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Digestive gland ultrastructure of the tunicate, *Halocynthia roretzi* (Ascidiacea: Pyuridae) in relation to function

Yun Kyung Shin[1], Jung Jun Park[1], Jeong-In Myeong[1], Mi Ae Jeon[2], Jung Sick Lee[2*]

[1]*Aquaculture Management Division, Aquaculture Research Institute, NFRDI, Busan 619-902, Republic of Korea*

[2]*Department of Aqualife Medicine, Chonnam National University, Yeosu 550-749, Republic of Korea*

PEER REVIEW

Peer reviewer

Roberto Najle, Doctor in Animal Science, University Professor, Researcher.
Tel: 054-249-4439850
Fax: 054-249-4439850
E-mail: robertonajle@gmail.com

Comments

This is a research paper where the authors characterize the cellular level (light microscopy) and ultracellular (transmission electron microscopy) epithelial cells of the digestive gland of the tunicate, *H. roretzi* and compared with results obtained in other species of ascidians, finding differences in the types of epithelial cells. Also describes some of the features found in the gland such as the storage of glycogen and lipid digestion and detoxification.
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ABSTRACT

Objective: To examine the glycogen storage and lipid digestion, as well as detoxification of the digestive gland of the tunicate, *Halocynthia roretzi*.
Methods: Tunicates used in this study were collected from tunicate aquafarm located in Hansan Bay on the southern coast of Korea. Light and electron microscopy was performed.
Results: The digestive gland was divided into the tubular and blind ampulla portions. Fine vacuolar granules were present, and lipofuscin granules were confirmed in the cytoplasm of the epithelia of the blind ampulla portion. Epithelial cells of the blind ampulla portion could be classified into three types.
Conclusions: The results indicate that these three types of cells are involved in glycogen storage, lipid digestion and detoxification.

KEYWORDS
Detoxification, Digestive gland epithelia, Glycogen, *Halocynthia roretzi*, Lipid

1. Introduction

Class Ascidiacea is a group of primitive chordates belonging to subphylum Urochordata, with approximately 2000 species distributed worldwide. They mostly have marine habitats, and are filter feeders that live in a sessile state either alone or as part of a colony during most of their adult stage[1]. A total of 16 species of 5 genera (*Boltenia*, *Halocynthia*, *Herdmania*, *Microcomus*, *Pyura*) of family Pyuridae of class Ascidiacea have been reported in Korea[2].

Although there are not many ultrastructural researches on the digestive system of tunicate, most studies are on the pyloric gland. In all tunicates, there is a network of digestive glands on the outer walls of the intestine. The digestive gland of tunicate is the organ that performs osmoregulation, digestion and excretory function[3]. Researches on the digestive functions of the gland of tunicate have concentrated mostly on the glycogen metabolism and storage[3-6].

However, the digestive gland of the tunicate has been determined to perform lipid digestion and detoxification functions similar to those of the liver in vertebrates or the analogous organ of the hepatopancreas of aquatic

*Corresponding author: Dr. Jung Sick Lee, Department of Aqualife Medicine, Chonnam National University, Daehak-ro 50, Yeosu 550-749, Republic of Korea.
Tel: +82-1089103172, +82-616597172
Fax: +82-616597179
E-mail: ljs@jnu.ac.kr
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invertebrates, thereby necessitating research on this aspect[3,7]. This research employed ultrastructural study to examine the glycogen storage and lipid digestion, as well as detoxification of the digestive gland of *Halocynthia roretzi* (*H. roretzi*).

2. Materials and methods

2.1. Specimens

Tunicates used in this study were collected from tunicate aquafarm located in Hansan Bay on the southern coast of Korea (34°46′36.8″ N, 128°28′13.7″ E). Morphological characteristics of the specimens were measured by using vernier calipers and electronic scale. Twenty specimens used in this study were adults with body heights of 10.2 cm (body heights: 9.4–10.8 cm) (Figure 1A). Specimens for histological analysis were produced by extracting the digestive gland following removal of the tunic (Figure 1B).

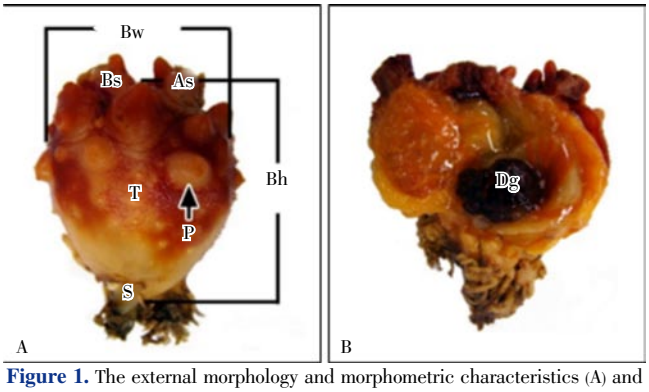

Figure 1. The external morphology and morphometric characteristics (A) and specimen sampling area (B) of the tunicate, *H. roretzi*.
As: Atrial siphon; Bh: Body height; Bs: Buccal siphon; Bw: Body width; P: Process; S: Stolon; T: Tunic; Dg: Digestive gland.

2.2. Light microscopy

Specimen preparation for light microscopy was performed according to the methodology of Drury and Wallington[8]. Specimens were fixed in aqueous Bouin's solution and were rinsed in running water and then dehydrated through a graded ethanol series (70%–100%). The specimens were then embedded in paraplast (McCormick, USA), and subsequently sectioned at 4–6 μm thickness using a microtome (RM2235, Leica, Germany). Specimens were stained with Mayer's hematoxylin and 0.5% eosin (H & E), Masson's trichrome stain, alcian blue and periodic acid–Schiff solution (AB–PAS, pH 2.5), and aldehyde fuchsin–alcian blue (AF–AB, pH 2.5) reaction. Long Ziehl–Neelsen stain was performed to confirm the presence of lipofuscin granules.

2.3. Transmission electron microscopy (TEM)

Specimen preparation for electron microscopy was performed according to the methodology of Cormack[9]. Specimens were fixed in 2.5% glutaraldehyde solution (pH 7.2, 0.1 mol/L phosphate buffer) for 2–4 h at 4 °C and rinsed in 0.1 mol/L phosphate buffer, after which they were post-fixed in 1% osmium tetroxide (OsO_4) solution for 2 h at 4 °C. After fixation, the specimens were washed with 0.1 mol/L phosphate buffer 4 times for 2 h and dehydrated with graded series of ethanol. Specimens were embedded in Epon 812, cut to ultrathin sections (70 nm in thickness) and placed on copper grids (200 mesh) in order to double-stain with uranyl acetate and lead citrate. Specimens were examined using a TEM (LIBRA 120, Zeiss, Germany).

3. Results

3.1. Light microscopical structure

The digestive gland of the tunicate could be classified into tubular and blind ampulla portions. The epithelial layer was a single layer, with the tubular portion composed of cuboidal epithelia, while the epithelial layer of blind ampulla portion was also composed of columnar epithelia (Figure 2A). A striated border was developed on the free surface of the epithelial layer. As the result of AB–PAS (pH 2.5) reaction, while the striated border of the tubular portion displayed a blue color, the striated border of the blind ampulla portion displayed a red color (Figure 2A). The cytoplasm of epithelial cells of the blind ampulla portion contained fine vacuoles that displayed negative reaction upon H & E stain, Masson's trichrome stain, AB–PAS (pH 2.5) reaction and AF–AB (pH 2.5) reaction (Figures 2A–2D). In addition, lipofuscin granules that reacted to crimson color in the Long–Ziehl Neelsen stain were observed in the cytoplasm of some of the cells (Figure 2E).

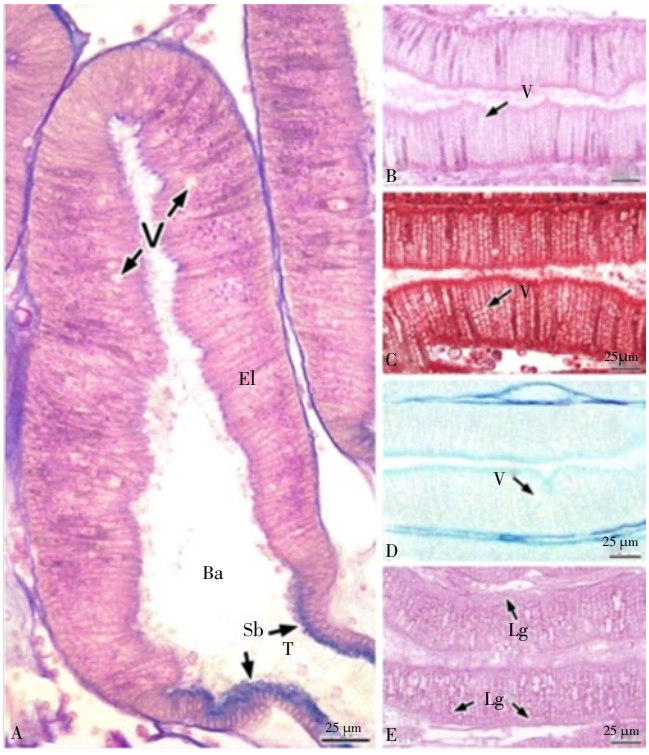

Figure 2. Light microscopical feature of the digestive gland of the tunicate, *H. roretzi*.
A: Digestive gland consists of tubule (T) and blind ampulla (Ba) portion, AB–PAS (pH 2.5) reaction; B–D: Small vacuoles (V) in the cytoplasm of epithelium, B: H & E stain, C: Masson's trichrome stain, D: AF–AB (pH 2.5) reaction; E: Lipofuscin granules (Lg) in the cytoplasm of epithelium, Long Ziehl–Neelsen stain. El: Epithelial layer; Sb: Striated border.

3.2. Transmission electron microscopical structure

As the result of TEM observation, epithelial cells of the tubular portion were cuboidal shape with the development of microvilli on the free surface. The cytoplasm of these cells contained lysosomes and vacuoles of a wide range of sizes filled with fibrous substances (Figure 3A).

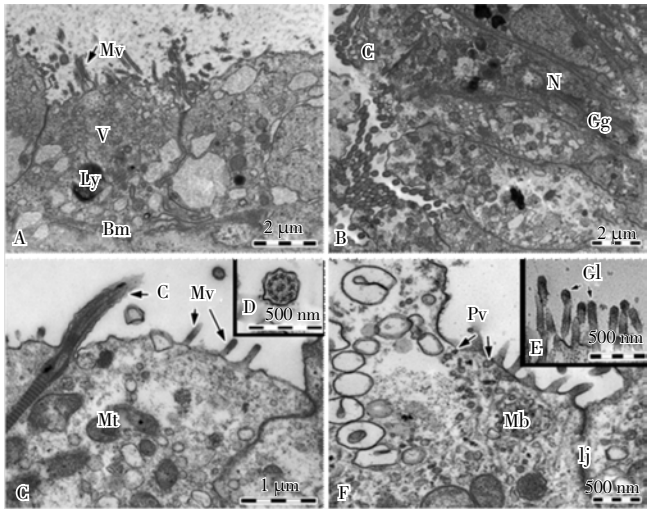

Figure 3. Transmission electron micrographs of the digestive gland epithelia of the tunicate, *H. roretzi*.

A: Epithelia in the tubular portion; B: Ciliated columnar epithelia in the blind ampulla portion; C: Free surface of ciliated columnar epithelium in the blind ampulla portion; D: Cross section of cilia (C) showing "9+2" microtubular structure; E: Microvilli (Mv) covered with glycocalyx (Gl); F: Pinocytotic vesicles (Pv), multivesicular body (Mb) and intercellular junction (ij) in the apical cytoplasm of ciliated columnar epithelium. N: Nucleus; V: Vacuole; Ly: Lysosome; Bm: Basal membrane; Cg: Glycogen granules; Mt: Mitochondria.

The epithelial layer of the blind ampulla portion is composed of columnar epithelia. However, these cells could be divided into one type of ciliated columnar epithelium and two types of non-ciliated columnar epithelium in accordance with the presence of cilia, electron density of cytoplasm, shape of the nucleus and ultrastructural characteristics of the cytoplasm (Figure 3B).

The ciliated columnar epithelium displayed higher electron density of the cytoplasm in comparison to the non-ciliated columnar epithelium, with the development of cilia and microvilli on the free surface (Figure 3C). The cilia displayed a "9+2" microtubular structure in cross section (Figure 3D) and the surface of microvilli was covered with glycocalyx of high electron density (Figure 3E). Numerous pinocytotic vesicles and multi-vesicular bodies were observed in the cytoplasm, in the vicinity of the free surface with scattered glycogen granules (Figure 3F). Multiple numbers of lysosomes and Golgi complex were developed in the mid cytoplasm (Figure 4A), and condensed vesicles with high electron density existed in the boundaries of cisternae of the Golgi complex (Figure 4B). Glycogen granules of high electron density were concentrated in the basal cytoplasm of the ciliated columnar epithelium (Figure 4C). Moreover, well-developed mitochondria, microsomes and glycogen granules were scattered throughout the basal cytoplasm of the other same shaped cells. Membrane interdigitations were well-developed on the lateral cell membrane near the basal membrane of these cells, and the infoldings were formed in portions of the basal membrane (Figure 4D).

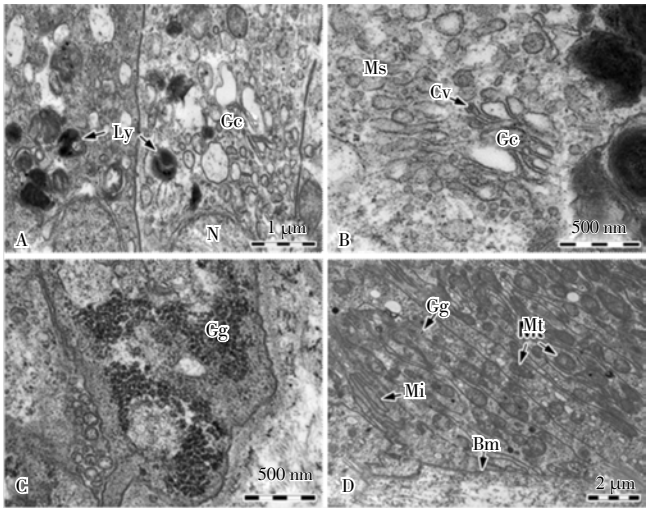

Figure 4. Transmission electron micrographs of mid and basal cytoplasm in the digestive gland epithelium of the tunicate, *H. roretzi*.

A: Golgi complex (Gc) and lysosomes (Ly) in the mid cytoplasm; B: Golgi complex with condensed vesicle (Cv) and microsomes (Ms) in the mid cytoplasm; C: Accumulated glycogen granules (Cg) in the basal cytoplasm; D: Mitochondria (Mt), membrane interdigitations (Mi) and basal membrane (Bm) infoldings. N: Nucleus.

The non-ciliated columnar epithelium can be divided into two types according to the ultrastructural characteristics of the nucleus and the cytoplasm. The first type of the non-ciliated columnar epithelium has development of microvilli on the free surface, and these microvilli were also covered with glycocalyx of high electron density. The nucleus was located in the basal area and had condensed heterochromatin in a circular shape in the vicinity of the karyomembrane (Figure 5A). The cytoplasm exhibited the development of oil droplets with a diameter of approximately 1 μm of low electron density, lysosomes, smooth endoplasmic reticula and mitochondria (Figures 5B and 5C).

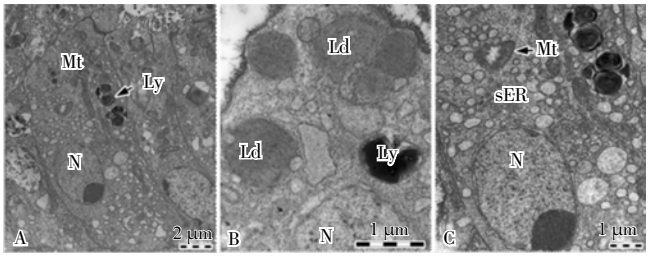

Figure 5. The first type of the non-ciliated columnar epithelium in the digestive gland of the tunicate, *H. roretzi*.

A: Non-ciliated columnar epithelium with heterochromatin condensed nucleus (N) in the blind ampulla portion; B: Section showing the several lipid droplets (Ld) and lysosome (Ly); C: Section showing the heterochromatin condensed nucleus and well developed smooth endoplasmic reticula (sER) and mitochondria (Mt) in the cytoplasm.

The second type of the non-ciliated columnar epithelium also displayed microvilli development on the free surface like the first type. Although the nucleus is located in the basal area, unlike the non-ciliated columnar epithelium of the first type, heterochromatin was scattered within the nucleus (Figure 6A). Pinocytotic vesicles and multivesicular body were observed in the cytoplasm in the vicinity of free surface (Figure 6B). Lysosomes with fibrous substance and multiple numbers of microsomes were observed in the mid cytoplasm (Figures 6C and 6D). Moreover, multiple numbers of Golgi complex, smooth endoplasmic reticula and lysosomes were distributed in the basal cytoplasm. These lysosomes contained characteristically homogeneous core granules of high electron density (Figure 6E).

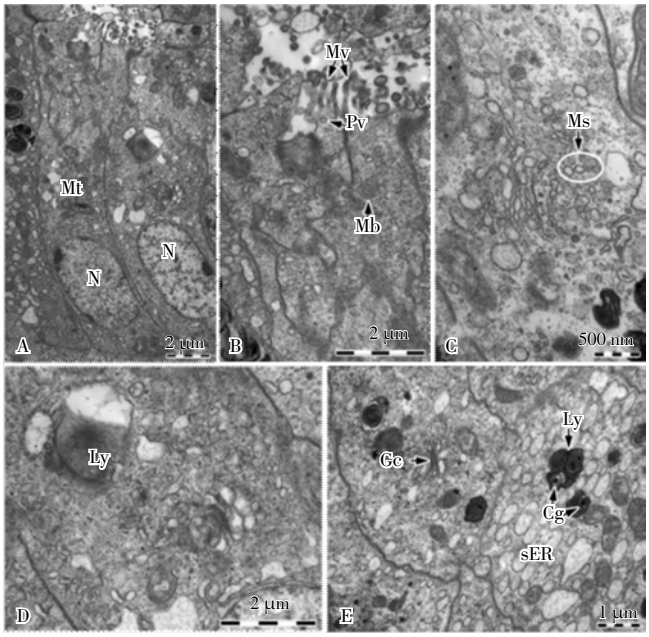

Figure 6. The second type of the non-ciliated columnar epithelium in the digestive gland of the tunicate, *H. roretzi*.
A: Two non-ciliated columnar epithelia with heterochromatin scattered nucleus; B: Microvilli (Mv), pinocytotic vesicles (Pv) and multivesicular body (Mb) in the apical cytoplasm; C: Numerous microsomes (Ms) in the cytoplasm; D: Lysosome (Ly) of low electron density with fibrous substance; E: Golgi complex (Gc), smooth endoplasmic reticula (sER) and several lysosomes with core granules (Cg) of high electron density. Mt: Mitochondria; N: Nucleus.

4. Discussion

The digestive glands of ascidian are structurally composed of tubules and blind ampullae[3]. The microscopic structure of the digestive glands of ascidian has been described in the *Botryllus schlosseri* (*B. schlosseri*). The epithelium of these glands is composed of a single cell type. The epithelium is cuboidal and has a few long cilia and numerous microvilli on the free surface[10]. The digestive glands of *Dendrodoa grossularia* (*D. grossularia*) are also composed of tubules and blind ampullae, and, although the shapes of epithelia are all columnar, the epithelia of the ampulla portion are longer[5,11]. As a result of this study, the digestive gland of *H. roretzi* could also be divided into the tubular and the blind ampulla portion. However, while the shape of the epithelium in the tubular portion was cuboidal, three types of columnar epithelia were confirmed in the blind ampulla portion, thereby displaying the difference between *B. schlosseri*[10] and *D. grossularia*[5].

Glycogen deposition, which is one of the digestion functions of the ascidian digestive gland, has been well-researched in observation of the electronic microscopic structure of *B. schlosseri*[10], *Styela clava*[4] and *D. grossularia*[5]. The common ultrastructural characteristics of these gland epithelia included the development of cilia and microvilli on the free surface, and the development of pinocytotic vesicles, lysosomes, endoplasmic reticula, mitochondria and glycogen granules in the cytoplasm. In this study, the ultrastructural characteristics that were confirmed in the *B. schlosseri*, *Styela clava* and *D. grossularia* were confirmed in the ciliated columnar epithelium of the digestive glands of *H. roretzi*. Accordingly, these cells were determined to participate in glycogen deposition. In addition, the development of mitochondria and infoldings of the basal membrane in the ciliated columnar epithelium of *H. roretzi* are determined to be structural characteristics that relate to the active transport of glycogen.

Mugnaini and Harboe reported that the digestive glands of ascidian have functional similarity to the liver of vertebrates[7]. Burighel and Cloney asserted that there is a need to pursue further research on this aspect[3].

Liver cells of vertebrates synthesize triglycerides, cholesterol and phospholipids, and store them in the form of large lipid droplets in the cytoplasm. Such ultrastructural characteristics of lipid synthesis are also displayed as numerous smooth endoplasmic reticula, mitochondria and large lipid droplets of low electronic density[12]. Lipid storage and metabolic functions were also reported in the digestive gland of marine mollusks. The digestive gland epithelium (digestive cell) of *Viviparus ater* performs hexosaminidase activity, and displays an ultrastructure which participates in lipid storage and metabolism, with the development of apical microvilli, pinocytotic vesicles, rough endoplasmic reticula, Golgi complex, lysosomes, mitochondria and oil droplets[13]. In addition, lipid digestion was confirmed in the cytochemistry and ultrastructure of the digestive gland epithelium of *Aplysia punctata*[14]. In the digestive glands of *Mytilus galloprovincialis*, digestive cells (digestive gland epithelium) which perform lipid digestive functions are distinguished. The digestive cells contain granules that display positive reaction in N-acetyl-β-hexosaminidase. Moreover, the digestive cell was confirmed to participate

in lipid metabolism through observation of Golgi complex, lysosomes, residual body and lipid inclusions under the electron microscope[15]. In this study, the presence of cells among the epithelial cells containing vacuoles that display negative reaction in H & E stain, Masson's trichrome stain, AB-PAS (pH 2.5) reaction and AF-AB (pH 2.5) reaction were confirmed upon light microscopic examination of the epithelium of digestive gland of *H. roretzi*. In addition, as the results of TEM observation, development of microvilli on the free surface, and development of smooth endoplasmic reticula, mitochondria and oil droplets in the cytoplasm of non-ciliated columnar epithelia were observed. Such ultrastructural characteristics are similar to those of the liver cells of vertebrates and digestive gland cells of marine mollusks which display lipid digestion.

Detoxification is another important function of liver cells in vertebrates. The detoxification function in various types of cells, including liver cells, is explained by means of the activation of smooth endoplasmic reticula, mitochondria and lysosome, and ultrastructural characteristics that relate to the accumulation of lipofuscin granules[16-18]. The ultrastructural characteristics of lysosomes that relate to the detoxification functions in the digestive glands of marine mollusks are very similar to those of vertebrates. Lysosomes also convert foreign substances infused into the organism from outside, including marine pollutants, into heterophagosomes, by dissolving them with hydrolytic enzymes. Macromolecular lysosomes that remain in non-disintegrated state with incomplete dissolution at this time are referred to as the residual body or lipofuscin. Pipe and Moore[19] explained the effect of phenanthrene in the digestive cells of periwinkle, *Littorina littorea* through the ultrastructural changes of lysosomes and distribution of β-glucuronidase, which is a lysosomal enzyme. The effect of stress arising from heavy metals or organic compounds was also explained through the lysosomal and lipid alterations and lipofuscin in green mussel, *Perna viridis*[20], *Mytilus galloprovincialis*[21], *Scrobicularia plana* and *Tapes semidecussatus*[1] and *Gomphina veneriformis*[22]. In this study, cells among the epithelial cells which contain lipofuscin granules (stained crimson as the result of Long Ziehl-Neelsen stain) were confirmed by light microscopic examination of the digestive gland of *H. roretzi*. In addition, as the result of TEM observation, lysosomes with homogeneous core granules of high electron density and smooth endoplasmic reticula were characteristically developed in the cytoplasm of the non-ciliated columnar epithelium. Such ultrastructural characteristics were similar to the characteristics of liver cells of vertebrates and the digestive gland epithelium of marine mollusks which display detoxification function.

Three types of cells of the digestive gland of *H. roretzi* are presumed to be involved in the glycogen storage, lipid digestion and detoxification. The main functions of the three types of cells in the digestive glands will differ according to cell type, although all the types of cells perform all of the three aforementioned functions rather than having functions which are different from one other.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

Research on the digestive system of tunicates is mostly directed to the pyloric gland and those related to the digestive gland of tunicates have focused primarily on metabolism and glycogen storage. The hepatopancreas or digestive gland in aquatic invertebrates meets some similar functions to the liver in vertebrates such as lipid digestion and play an important role in detoxification of chemicals and drugs. This paper examines the storage of glycogen and lipid digestion and detoxification of the digestive gland of the tunicate, *H. roretzi*.

Research frontiers

This paper distinguishes morphologically and structurally different portions of the digestive gland (tubular and blind the ampulla) and by light microscopy and electron microscopy found that the three types of epithelial cells of blind the ampulla portion are involved in storing glycogen, lipid digestion and detoxification. Good prepared for light and electron microscopy.

Related reports

Although ascidian glands are structurally composed of tubules and blind blisters. Other authors have reported that epithelial cells in *Botryllus schlosseri* differ from *Dendrodoa grossular*. This difference in the types of epithelial cells also has been found in this work, since three types of columnar epithelia were confirmed in blind the ampulla portion.

Innovations and breakthroughs

The ultrastructural features found in the epithelial cells of the digestive gland of *H. roretzi* were similar to the characteristics of liver cells of vertebrates and the epithelium of the digestive gland of marine mollusks showing detoxification function. The three types of cells are involved in the storage of glycogen, lipid digestion and detoxification. All cell types perform the three functions mentioned above instead of having functions that are different from each other.

Applications

This study provides scientific data on the ultrastructure of the digestive gland of *H. roretzi* (Ascidiacea: Pyuridae) in relation to the functions the glycogen storage and lipid digestion, as well as detoxification. Finding a great similarity in these functions to what happens in liver cells of vertebrates and the epithelium of the digestive gland of marine molluscs.

Peer review

This is a research paper where the authors characterize the cellular level (light microscopy) and ultracellular (transmission electron microscopy) epithelial cells of the digestive gland of the tunicate, *H. roretzi* and compared with results obtained in other species of ascidians, finding differences in the types of epithelial cells. Also describes some of the features found in the gland such as the storage of glycogen and lipid digestion and detoxification.

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