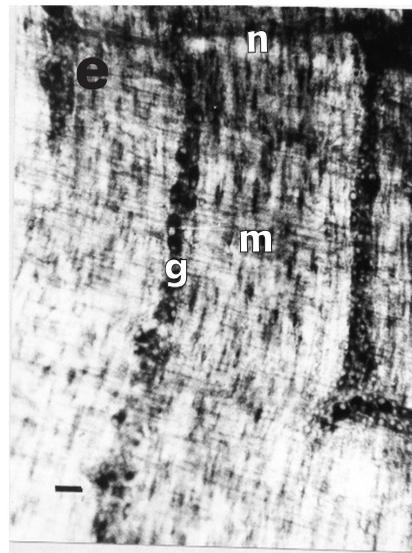
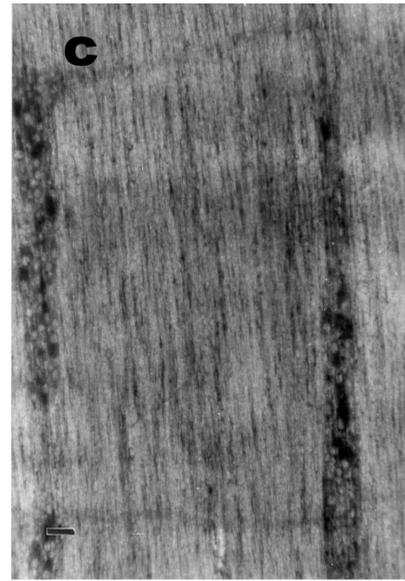
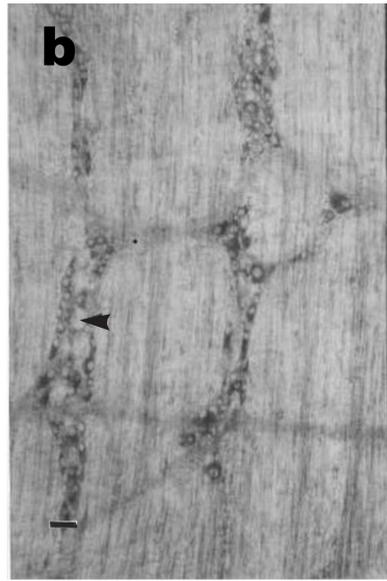
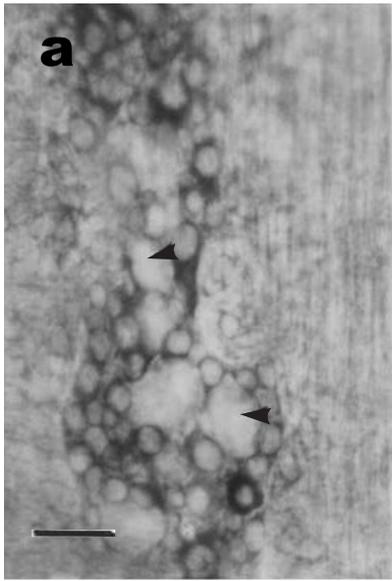
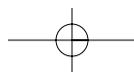


Table 1. Morphometrical data are presented as mean \pm SEM and have been compared using two-tailed *t* test for unpaired data. Rat body and abdominal fat pads weights are lower in RD rats at 4 and 8 months old. Intestinal weights were lower in RD rats at 8 months old; conversely, no differences were detected in 4 months old rats.

	MORPHOMETRY 8 months old rats		<i>P</i>
	restricted diet rats (n=10)	non-restricted diet rats (n=10)	
rat BW (g)	238.25 \pm 11.33	396.65 \pm 10.09	<i>P</i> < 0.001
abdominal fat pads (g)	7.83 \pm 1.01	18.86 \pm 0.24	<i>P</i> < 0.001
INTESTINAL TRACT			
Small intestine (g)	7.67 \pm 0.27	8.75 \pm 0.32	<i>P</i> < 0.05
Small intestine (cm)	109.8 \pm 1.85	114.73 \pm 2.57	n.s.
Colon (g)	1.89 \pm 0.03	2.10 \pm 0.08	<i>P</i> < 0.05
Colon (cm)	16.42 \pm 0.35	16.28 \pm 0.51	n.s.

	MORPHOMETRY 4 months old rats		<i>P</i>
	restricted diet rats (n=4)	non-restricted diet rats (n=5)	
rat BW (g)	317.00 \pm 10.02	448.00 \pm 5.81	<i>P</i> < 0.001
abdominal fat pads (g)	13.17 \pm 1.48	28.29 \pm 1.29	<i>P</i> < 0.001
GASTROINTESTINAL TRACT			
Small intestine (g)	9.81 \pm 0.96	9.85 \pm 1.09	n.s.
Small intestine (cm)	111.75 \pm 2.17	118.60	<i>P</i> < 0.05
Colon (g)	2.36 \pm 0.18	2.43 \pm 0.15	n.s.
Colon (cm)	16.75 \pm 0.25	19.50 \pm 0.75	<i>P</i> < 0.05

Table 2. Plasma parameters data are presented as mean \pm SEM and have been compared using two-tailed *t* test for unpaired data. Note that glycaemia are scarcely higher than normal values. No differences were found in cholesterolemia at 4 and 8 months old rats in RD and ALD rats, but, triacylglycerides were lower in RD rats.

	PLASMA PARAMETERS 8 months old rats		<i>P</i>
	restricted diet rats (n=10)	non-restricted diet rats (n=10)	
glucose (g/l)	1.21 \pm 0.04	1.29 \pm 0.07	n.s.
cholesterol (g/l)	1.25 \pm 0.03	1.16 \pm 0.10	n.s.
triacylglycerides (g/l)	0.78 \pm 0.08	1.85 \pm 0.38	<i>P</i> < 0.001

	PLASMA PARAMETERS 4 months old rats		<i>P</i>
	restricted diet rats (n=4)	non-restricted diet rats (n=5)	
glucose (g/l)	1.29 \pm 0.08	1.14 \pm 0.09	n.s.
cholesterol (g/l)	1.05 \pm 0.03	0.68 \pm 0.16	n.s.
triacylglycerides (g/l)	1.02 \pm 0.11	2.46 \pm 0.38	<i>P</i> < 0.001

Figure 1. Auerbach plexus from β rats, stained with histochemical NADH technique.

- a)** jejunum, ALD, 60 days old rats. NADH+ neurons are seen with eccentric uncolored nucleous, empty spaces (arrowhead) named as neuronal "ghost". As background, smooth muscle fibers could be observed as paralell lines. (bar = 50 μ m).
- b)** distal colon, ALD, 60 days old rats, ganglia along transversal smooth muscle (paralell lines, composed with NADH neurons and empty spaces (arrowhead) named as neuronal "ghost". Nerve fibers (NADH+) interconnect ganglia. (bar = 150 μ m).
- c)** jejunum, RD, 120 days old rats, mesh-like structure is observed, ganglia are disposed along transversal smooth muscle (paralell lines), ganglia are interconnect with nerve fibers (bar = 150 μ m).
- d)** jejunum, DAL, 120 days old rats, disruption of mesh-like structures are seen, some neurons still remain clustered (bar = 150 μ m).
- e)** jejunum, RD, 8 months old rat, similar to fig. "c": muscle (m) is seen as paralell lines, fiber nerves (n) interconnect ganglia, (bar = 150 μ m).
- f)** jejunum, DAL, 8 months old rat, disruption of mesh-like structure is observed, neurons (arrowhead), some remain clustered, muscle (m). (bar = 150 μ m).
- g)** distal colon, ALD, 12 months old rats, some isolated or small groups of neurons are seen, nerve fibres and muscle fibres are weakly stained with NADH, (bar = 150 μ m).
- h)** proximal colon, ALD, 18 months old rats, total disruption of mesh like structure could be observed, scarce isolated neurons could be seen (arrowhead), in artery (A) clotted NADH+ could be observed (bar = 150 μ m).
- i)** jejunum, DAL, 18 months old rats. Total disruption of mesh-like structure is observed, remain some isolated or small groups of neurons, no nerve tracts (NADH+) are observed, (bar = 200 μ m).

Table 3. Neuronal counts expressed as neurons/mm² are presented as mean \pm SEM and have been compared using two-tailed *t* test for unpaired data. A reduction in neurons are detected in ALD 8 months old rats.

Neuronal counts 8 months old rats	Neurons/mm ²		
	RD	ALD	
small intestine	59.97 \pm 8.45	38.15 \pm 7.42	<i>P</i> < 0.05
proximal colon	65.85 \pm 1.83	19.14 \pm 5.99	<i>P</i> < 0.05
distal colon	39.08 \pm 8.9	13.47 \pm 3.56	<i>P</i> < 0.05

4 months old rats	Neurons/mm ²		
	RD	ALD	
small intestine	57.33 \pm 7.44	58.15 \pm 10.42	n.s.
proximal colon	55.85 \pm 1.83	49.14 \pm 5.99	n.s.
distal colon	36.08 \pm 8.9	33.47 \pm 3.56	n.s.

Discussion

Gastrointestinal problems are reported in the elderly,²¹ diabetic²² and obese persons.²³ With the aim to make an approach for the understanding of these phenomena, we study morphological data in a line of obese rat along ages. Secondarily, modifications in animals fed with a restricted diet are reported.

At adult age, β rats in DAL are obese, hypertriacylglycerolemic, normocholesterolemic, and hyperglycaemic (model of type 2 diabetes).¹³ Hyperinsulinemia is present since postpubertal age.¹² As described by Gabella²⁴ in small intestine, the myenteric plexus of the adult rat ganglia was found to be elongated along the circular muscle coat, interconnected by thick nerve bundles and fine fibres.¹⁹ With NADH histochemistry, no differences were reported as regards to structure in aged rats, but are accompanied with a decrease in neurons.^{19;25} In our animal model in *ad lib-fed* rats, mesh-like structure is disrupted with ageing. Results obtained from β rats with diet restriction produced a minor body, lower abdominal fat pads and gut weights (Table 1). Total body weight gain in RD lagged behind *ad libitum* controls.^{3,26} According to Ferraris and Carey,²⁷ chronic diet restriction led to a dramatic decrease in body weight with relatively modest effects on intestinal structure.

Differences in myenteric plexus were apparent between young and old restricted fed Sprague Dawley rats¹⁹ stained with NADH. NADH positive neurons densities were lower in *ad libitum* than in restricted fed Sprague-Dawley rats.²⁸ According to

our results, remarkable differences could be pointed out. In β restricted fed rats we found that myenteric plexus structures were similar to those described by several authors for control rats. Instead, in non-restricted diet the described mesh-like plexual structure is absent. Hyperacyltryglycerolemia is detected in β rats since postpubertal age.¹³ When restriction began at 60 days old in β rats, at 120 days old disruption of mesh-like structure was lower than *ad libitum* fed rats. Factors affecting neurons of Auerbach plexus in obese rats are still unknown. But, dietary restriction (DR) reduces oxidative stress.²⁹ A colon chemically denervated by topical serosal application of benzalconium chloride induced a significant reduction of neurons,²⁷ without a concomitant alteration in muscle contractility,²⁸ resembling our ALD aged rats. Changes in Auerbach plexus structure (disruption of mesh-like appearance) in this line of β rats were quite different from "normal" control rats suggesting dimetabolism effects probably influenced by genetics (obesity, hypertriacylglycerolemia, normocholesterolemia). Hyperglycaemia might not play an important role. In *eSS* rats (lean, spontaneous type 2 diabetes model).³² Auerbach plexus was seen as a mesh-like structure similar to that described in Wistar rats.¹⁹

In short, according to our results, in obese β rats alterations of mesh-like structure of Auerbach plexus are observed since early in their life and disruption of those structures are severely affected according to ageing, restricted diet delays the alterations in Auerbach plexus structures and is more effective when restriction is started at postweaning age, and minor neurons and disruption of mesh-like structure in animal model anatomically support changes observed in gastrointestinal motility in obese patients.^{20,30}

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