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Comparison between Whole-Mount Preparations and Paraffin-Embedded Sections in the Study of the Myenteric Plexus

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The present study aimes to compare the whole-mount preparation of the colorectal region with the paraffin-embedded sections of the same area for histology studies. It focuses on analyzing the sectional area of myenteric ganglia and the sectional area of neuronal perikarya in the colorectal region of the rat. 82% of the ganglia obtained by cross-sectional cut had sectional area sizes ranging from 500 μm^2 to 4500 μm^2. Conversely, 72% of the ganglia observed by tangential cut ranged from 4500 μm^2 to 10500 μm^2. In whole-mount prepared slides, 40% of them were in the range of 10500 μm^2 to 20500 μm^2 while 80% of perikarya in the cross-sectional cut paraffin-embedded slides had a sectional area within 50 μm^2 to 150 μm^2. In tangential cut, 70% of the neuronal bodies had similar sectional sizes. Neuronal somata ranging from 200 μm^2 to 450 μm^2 were observed more frequently (57%) in whole-mount preparations.

Key words: morphometry, colon, rectum, myenteric plexus, myenteric ganglion

Introduction

The enteric nervous system is composed of a considerable number of cells which provide the independent innervation of the gastrointestinal (GI) tract, including smooth muscles of its wall, the epithelial tissues, blood vessels and endocrine cells associated with it [2, 5]. Enteric neurons are arranged in interconnected ganglia, thus forming a complex meshwork described as a polysynaptic circuit [10]. According to

current knowledge, the total number of enteric neurons is around 500 million, that is almost equal to the number of neurons in the spinal cord [2]. The ganglia between the longitudinal and circular muscle layers form the myenteric plexus. Enteric neurons and closely associated glial cells are compressed between muscle layers which can change their dimensions when observed by the longitudinal or the transverse axis [3].

The overall shape of the myenteric plexus is three-dimensional, which makes standard methods such as cryosections and paraffin-embedded sections not suitable for examination of the whole nerve plexus because only a fraction of it could be observed [5, 6]. Due to its specific shape, the usage of cross-sectional cut compared with tangential sectioning could bring a much more comprehensive information about the size of neuronal perikarya in the myenteric plexus [3].

On the other hand, whole-mount (WM) preparations have been used by many authors to provide a three-dimensional view of the meshwork of enteric ganglia and to give a much better ability for examination of the myenteric plexus and evaluation of the maximal size of the neuronal soma [2, 3, 6, 7, 9].

The present study aimed to measure the cross-sectional area of myenteric ganglia and the cross-sectional area of neuronal perikarya obtained by paraffin-embedded tissue (PET) at the light microscope level, and the subsequent cutting of cross-sectional (CS) and tangential sections (TS) and to compare them with whole-mounted preparations.

Material and Methods

The study was performed on six adult (18-month-old) male Wistar rats with an average weight 350-400 g. The animals have been delivered from the vivarium of the Faculty of Medicine at Trakia University-Stara Zagora. The housing of the animals has been conducted under an artificial 12-h light/dark cycle and at a temperature of 22 °C. Water and food pellets have been given *ad libitum*. The experimental procedure was approved by the Commission for Ethical Treatment of Animals at the Bulgarian Food Safety Agency and all experiments were carried out in full agreement with the Directive 2010/63/EU on the protection of animals used for scientific purposes. For the morphometric analyses, all rats were anaesthetized with 87 mg ketamine/kg of body weight and 13 mg xylazine/kg after simultaneous intraperitoneal injection and then transcardially perfused first with cold 0.05 M phosphate-buffered saline (PBS) and after that with cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.36. Three tissue segments were collected from the proximal colon, distal colon, and rectum. For the PET (CS) and the PET (TS) sections the tissue samples were postfixed in the same fixative for 24 h and subsequently washed first in tap water, followed by distilled water, dehydrated, and embedded in paraffin. Paraffin blocks were cut into 6 μm tissue sections and mounted on chrome-gelatinized glass slides and then processed for hematoxylin and eosin staining.

We used colonic tissue fixed with 4% PFA in 0.1M PB for the whole-mount preparations. It was dissected into the following segments: caecum, proximal colon,

distal colon, and rectum. Each of the samples was cut into separate pieces with a length of roughly 2 cm. During the whole mount preparation, the samples were held in cold PBS. The separation of the myenteric plexus from the mucosa and the circular muscle layer was done under a stereoscopic microscope (ZEISS OPTON 47 50 57) with microdissection tools. A research microscope Leica DM1000 equipped with a digital camera Leica DFC 290 was used to obtain images of objects in the slides. All images were processed with Adobe Photoshop CC software.

The graphic analysis, as well as the measurement of the sectional area of the myenteric ganglia and neurons, were performed with the software ImageJ (National Institutes of Health, Bethesda, MD, USA). Statistical analysis was performed by GraphPad Prism®6 software (San Diego, CA, USA) and Kruskal-Wallis One Way Analysis of Variance by Ranks test. Statistically significant differences were considered if p-values were <0.05.

Results

In this study, we have measured the sectional area of the ganglia and the neuronal perikarya in the myenteric plexus located at the colorectal region. The sections used in the measuring were from PET (CS), PET (TS) and whole-mount preparations (**Fig. 1**). The examined segments were collected from the proximal colon, distal colon and rectum and routinely stained with the haematoxylin and eosin staining technique. This staining method allowed us to observe in detail the ganglia and the perikarya from the myenteric plexus by fully delineating it from the nearby structures thus permitting us to measure it more precisely. For statistical purposes, we performed the non-parametric Kruskal–Wallis test to study the sectional area of the myenteric ganglia and the perikarya in cross-sectional, tangential and whole-mount preparations.

The measurements of the sectional area of the myenteric ganglia showed statically significant differences in the three examined methods proven by the non-

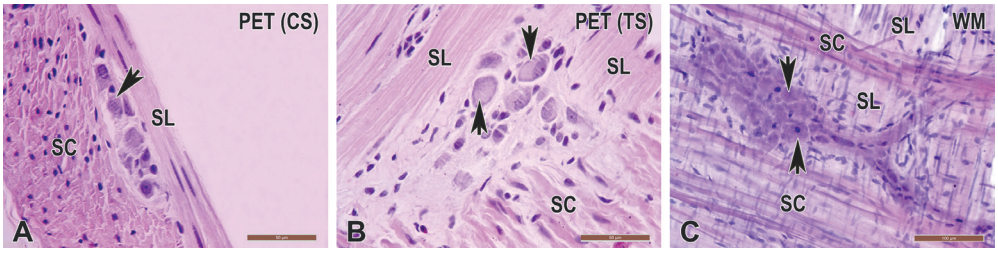

Fig. 1. Hematoxylin and eosin-stained sections visualizing the myenteric ganglia and the neuronal perikarya obtained by paraffin-embedded tissue (PET) and the subsequent cutting of cross-sectional (CS) **(A)** and tangential sections (TS) **(B)** and whole-mounted preparation **(C)**. Arrows point out neurons. SC, stratum cyrculare; SL, stratum longitudinale. Scale bars: 50 µm.

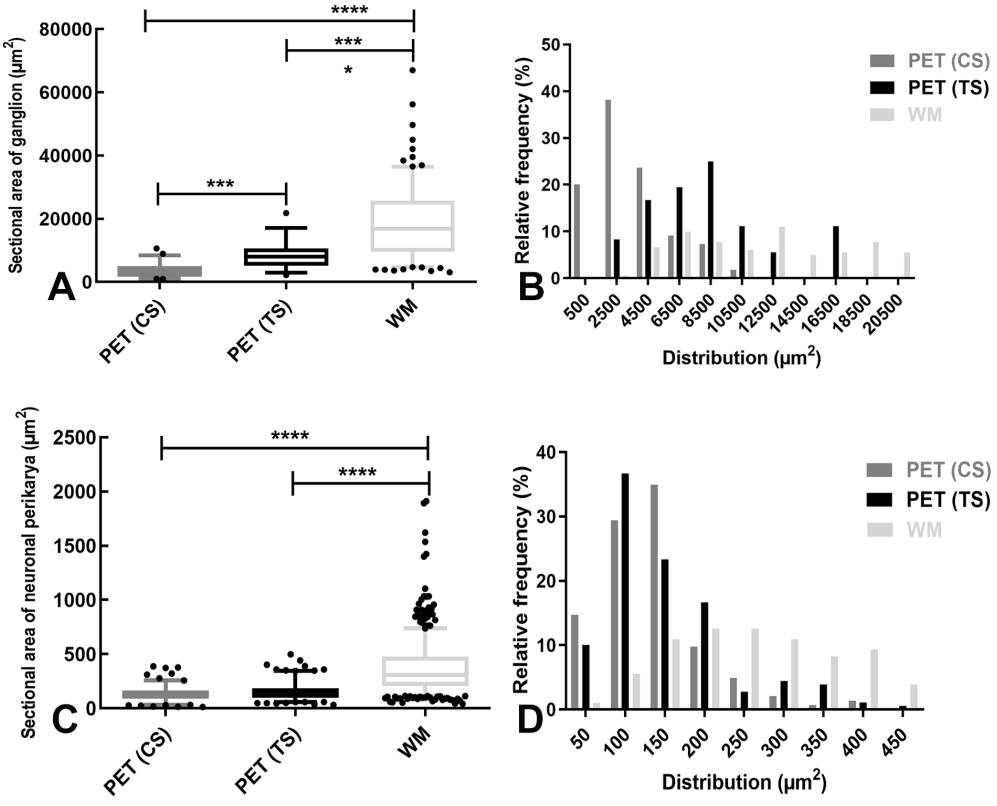

Fig. 2. Box plot diagrams showing statistical comparison of the sectional area of ganglia **(A)** and the sectional area of neuronal perikarya **(C)** in paraffin-embedded tissue (PET) and the subsequent cutting of cross-sectional PET (CS), tangential section PET (TS) and whole-mount (WM) preparations from the rat myenteric plexus. The data are presented as box plots, where the line within the box represents the median and the boxes represent the second and third quartiles (25–75%). Individual points are the corresponding values. The data compared using the Kruskal-Wallis test, where p*** < 0.001, p**** < 0.0001. Histograms showing the relative frequency in percent of a distribution of values for the cross-sectional area of ganglia **(B)** and cross-sectional area of neuronal perikarya **(D)**.

parametric Kruskal-Wallis test H(2)=127.3, p<0.0001 (**Fig. 2A**). Difference of the median (Med) of the ganglia obtained by the three methods applied for preparation of the large intestine was presented [paraffin-embedded tissue cross-sectional cut (Med = 3222 µm^2), paraffin-embedded tissue tangential section (Med = 8010 µm^2) and whole-mount preparations (Med = 16847 µm^2)]. Cross-sectional cut revealed that 82% of the myenteric ganglia had sectional area sizes in the field of 500 µm^2 to 4500 µm^2. In tangential cut, the sectional area in 72% of the ganglia ranged from 4500 µm^2 to 10500 µm^2, and 40% of the whole-mount it varied between 10500 µm^2 to 20500 µm^2 (**Fig. 2B**). The sectional area of the neuronal perikarya measured by the three

methods also showed statically significant differences determined by the same test $H(2)=352.6$, $p<0.0001$. The medians of the perikarya obtained by the three cutting techniques was different: PET (CS) (128.5 μm^2), PET (TS) (132.8 μm^2), and whole-mount preparations (309.7 μm^2) (**Fig. 2C, D**). The total number (n) of the examined ganglia was 272, and the number of the examined neuronal bodies was 991.

Discussion

In the present study, we compared tissue preparations to examine the morphological differences of the Auerbach's plexus by three cutting directions (transversal, tangential and whole-mount preparations). Our results showed a statistically significant difference between the ganglia area and neuronal perikarya obtained by paraffin-embedded sections and whole-mount prepared slides. The three dimensions of the network forming the myenteric plexus do not allow us to thoroughly study its complexity by the standard methods of preparations, a conclusion drawn by many authors [5, 6].

Previous studies have reported that the most common neuron soma area ranges from 200 to 400 μm^2 [8]. By using whole mount preparations, we obtained similar data demonstrating that in 57% of the neuronal perikarya the surface area varies from 200 μm^2 to 450 μm^2. On the contrary, in the transverse and tangential plane sections, more than 70% of the neuronal cell bodies have sectional areas ranging from 50 μm^2 to 150 μm^2. Therefore, whole-mount preparation slides could give much more accurate results in terms of surface morphology. However, the procedure is more time-consuming, it requires a specific preparation technique and trained investigators with good microdissection skills to achieve reliable results. Last but not least, a greater number of experimental animals is needed contrary to animals used in the 3R principles. As reported by other researchers, during whole-mount preparations, it is not a rare occurrence to rupture the longitudinal muscle layer and even to damage the myenteric plexus [4, 5]. We have faced that problem many times in the tissue preparations. In contrast, standard techniques with paraffin embedding are well established and they can be performed on a routine basis. However, they generally tend to demonstrate the neuronal cell bodies and ganglia with sectional areas far smaller than the those in whole-mount slides.

Conclusions

The present study shows that the whole mount preparation technique allows a more precise measurement of myenteric ganglia and their connectivity. On the other hand, the paraffin-sectioning procedure could be a practical solution, which reduces the number of experimental animals and allows for multiple histological processing of the same tissue. One has to keep in mind the possible pitfalls of underestimating

neuronal and ganglion sizes when using paraffin sectioning procedure. A stereological approach is justified when accurate and precise measurements are needed.

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