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Aging of the Myenteric Plexus in the Rat Colorectal Region

Nikolay Genov[1*], Nikolay Dimitrov[1], Dimitrinka Atanasova[1,2]

[1] *Department of Anatomy, Faculty of Medicine, Trakia University, Stara Zagora, Bulgaria*
[2] *Institute of Neurobiology, Bulgarian Academy of Sciences, Sofia, Bulgaria*

*Corresponding author e-mail: nikolay.genov@trakia-uni.bg

In senile organisms various changes occur at the level of the gastrointestinal tract. Not that many studies have been focused on the changes that develop in the connective tissue capsule positioned around the ganglia of the myenteric plexus. The aim of this study is to demonstrate the changes of the connective tissue around the ganglia of the Auerbach plexus. We have studied the colon of Wistar rats and have divided the animals into four groups: 3-months, 18-months, 28- and 48-months. Our study has demonstrated a 65% increase of the area of collagen fibers around the myenteric plexus in the 18- and 48-months old rats in comparison with the 3-months. The Orcein staining for elastic fibers has shown a 40% increase of their area around the myenteric ganglia of the proximal colon of the 18-months animals compared with the 3-months.

Key words: myenteric plexus, rat, colon, colorectal region, connective tissue, collagen fibers, elastic fibers

Introduction

The enteric nervous system (ENS) is a complex network of neurons that plays a major role into gastrointestinal motility, secretion, nutrient uptake [3, 6]. The biggest portion of the cells of the ENS is making the so-called myenteric plexus [10]. In the literature there are various sources that are presenting the existence of a collagen capsule surrounding the ganglia of the myenteric plexus [8]. A lot of studies are pointing at the existence of connective tissue molecules surrounding the ganglia of the Auerbach plexus [2, 6]. In senile organisms a general increase of the amount of that tissue is noticed [8].

The aim of the present study was to determine the overall change of the area of connective tissue elements, collagen and elastic fibers, around the ganglia of the myenteric plexus at the level of the rat large intestine in the different age groups.

Material and Methods

The scientific experiments were conducted on 20 Wistar rats divided into four groups: 3-, 18-, 28- and 48 months old. Their average weight was 180-450 g. The 3-months-old rats were delivered from the vivarium of the Faculty of Medicine at Trakia University – Stara Zagora. The others were obtained from project 13/17 of Trakia University – Stara Zagora, №174.

The animals were housed under an artificial 12-h light/dark cycle and at a temperature of 22 °C. Water and food pellets were supplied *ad libitum*. The experiments in this study were approved by the Research Ethics Committee at the Medical Faculty of Trakia University and the Commission for Ethical Treatment of Animals at the Bulgarian Food Safety Agency. All the experiments were carried out in full agreement with the Directive 2010/63/EU on the protection of animals used for scientific purposes.

All rats were anesthetized with 87 mg ketamine/kg of body weight and 13 mg xylazine/kg after simultaneous intraperitoneal injection and transcardially perfused first with cold 0.05 M phosphate buffered saline (PBS) and after that cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.36. The large intestine of the examined animals was dissected and divided into *caecum* (CAE), *proximal colon* (PC), *distal colon* (DC) and *rectum* (REC). The tissue samples were than immersed into the same fixative overnight at 4 ºC. After that we followed the standard procedure for embedding the tissue in paraffin. The paraffin blocks were cut into 6 μm sections and were mounted onto chrome-gelatinized glass slides. For the purpose of the experiment we have stained the slides with the following standard histological stains: Azan, van Gieson and Orcein.

The microphotographs have been made with research microscope Leica DM1000 equipped with a digital camera Leica DFC 290. The images were processed with Adobe Photoshop 24.1.0 to achieve better contrast of the stained slides.

The free graphic analyzing software ImageJ (*National Institutes of Health, Bethesda, MD, USA*) was used to perform the morphometric analysis of the connective tissue capsule. In order to make the measurements, each of the microphotographs has been converted into an 8-bit image by the plug-in *Colour Deconvolution2.* That helped us to measure the percentage of the stained collagen and elastic fibers as part of the whole area of the ganglia of the myenteric plexus.

Statistical analysis was performed by GraphPad Prism® 8 software (San Diego, CA, USA). The comparison of the results has been conducted with Kruskal-Wallis Analysis as well as Ordinary one-way ANOVA followed by the multiple comparisons Dunn tests or Sidak's multiple comparisons test for the ordinary one-way ANOVA. Statistically significant differences were considered if p-values were <0.05.

Results

The connective tissue around the ganglia of the Auerbach plexus was presented with Azan, Van Gieson and Orcein staining. The usage of Azan classical histological stain presented the collagen fibers as a green blue network. After measuring the percentage of the collagen fibers as part of the whole area of the ganglia, the Kruskal-Wallis test showed no statistically significant difference between the examine groups: 3m Mdn = 20.98, 18m Mdn = 27.20, 28m Mdn = 23.42; $H(2) = 3.157$, $p = 0.2063$.

The acidic fucsin of the Veigert-van Gieson stain colored the collagen fibers in red. The Kruskal-Wallis test showed statistical significant difference between the percentage of the collagen fibers compared to the area of the ganglia in between the different groups of the examined animals: PC 3m (Mdn = 3.55), PC 18m (Mdn = 9.993), PC 48m Mdn = 10.23, DC 3m Mdn = 7.275, DC 18m Mdn = 31.86, DC 48m Mdn = 13.65, REC 3m Mdn = 4.5, REC 18m Mdn = 18.02, REC 48m Mdn = 28.31; $H(8) = 48.99$, $p < 0.0001$. Dunn's multiple comparison test showed that the significant change occurs in between DC 3m and DC 18m ($p < 0.005$), REC 3m and REC 18m ($p < 0.05$), REC 3m and REC 48m ($p < 0.001$) .

Orcein stained the elastic fibers around the ganglia of the myenteric plexus in dark brown color. There were statically more elastic fibers in the aged groups: PC 3м Mdn = 8.106, PC 18м Mdn = 13.81, DC 3м Mdn = 5.425, DC 18м Mdn = 7.550, REC 3м Mdn = 13.32, REC 18м Mdn = 8.832; $H(5) = 15.22$.

Frequency distribution showed that the percentage of the collagen fibers around the myenteric plexus ganglia in 18m and 48m PC of Wistar rats has increased with 65% compared with the 3 months of age. At the level of the distal colon the percentage of the collagen capsule compared to the whole area of the ganglia has increased with 75% in comparison with the control. At the level of the rectum collagen capsule has increased with 71% for the first 15 months of the animals' life.

Orcein stained showed an increase of almost 40% of the elastic fibers around the ganglions of the Auerbach plexus of the 18 months rats compared with the 3 months old. This has been identified at the level of the proximal colon.

Discussion

In this study we attempt to present the existence of a structure around the ganglia of the myenteric plexus and to show its change with age. In some literature references the ganglia of the Auerbach's plexus are shown as structures without a capsule but rather directly positioned between the muscle layers of the intestines having a thin layer of fibroblast-like cells [11]. In others they are proving the existence of a capsule made of collagen and elastic fibers that are even piercing in between the perikaryons of the neurons of the myenteric plexus [8, 13]. The collagen structures around the ganglia of the Auerbach's plexus form a basement membrane (BM) [4], a key component of which is collagen type IV [7]. Collagen is one of the most abundant glycoproteins in the human body and is also a key component of the extracellular matrix (ECM) [12]. The usage of Azan and van Gieson stain helped us identify a well-defined collagen structure around the ganglia of the myenteric plexus at all of the levels of the large intestine. Some researchers are pointing to van Gieson as an exemplary method for representing the accumulation of collagen type I [9]. Recent studies show the existence of 28 different subtypes of collagen, some being fibril-forming (I, II, III) and others being network-forming, such as collagen type IV [14]. The increased number of stained structures around Auerbach's plexus by van Gieson in the aged animals could be related to the fibrotic shift of the collagen in the BM of the myenteric plexus.

In literature we have found that with age the amount of collagen and elastic tissue around the myenteric plexus increases [8] and other sources have pointed out that the total deposition of collagen in the aging intestines increases with about 16% [1].

Our study has proven the existence of a collagen capsule around the neurons of the myenteric plexus and has given a detailed characteristic of it at the different levels of the large intestine. We have identified the change of the collagen and elastic fibers with age but in contrast with literature the increase was significantly bigger and statistically significant on almost all the levels of *intestinum crassum*. That increase of the connective tissue deposition around the ganglia of the myenteric plexus could be in direct connection with the decreased motility of the intestines which has been presented as one of the main changes that occur at the level of the aging colon [12].

The method we used to conduct our morphometric analysis could be criticized but we are looking forward to use electron microscopy as well as confocal microscopy to define the changes that occur at molecular level as well as to study the specific proteins that increase or decrease at the aging myenteric plexus.

Conclusions

This is one of the few studies of that focuses on the connective tissue capsule positioned around the myenteric plexus. We have identified a significant change in the collagen and elastic fibers around the ganglia of the aging animals. Further studies are needed to determine the specific type of collagen that accumulates around the plexus. More work is required to evaluate the clinical significance of the observed change.

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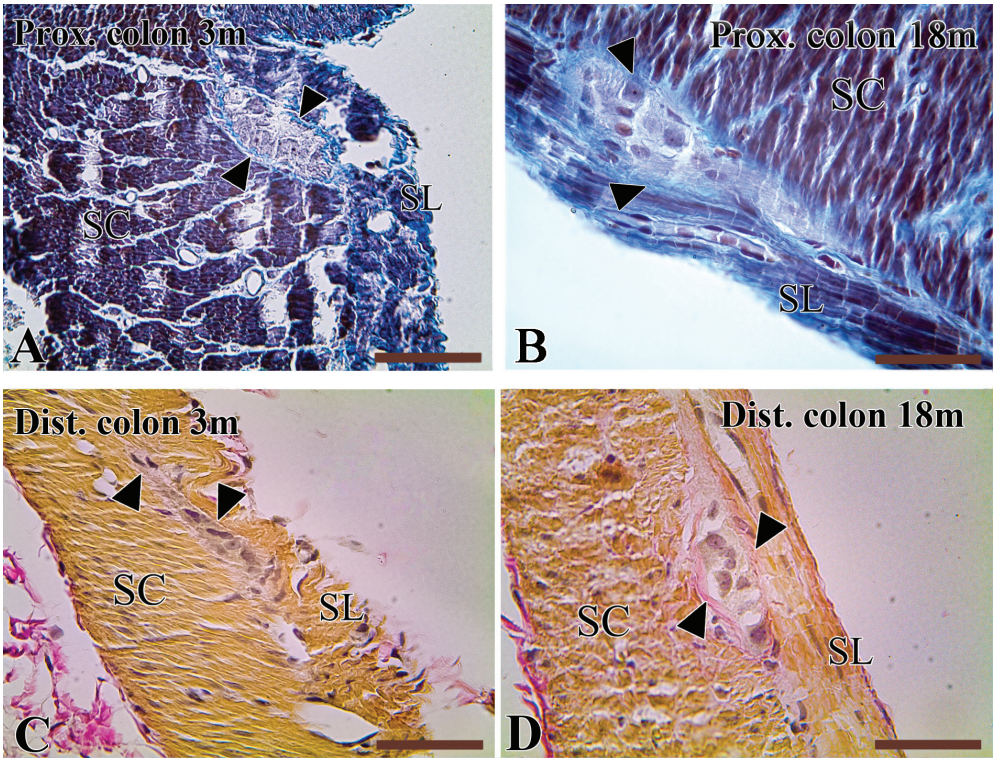

Figure 1. Azan (**A-B**) and van Gieson (**C-D**) staining of sagital cut of proximal colon (**A-B**) and distal colon (**C-D**) of a 3 month (**A, C**) and 18 month (**B, D**) Wistar rat. Arrowheads indicate the collagen fibers that are encapsulating the ganglia of the myenteric plexus. SC-*stratum longitudinale*, SC- *stratum circualare*. Scale bars: 50 μm.

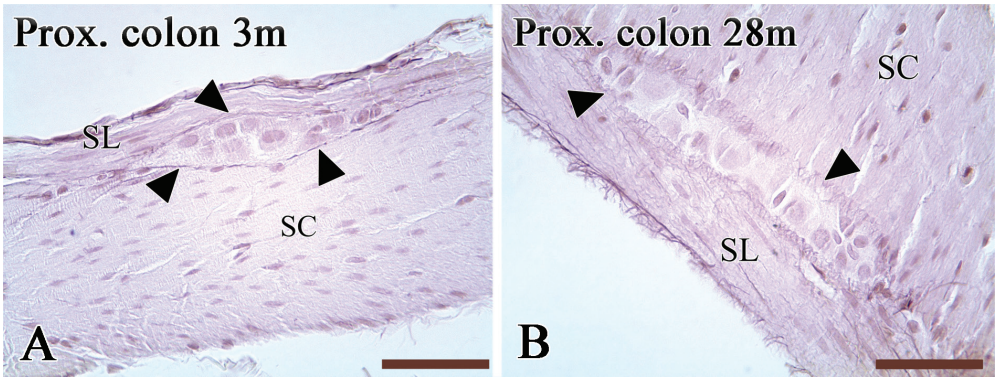

Figure 2. Orcein staining (**A-B**) of sagital cut of proximal colon of a 3 month (**A**) and 28 month (**B**) Wistar rat. Arrowheads indicate the elastic fibers that are positioned around the ganglia of the Myenteric plexus. SC-*stratum longitudinale*, SC- *stratum circualare*. Scale bars: 50 µm.

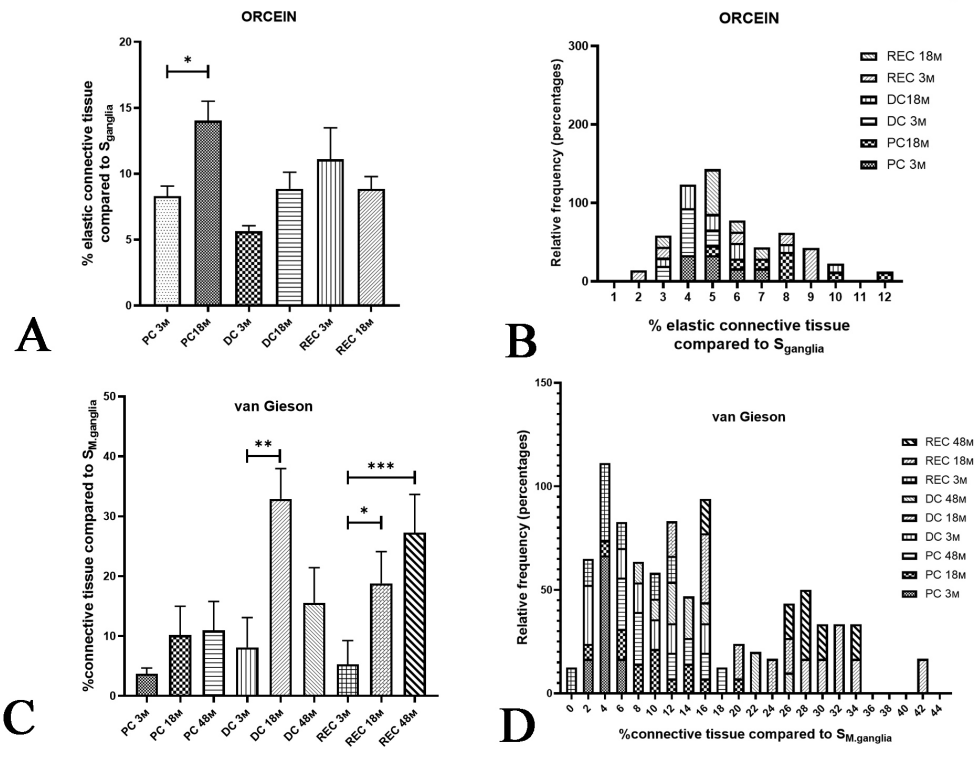

Figure 3. Histograms showing statistical comparison of the percentage of the area of the elastic fibers stained with Orcein **(A)** and collagen fibers stained with van Gieson **(B)** surrounding the ganglia and the whole area of the ganglia from the rat myenteric plexus. The line represents S.E.M. The data is compared using the Sidak's multiple comparisons test and Kruskal-Wallis test, where p* < 0.05, p** < 0.01, p*** < 0.001. Histograms showing the relative frequency in percent of a distribution of values for the percentage area of elastic fibers **(B)** and collagen fibers (**D**).