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Original scientific paper

The Notochordal Sheath in Amphioxus – An Ultrastructural and Histochemical Study

Ivana Bočina[1] and Mirna Saraga-Babić[2]

[1] Department of Biology, Faculty of Natural Sciences, University of Split, Split, Croatia
[2] Department of Anatomy, Histology and Embryology, School of Medicine, University of Split, Split, Croatia

A B S T R A C T

The notochord and notochordal sheath of 10 adult amphioxus were investigated ultrastructurally and histochemically. The notochord in amphioxus consists of parallel notochordal cells (plates) and each plate consists of parallel thicker and thinner fibrils and numerous profiles of smooth endoplasmic reticulum situated just beneath the cell membrane. Histochemical staining shows that the notochordal plates resemble neither the connective tissue notochordal sheath nor the typical muscular structure myotomes. The notochordal sheath has a complex three-layered organization with the outer, middle and inner layer. The outer and middle layer are composed of collagen fibers of different thickness and course, that correspond to collagen type I and collagen type III in vertebrates, respectively, and the inner layer is amorphous, resembles basal lamina, and is closely attached to the notochord by hemidesmosome junctions. These results confirm the presence of collagen fibers and absence of elastic fibers in amphioxus.

***Key words**: amphioxus, notochord, notochordal sheath, electron microscopy*

Introduction

The notochord is a form of the inside skeleton, which exists as an embryonic structure in all vertebrates, including men. In most species, the notochord appears during the primary embryonic induction and causes induction of the neural tube and vertebral column[1–3]. It also specifies the type of overlying neural tissue maintains integrity of the somites and stimulates their chondrogenesis[4]. During the mouse, chicken and human development, the close position of the notochord to the neural tube seems to be important for normal formation of all axial organs: neural tube, notochord and vertebral column[5–8]. After accomplishing its inductive role, the subsequent degeneration of the notochord reflects the end of its influence on the neural tube and vertebral column. In adult humans it remains only in the area of nucleus pulposus of the intervertebral disc[8–9].

In ascidians, the notochord is also present during the whole larval period[10], while the only organism that keeps the notochord as the final skeletal support during the whole life is the amphioxus[11]. The amphioxus (*Branchiostoma lanceolatum* L.) belongs to the *Cephalochordata* subphyllum. The name cephalochordate, meaning »head chordate«, refers to the unique continuation of the notochord over the anterior end of the neural tube[12].

The role and structure of the notochord have been extensively studied in ascidian, amphibian and human embryos[8,13–15]. It was found[16] that the expression of Brachyury genes during embryonic and larval development of amphioxus is significantly similar to those found in other vertebrates. In most species the notochord is formed of vacuolated cells and abundant notochordal sheath[8,17,18]. However, the notochord in amphioxus seems to have a different and very peculiar structure when compared to others species. The morphology of the notochord in amphioxus was first described by Schneider, which dates back to 1902. Under light microscope, the notochord of amphioxus consisted of connective tissue plates (notochordal plates) and Müller's tissue. Later investigations on the notochordal lamellae by the transmission electron microscopy[20–25] gave a completely new view about the nature of the notochord. In ultrathin sections, it appeared that the lamellae contain two kinds of filaments: the thick ones that displayed a periodic cross- striation and the thin ones without any cross-striation, homologous to actin filaments[26]. As solubility characteristics[27] and cross--striation[28] are quite special for paramyosin (or tropomyosin A), it seemed justified to believe that the thick notochordal filaments are made up of a paramyosin-like

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protein, and accordingly, that the lamellae are real muscles. The latter conclusion received experimental support from movie and electrophysiological recordings[25,26,29].

Recent genetic investigation revealed simultaneous expression of vertebrate skeletal and smooth muscle-type genes in the amphioxus notochord, as well as genes for extracellular matrix proteins associated with the notochordal sheath[30]. Moreover, it was shown that amphioxus notochord has unique intermediate type of actin, something between muscle-type and cytoplasmic type of actin[30,31]. Therefore, unlike in other species where the notochord has a structure of connective tissue, the amphioxus notochord seems to be a contractile skeletal organ. For a long time the notochordal sheath was considered as an elastic envelope[32]. Ultrastructurally, notochordal sheath of amphioxus consists of mostly spiral collagen fibres[26]. Some of the collagen fibers of amphioxus notochordal sheath are circular and longitudinal in orientation[20,33,34]. Although Müller's cells mostly occupied dorsal and ventral edges of the notochord, some of them were found on the lateral attachments of the notochordal lamellae to the sheath[26]. Anyway, histochemical investigations on amphioxus notochord and its sheath were very rare and incomplete[35].

In order to gain insight into the nature, composition and unique structure of the amphioxus notochord and its sheath, we compared their ultrastructure with the neighboring skeletal muscles of the myotomes, and described in more detail the very complex three-layered structure of the notochordal sheath. Using a combination of photochemical methods in tissue sections we confirmed clear difference in staining properties between the connective tissue notochord sheath and the specific muscular structure of the notochord plates.

Materials and Methods

Investigation was done on ten adult specimens of amphioxus *Branchiostoma lanceolatum* L. which were caught in the Adriatic sea at the average depth of 5 m. The length of all specimens was measured and the mean average of the length was 27.47 mm. The animals were kept in seawater until fixation.

Light microscopy

The specimens were fixed in 4% formaldehyde and then cut into 3–5 pieces, depending upon the length of the specimen. The samples were then dehydrated and embedded in paraffin or 2% celloidin[36].

Celloidine sections

Embedding in celloidin enables good preservation of structure in tissues and organs. Using serial celloidin sections, a three dimensional reconstruction of the organ systems and their relationship was done. Celloidin sections were cut at 10 μm and then stained with haematoxylin and eosin.

Paraffin sections

Paraffin sections were cut at 6μm and stained using the following histochemical methods in order to distinguish muscular from connective tissue, or to selectively stain different connective tissue fibres.

Verhoeff-Van Gieson technique

The first step in the procedure is an overstaining of the sections with a soluble lake haematoxylin-ferric chloride-iodine. Sodium-thiosulfate is used to remove the excess iodine from the solution. The Van-Gieson solution of acid fuchsin and picric acid is used to counterstain. The technique is useful for staining collagen and elastic fibers. Collagen fibers (if present in the tissue) should be stained red, while elastic fibers should be stained dark blue to black.

Mallory's method

Sections were first stained with haematoxylin, then left in acid fuchsin orange G solution, and transferred into 1% phosphotungstic acid. After rinsing in distilled water, sections were directly transferred into aniline blue (or light green) solution and then rinsed with acetic acid. With this method connective and muscular tissue could be clearly distinguished: tissue components containing muscle fibers should be stained red, while components containing connective fibers should be stained blue (if anilin-blue is used) or green (if light- green is used).

Gomori's methenamine silver method

The sections were stained with silver-methenamine in a boiling bath. They were rinsed in 1% sodium-acetate, 0.5% sodium thiosulfate and also in phosphate buffer solution. This method was used to stain black reticulin fibers in connective tissue[36].

Electron microscopy

The small pieces of tissue (2 x 4 mm) were dissected from the head region, middle part of the body, and the tail region of the animal. The tissue was fixed in 3.5% paraformaldehyde in 0.1 M phosphate buffer solution (pH 7.3) during 24 hours on 4 °C; and then in 3% glutaraldehyde in 0.1 M phosphate buffer solution (pH 7.2) during 2 hours. The postfixation was done in 2% osmium tetroxide in the same buffer solution[36]. The tissue was embedded in Epoxy resin and cut transversally. Semithin sections (0.5–1 μm thick) were stained with toluidine blue and then examined under light microscope. The ultrathin sections (0.05 μm) were made from the chosen area of interest. They were stained with uranyl acetate and lead citrate. The electron microscope Zeiss EM 10A was used for examination of ultrathin sections.

Results

Light microscopy

We analysed serial celloidin sections stained with haematoxylin and eosin to define the three dimensional

structure of the notochord and its relationship with different organ systems. From the very first cross sections in the head region it was seen that this part of the amphioxus body consisted of one-layered epidermis with underlying connective tissue, muscles and the notochord (Figure 1A). Notochord had an elliptic form and looked like a compact tissue enveloped by a connective tissue sheath. In sections more caudal to this region, the neural tube appeared. In these sections notochord became round shaped with a small indentation on its dorsal side. At place of attachment to the neural tube, the notochordal sheath fused with the tube sheath. At lateral sides of the notochord and neural tube, muscles were organized in blocks called myotomes. A thin connective myosepta separated myotomes from each other an attach to the notochordal sheath (Figure 1A).

Histochemical analysis

Verhoeff-Van Gieson technique

This technique was used in order to distinguish collagen from elastic fibers within the connective tissue sheath. Longitudinal section through the notochord shows numerous parallel organized notochordal plates, attached to the sheath. The notochordal sheath is stained red, thus confirming the presence of collagen fibers and the connective tissue nature of the sheath. As there is no trace of dark blue to black staining, it seems that elastic fibers are missing in the notochordal sheath. Notochordal plates are nonspecifically stained light brown. Dorsal and ventral attachments of the plates to the notochord are stained dark brown to black. Such staining indicates that the attaching ends and the middle parts of the plates probably differ in structure, this is also confirmed by electron microscopy (Figure 1B).

Mallory's method

We used this method to differentiate muscular from connective tissue. Longitudinal section of the notochord and its sheath shows that the notochord itself is not specifically stained either for muscles, or for connective tissue. Each notochordal plate (notochordal cell) consists of stripes of different color: from blue to pink and orange. This means that although mostly stained red (pink and orange), which is characteristic of muscle tissue staining,

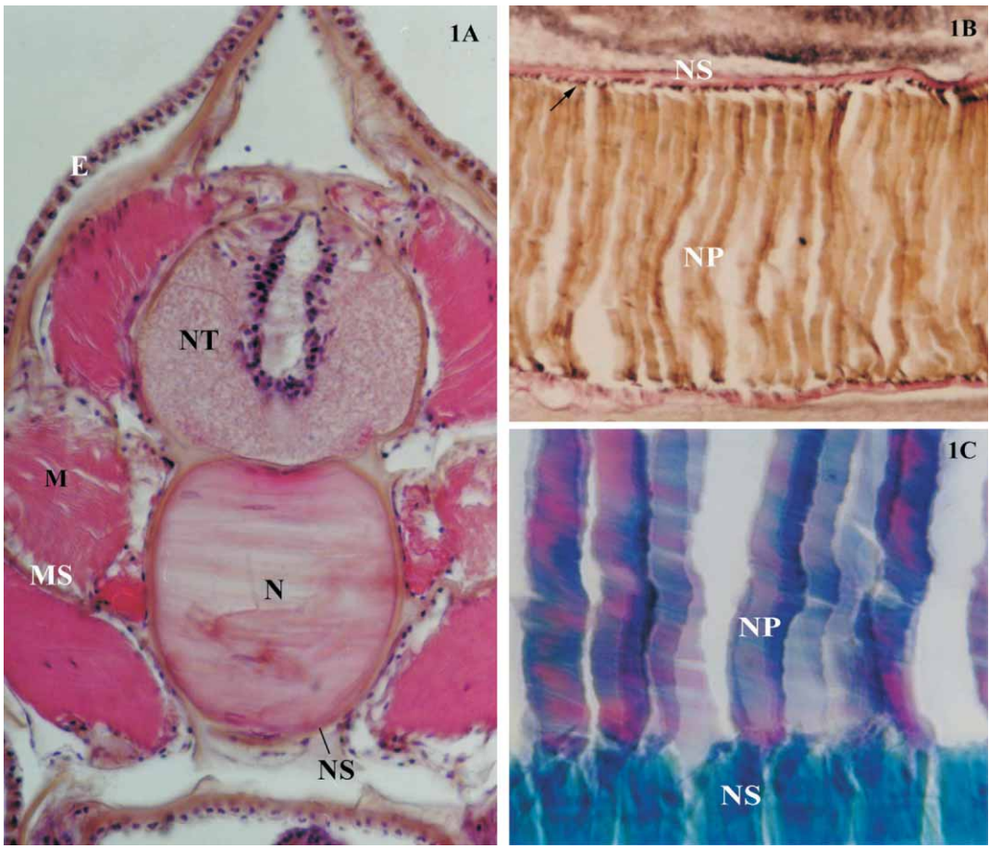

Fig. 1. A) Cross section through amphioxus adult. N – notochord, NT – neural tube, NS – notochordal sheath, M – myotomes, MS – myosepta, E – epiderma. B) Amphioxus notochord in longitudinal section. NP – notochordal plates, NS – notochordal sheath, ↑ – attachment of the plates to the notochordal sheath. C) Detail of amphioxus notochord in longitudinal section. NP – notochordal plates, NS – notochordal sheath. Notice the stripy appearance of the notochordal plates.

some parts of the notochordal plates (blue stripes) might be of different (connective tissue) nature. The notochordal sheath is stained green, meaning it is made of connective tissue. According to the results obtained by this method, the amphioxus notochord shows unique features, not found in any typical tissue, while notochordal sheath is pure connective tissue made probably of collagen fibers (Figure 1C).

Gomori's methenamine silver method

This method is used to stain black reticulin fibers in connective tissue. All connective tissue structures in the section of amphioxus are stained black: skin, myosepta and notochordal sheath, while the notochord stains red, as it does not contain reticulin fibers (not shown).

Electron microscopy

The ultrastructure of the notochord

In electron microscope the notochord consists of parallel notochordal plates, covered by a cell membrane. On its surface, each plate contains numerous »bubbles« situated just beneath the cell membrane. These »bubbles« may be profiles of sarcoplasmic reticulum. Neighboring plates are separated one from each other by free spaces. In each plate, numerous parallel fibrils are positioned vertically to the long axis of the plate (parallel to the body axis) (Figure 2A). In higher resolution, notochordal plates consist of parallel thicker (cross-striated) and thinner fibrils (Figure 2B). The notochord and the muscular tissue of myotomes in amphioxus have very similar appearance in lower resolution. Muscle fibers are also arranged in parallel and surrounded by cell membrane containing numerous »bubbles«. The »bubbles« protrude into free spaces between fibers, and they seem to be also the sarcoplasmic reticulum (Figure 2C). However, in higher resolution, the myotomal muscles clearly display the typical structure of skeletal muscles in vertebrates, showing cross-striation with formation of anisotropic and isotropic bands. The »bubbles«, resembling sarcoplasmic reticulum, are situated just beneath the membrane of each muscle fiber (Figure 2D).

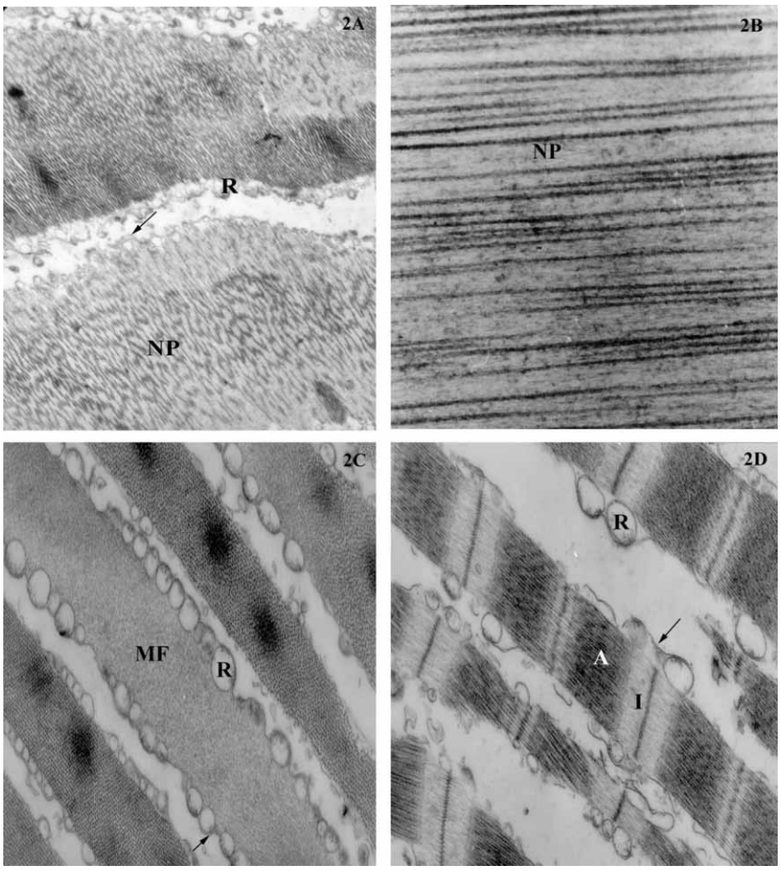

Fig. 2. A) Longitudinal section through the amphioxus notochord. NP – parallel notochordal plates separated by free spaces, R – profiles of the smooth endoplasmic reticulum situated beneath the cell membrane. B) Detail of Fig. 2A. NP – notochordal plates containing parallel thick and thin fibres. C) Longitudinal section through the myotomal muscles. MF – parallel muscle fibres, R – numerous profiles of smooth endoplasmic reticulum, ↑ – cell membrane. D) Detail of Fig. 2C. A – anisotropic and I – isotropic bands of myotomal muscles, ↑ – cell membrane, R – smooth endoplasmic reticulum

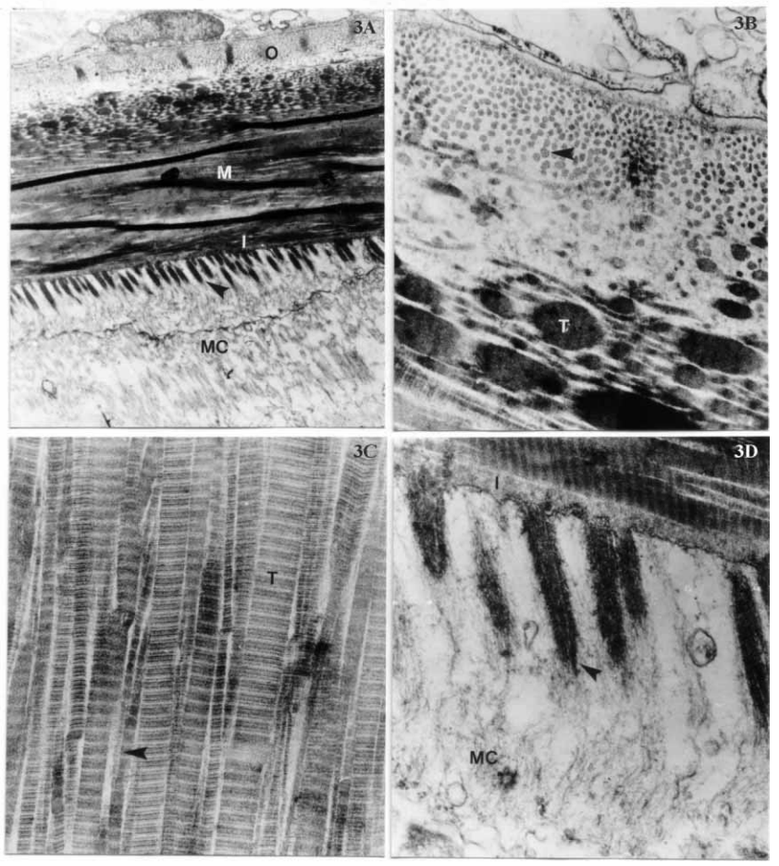

Fig. 3. A) The notochordal sheath shows a three-layered organization. OL – outer layer, ML – middle layer, IL – inner layer, MC – Müller's cells, (arrowhead) – hemidesmosome-like junctions. B) Detail of the outer layer of the notochordal sheath. (arrowhead) – thin collagen fibres, T – thick collagen fibres. C) Detail of the middle layer of the notochordal sheath. T – thick and striated collagen fibres, (arrowhead –) thin and branched reticular fibres. D) Detail of the inner layer (IL) of the notochordal sheath. MC – Müller's cells, (arrowhead) – hemidesmosome-like junctions.

Ultrastructure of the notochordal sheath

The notochordal sheath surrounds the whole notochord without interruptions. Ultrastructurally, the notochordal sheath shows three-layered organization, thus forming outer, middle and inner layer. Müller's cells are bound to the sheath by hemidesmosome junctions (Figure 3A). In cross-section, the outer layer of the sheath consists of the thick and thin collagen fibers (Figure 3B). In the middle layer, collagen fibers are first oblique and then longitudinal, which indicates their spiral course. The collagen fibers are not of the same thickness, but they are all cross-striated. Some fibers are thick and straight, they are similar to collagen type I in vertebrates. The others are thin and branched, and they probably correspond to collagen type III, e.g. reticular fibers (Figure 3C). The inner layer is the thinnest layer of the sheath. It is amorphous and granular in structure (like basal lamina) and it is closely attached to the notochord. Just beneath the inner layer of the sheath lays the peripheral part of the notochord, made of Müller's tissue. The cytoplasm of Müller's cells is bound to the inner layer of the sheath by hemidesmosome junctions (Figure 3D).

Discussion

The peculiar morphology, biochemical composition and recently also the expression of numerous genes in the amphioxus notochord have been investigated for more than hundred years. Amphioxus belongs to an invertebrate group, evolutionarily closest to the vertebrates[37,38]. It is believed that the mechanisms of notochordal induction and development are conserved in all chordates[15], and are characterized by subsequent formation of a large vacuole within the notochordal cell[30]. In amphibians, notochordal cells are gradually filled with vacuoles, while huge amount of extracellular matrix surrounds the notochord thus forming the notochordal sheath[17]. The vacuolization of notochordal cells is present also in other vertebrates[11] and in human development[8]. During subse-

quent developmental stages, the notochordal cells gradually transform from predominantly epithelial type to the more mesenchymal (connective) type of cells. Immunohistochemically, expression of both, the cytokeratins and vimentins temporarily characterizes human notochordal cells[39].

Unlike in all other species, the amphioxus notochordal cells are a type of muscle cells, e.g. they are unique due to occurrence of myofilaments in their cytoplasm. Muscle-related genes including both, genes for skeletal and smooth muscle type genes were simultaneously expressed in the notochord[30]. Morphologically, notochordal cells also showed mixed features of skeletal and smooth muscles. In the light microscope, centrally located nuclei of notochordal cells in our samples resembled smooth muscle nuclei. In electron microscope, each notochordal cell contained thick and thin filaments, and under the notochordal cell membranes we noticed numerous profiles of smooth endoplasmic reticulum. Contrary to those findings, in smooth muscles of vertebrates organelles usually lie around the centrally located nucleus[40]. The notochordal cells of amphioxus contained parallel filaments oriented vertically to the long axis of the cell, thus causing stripy appearance in our tissue sections. However, those stripes did not correspond to the cross-striation seen in the typical skeletal muscles of surrounding myotomes.

Histochemically, the notochord of amphioxus showed unspecific staining characteristics: it is mostly stained like muscles, but at places it acquired characteristics specific for connective tissue. Previous histochemical studies only described distribution of connective tissue in the entire amphioxus body, indicating also the importance of mucopolysaccharides in these tissues[35]. The predominant mucopolysaccharide in the amphioxus skin and notochord were shown to be hyaluronic acid and dermatan sulphate[41].

In our samples, the notochordal cells were attached to the notochordal sheath by hemidesmosome junctions who displayed staining properties different from both the notochordal cells (plates) and the notochordal sheath. In higher vertebrates, the representative cells that have mixed properties of two types of tissues (epithelial and muscular) are the myoepithelial cells, which contain both myofilaments (actin, tropomyosin and myosin) and intermediate filaments (cytokeratins). Similar to notochordal cells they are also bound to neighbouring secretory cells by desmosomes[40]. Although of epithelial origin, the myoepithelial cells can contract and therefore represent a morphological and functional hybrid between epithelial and muscle cells types. Further immunohistochemical investigations using intermediate filament antibodies on amphioxus notochordal cells should clear up whether these cells besides muscular features share characteristics with some other type of tissue (e.g. epithelial).

The notochordal sheath was considered to be a product of the chordal plates (notochordal cells) and Müller's tissue[19]. It was also described as an elastic envelope[32]. While Young (1981) gave only the general plan of amphioxus with no description of notochord and its sheath, Flood (1975) described the ultrastructure of the notochord in amphioxus as connective tissue embedded in granular matrix, but no stratification of the sheath was observed. The only data on the stratified structure of the notochordal sheath are those described in amphibians: it is a multilayered structure containing the poorly organized matrix and six layers of collagen fibres[17]. In the chicken embryo, the notochord is surrounded by collagen identified as type X1(II) and by sulphated glycosaminoglycans[17]. According to our results, the notochordal sheath in amphioxus is a complex three-layered structure, containing in, outer, and middle parts collagen fibres, which due to their morphological appearance, probably correspond to collagen type I and III in the vertebrates. The innermost layer in the amphioxus notochordal sheath is amorphous and resembles the basal lamina[26]. Ultrastructurally, we did not detect any elastic fibers neither in the notochord itself nor in its sheath. Histochemically, the notochordal sheath in our samples also showed staining features specific for collagen in connective tissue, but without any trace of elastic fibers. Recent genetic investigations showed presence of genes encoding extracellular matrix proteins in the notochordal sheath[30]. These results suggest the presence of collagen I, II and III in the sheath, as indicated by our morphological study. Myosepta attached to the surface of the notochordal sheath, seem to play a role in the transmission of muscular (myotomal) forces to axial (notochordal) structures during swimming[43].

In conclusion, the amphioxus notochord is structurally and histocemically a muscle tissue, with mixed properties of both skeletal and smooth-muscle type. The amphioxus notochord seems to differ by its embryological role and final destiny from the notochord of all other species. It develops as an outfold of the archenteron roof, being at the beginning an epithelial rod of cells. During further development it acquires characteristics of muscle tissue, while in vertebrates it transforms into a mesodermal skeletal tissue structure. Whether notochordal cells retain some epithelial/mesenchymal characteristic in amphioxus adulthood remains to be elucidated. The amphioxus notochordal sheath is a three-layered connective tissue envelope that probably contains collagen, but not elastic fibers. The exact location and type of the collagen fibres within this complex structure and in the tissue sections is still obscure and requires further analysis.

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I. Bočina

Department of Biology, Faculty of Natural Sciences, University of Split, Teslina 12, 21000 Split, Croatia
E-mail: bocina@pmfst.hr

OVOJNICA SVITKA U KOPLJAČE – ULTRASTRUKTURNO I HISTOKEMIJSKO ISTRAŽIVANJE

SAŽETAK

Svitak i njegova ovojnica proučavani su ultrastrukturno i histokemijski u 10 odraslih kopljača. Svitak kopljače sastoji se od paralelnih stanica (ploča) svitka, a svaka ploča sastoji se od paralelnih debljih i tanjih vlakana i brojnih oblika glatke endoplazmatske mrežice smještenih ispod stanične membrane. Histokemijsko bojanje pokazuje da ploče svitka ne nalikuju niti vezivnom tkivu ovojnice svitka, ni tipičnoj mišićnoj strukturi miotoma. Ovojnica svitka ima složenu troslojnu građu; sastoji se od vanjskog, srednjeg i unutarnjeg sloja. Vanjski i srednji sloj sastoji se od kolagenih vlakana različite debljine i smjera pružanja, što odgovara kolagenu tipa I i kolagenu tipa III u kralježnjaka, dok je unutarnji sloj amorfan, nalikuje bazalnoj lamini, i usko je vezan za svitak pomoću hemidezmosoma. Rezultati potvrđuju nazočnost kolagenih vlakana i odsutnost elastičnih vlakana u kopljače.