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Special dyeing, histochemistry, immunohistochemistry and ultrastructure: A study of mast cells eosinophilic granules cells (MCs/EGC) from centropomus parallelus intestine

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ABSTRACT

Intestine mast cells/eosinophilic granule cells (MCs/EGC) of the marine species Centropomus parallelus (fat snook) were first studied using light and electron microscopy techniques. Mast cells are cells from the connective tissue found in almost all organs and tissues of vertebrates. In fish, they appear in greater numbers in parts of their bodies that are exposed to their environment, such as skin, gills and intestine. The granules in fat snook's mast cell contain a variety of substances, such as histamine, heparin, chondroitin sulfate, serotonin, proteases and cytokines. The present study of intestine MCs/EGC was carried out in 20 specimens of fat snook. Samples of tissue were fixed in Bouin solution and in buffered formalin. Ferric hematoxylin - Congo red, pH6 acridine orange, pH2.5 and pH0,5 Alcian Blue (AB), toluidine blue, PAS, AB + PAS and immunohistochemistry protocols were used. In the mucosa and submucosa layers, MCs/EGCs granules with basic contents were evidenced by Congo red staining, and with acid contents granules were identified through pH 2.5 and 0,5 AB, and acridine orange. Basic and acid contents were simultaneously evidenced using ferric hematoxylin - Congo red stain. Metachromasia was observed in both mucosal and submucosal mast cells. Neutral glycoproteins were evidenced by using PAS protocol, glycosaminoglycan through AB and both simultaneously through AB + PAS. In immunohistochemistry assays, MCs/EGC were positive for tryptase, chymase and serotonin. As in mammals, the study of samples fixed in modified Karnovsky for transmission electron microscopy evidenced that most of the MCs granules were spherical and showed varying electron density, as described in previous reports on other teleost fish species. The metachromasia observed and the identification of tryptase, chymase and serotonin suggest a great similarity between fat snook's MCs/EGC and those described in the mucosa of mammals.

1. Introduction

Mast cells (MCs) are connective tissue myeloid cells found in almost all tissues and organs of vertebrates. In fish, they are particularly present in the parts of the body exposed to the environment, such as skin, gills and intestine, where they are frequently seen near blood vessels and nerve fibers indicating their key role in recovery from injuries [1–5]. In mammals, mast cells have been so far adequately well described, and generally, also involved in biological processes such as gastrointestinal motility, leukocyte recruitment, increase in vascular permeability and mucus production by goblet cells [6]. Mediators produced by MCs include small molecules (histamine and serotonin), cytokines, proteases, lipids (leukotrienes and prostaglandins), and proteoglycans (heparin, chodroitin sulfate, etc) [4,7–10]. Some of these mediators are in store within granules (histamine, proteases,

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glycosaminoglycans, and TNFα) and thus can be released within seconds or minutes, whereas others must be synthesized (lipid mediators and most cytokines), which results in minutes up to hours between triggering stimulus and secretion, since synthesis may involve simple processes up to complex RNA transcription [10]. In teleosts, some authors – despite some differing descriptions as to the morphology of such cells – have reported structural and functional similarities between fish and mammal mast cells [5,11–14]. Mast cells have been reported to be involved in immunological mechanisms, as they are increased in number in gills, intestine and skin of animals infected with parasites [11,15,16], with such increase frequently occurring near the vessels of various organs [11,15,17,18], where they pormote increased vascular permeability, degranulate upon activation by degranulating agents, releasing and secreting substances involved in inflamatory response [15,19–23]. In addition, mast cell granules contain different chemical compounds such as histamine, piscidin, serotonin, tryptase and chymase [14,22,24–26].

In mammals, despite their similarity in different body tissues, mast cells are classified as different populations [4,27]. For instance, MCs in the mucosa (MMC) and in the connective tissue (CTMC) are referred to as two different specific cells [7,28]. Such different classification originates from the fact that each subtype of mast cells characteristically expresses different types of proteases, mainly tryptase and chymase [7,27]. Different proteases are stored in the cytoplasmic granules and released into the extra-cellular environment when MCs are stimulated by inflammatory mediators which play a major role in the mechanisms involved in fighting pathogens, such as granulocytes recruitment and tissue remodeling [1,29–32]. Tryptase is the main human MCs protease whose use seems to come exclusively from stores in secretion granules where it is kept in its fully active form [1]. Its role is fundamentally linked to epithelial repairing, recruitment of granulocytes and activation of mast cells themselves [29,31]. Chymase in turn is an intestine peptide playing a vasoactive role and participating in tissue remodeling breaking down of extra-cellular matrix proteins including collagen precursors [1,30]. Despite their acknowledged importance in mammal's physiology, the study of these proteases in fish is still incipient.

The cells focused on in this study have been referred to with different names over the years. More recent studies have used the term mast cells/eosinophilic granule cells (MCs/EGCs), since it describes the eosinophilic character of fish mast cells granules and, thus, makes a difference between fish mast cells and those in mammals [2,20].

The metachromasia produced by certain cationic dies is viewed as one of the main similarities between mammal and fish mast cells [12,33]. Furthermore, studies have shown that acute teleost's mast cells responses to harmful agents are similar to those observed in mammals, especially concerning vasomotor responses and vascular permeability induced by the release of granule content [13]. It seems, therefore, that a research approach similar to that used in studies focusing on mammal's mast cell would show the desired efficacy [2]. Even though teleost's MCs/EGCs have some homology to mammal's MCs, a variety of important morphologic and functional features, as well as the role played in fighting fish diseases, are not known in detail [2,13,21,24].

A particular feature of MCs/EGCs is their acid-basic character that may vary among fish species [2,5]. Such a feature has led researches to focus on the specific character of fish MCs granules in each species and determine comparisons between the target fish species and other fish species and between the target fish species and mammals [5,15].

Recently, the number of research works on fish MCs has increased notably [16,20,34]. Yet, the current knowledge of mammal MCs can benefit from research into fish MCs. Similarly the study of other myeloid cells in teleosts can provide relevant information about the diversity of cells and their roles, as well as, may favor the development of fish models as substitutes for mammal models that pose a higher degree of difficulty to carry out the intended study [25].

Acknowledging the importance of acquiring a more detailed knowledge on such an important cell involved in the immune system of vertebrates, the present study aimed at characterizing fat snook's intestine MCs/EGCs using various methods including histochemistry and immunohistochemistry protocols, and ultrastructure study.

2. Material and methods

2.1. Fish and tissue preparation

A total of 20 fat snook specimens (size = 26–29 cm and weight = 144–202 g), were kept in floating net cage in a lagoon of the estuary of Cananéia, São Paulo state – Brazil. In order to obtain study material, the animals were captured and anesthetized with 3% benzocaine (Sigma Chemical Co, St Louis, MO, USA) followed by immediate dissection of the gastro-intestinal tract. Part of the samples harvested from the pyloric caecum (PC), proximal intestine (PI), distal intestine (DI) and rectum (R) were fixed in alcoholic Bouin's solution and part in buffered formalin, dehydrated in ethanol solutions in series of increasing concentration, treated with xylol and embedded in paraffin. All animal experiments were approved by the Research Ethics Committee at UNIFESP/EPM (Report N° 0571/11), and treated according to institutional guidelines.

2.2. Histochemistry

Sections 5μm thin from each intestine area (PC, PI, DI and R) were stained according to different protocols – ferric hematoxylin - Congo red (identify acidic components and basic polyamino acids, respectively), Congo red [35], acridine Orange staining (pH 6,0) at 5% (identify heparina) [36], alcian blue (AB) at 1% (pH 2.5 and 0.5) (identify carboxylated and sulphated glycosaminoglycans, respectively) [37], periodic acid Schiff (PAS) (identify neutral glycoprotein) [38] and toluidine blue (TB) at 0.5% (pH3.5) in acid water (identify heparina) [35]. Sections were examined under light microscope AxionScope A1 (Carl Zeiss), and confocal microscope Leica TCS SP5.

2.3. Immunohistochemistry

Tryptase, chymase and serotonin were detected by using the conjugated streptavidin-peroxidase (LSAB) and followed by diamine-benzidine (DAB) development. Sections were incubated with hydrogen peroxide at 3% for 20 min in order to inactivate endogenous peroxide, rinsed in running distilled water and incubated with lean milk at 3% in phosphate-buffered saline solution (PBS) for 30 min at room temperature. Sections were then incubated overnight at 40C in wet chamber with 100 μL of mice monoclonal IgG anti-human mastocyte tryptase antibody (Clone AA1 – Dako, Corp; USA, dilution1:200), 100 μL of mice monoclonal IgG anti-human mastocyte chymase antibody (Clone CC1 – Dako, Corp; USA, dilution1:200), and 100 μL of mice monoclonal IgG anti-serotonin antibody (Clone 5HT-H209 – Dako, Corp; USA, dilution1:200). Following, the sections were rinsed in PBS and incubated with rabbit biotinylated secondary antibody (DiagnosticBioSystem DBS, USA, dilution 1:400) anti mice IgG for 30 min in room temperature and subsequently rinsed in PBS for 10 min and then treated with 2 drops of conjugated streptavidin-

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peroxidase (LSAB) for 30 min at room temperature. Development was carried out by immersion in diamine-benzidine at 0.06% in PBS solution for 5 min. After rinsing in running water, sections were stained with Carazzi hematoxylin and mounted in resin.

2.4. Transmission electron microscopy

Samples of approximately 5 mm collected from each study area of the intestine (CP, IP, ID and R) were immediately fixed in modified Karnovsky fixative solution – 2.5% glutaraldehyde and 2% paraformaldehyde in buffer solution of cacodylate 0.2 M and pH 7.2. Samples were resized, processed and embedded in araldite according standard protocols for transmission electron microscopy (TEM) study. Ultrathin section were stained with uranyl acetate (0.5%) and 3.0% lead citrate for contrast, and imaging was carried out using a transmission electron microscope Zeiss EM900 at the Department of Morphology and Genetics – UNIFESP.

3. Results

MCs/EGCs were found in the mucosa and submucosa of all study areas in the intestine of the fat snook as shown in Table 1.

3.1. Histochemistry

MCs/EGC were identified in both mucosa and submucosa. Morphologically these cells show, in the majority of cases, oblong shape, oblong or spherical nucleus and cytoplasmcontaining dark red granules after simultaneous staining with ferric hematoxilin and Congo red (Fig. 1). Furthermore, after staining, acidic and basic components were simultaneously identified in plasmic granules. Staining and histochemistry protocols in the target area showed MCs/EGCs with granules staining with Congo red indicating a basic content (Fig. 1b) and staining with Alcian blue pH 2.5 e 0.5 (Fig. 1c) revealing an acid character. The acid character of these latter granules was confirmed by the red fluorescence in acridin orange protocol (Fig. 1d). Neutral glycoproteins were evidenced by PAS staining (data not shown) and metachromasia occurred in pH 3.5 toluidine blue staining with mast cells in the submucosa showing stronger staining as compared with that showed by mucosa mast cells (Fig. 1e). The simultaneous occurrence of glycosaminoglycan and neutral glycoprotein were revealed by applying AB + PAS (Fig. 1f).

3.2. Immunohistochemistry

Sections treated with *anti*-tryptase, *anti*-chymase and anti-serotonin indicated MCs/EGCs in the mucosa and submucosa of the intestinal tract. Staining for *anti*-tryptase showed low intensity in both mucosa and submucosa sections (Fig. 2a). Contrasting with the intense staining produced by using *anti*-chymase in sections from both regions (Fig. 2b). Differently from both antibody staining findings, serotonin antibody staining was more intense in the submucosa sections as compared with that in the mucosa sections (Fig. 2c). In order to assure the specificity of each staining, all sections were incubated without the respective primary antibodies.

Table 1
Special staining, histochemistry and immunohistochemistry observations in fat snook intestine MCs/EGC.

	Mucosa	Submucosa
Ferric hematoxylin - Congo red	+	+
Congo red	+	+
Alcian blue pH 2,5	+	+
Alcian blue pH 0,5	+	+
Toluidine blue	±	+
PAS	+	+
AB + PAS	+	+
Acridine orange	+	+
Tryptase	±	±
Chymase	+	+
Serotonin	±	+

+: Strongly positive; ±: weakly positive.

3.3. Transmission electron microscopy

The ultra-structural examination of MCs/MGCs showed both spherical and irregular nucleus with euchromatin imaging over most of it. A nucleolus in the mast cell was observed only rarely. The cytoplasmic granules were spherical in form and showed varied electron density (Fig. 2d). Mast cells frequently surrounded blood vessels and were closely associated with collagen fibers and cells of the connective tissue (images not shown).

4. Discussion

Mast cells are cells of the immune system that can be found in the connective tissue. They mediate the inflammatory process and the secretion by the goblet cells in the epithelium. Modulating the immune response, they are the first cells to respond to exogenous stimuli exerted by bacteria, viruses, and pathogenic agents such as toxins [25,39,40]. In fish, these cells are referred to as mast cells with eosinophilic granules (MCs/EGCs) and seem to have morphology and functions similar to those of mammal mast cells [2,14,20,41]. Such similarity have prompted researchers to determine the content of cytoplasmic granules in order to have a deeper understanding of the role of such cells [15,17,42]. In this regard, research on teleost MCs/EGCs can contribute to a broader picture of the roles and mechanisms associated with mast cells in general [2,13].

4.1. Histochemistry

While MCs/EGCs have been reported as a finding in the intestine of several species of teleosts, the present study is the first to report on morphological, histochemical and immunohistochemical characteristics of MCs/EGCs in fat snook specimens. Similarly to what was observed in other teleost species, the fat snook's MCs/EGCs are more abundant in the submucosa than in the mucosa [22,43]. As in mammals, fish mast cell is considered to comprise a heterogeneous population mainly because of their diverse staining characteristics [2,5]. The cytoplasmic components in mast cells can be of acid or basic character [2,5] and stains differently in differents by previous authors in reference to the occurrence of eosinophilic content in the granules of mastocyte studied. When ascertaining metachromasia, the current authors used alcohol-based fixatives as they better preserve the metachromatic components of MCs/EGCs as compared with aqueous fixatives [2].

All staining and histochemistry protocols used in this study succeeded in evidencing MCs/EGCs in both mucosa and submucosa.

The basic character of cytoplasmic granules shown using Congo red staining protocol indicated the presence of proteic substance, a finding also observed in other species, such as *Oncorhynchus kisut* [44] and *Esox lucius* [12], as well as in salmonids [33]. Differences in the type of protein can occur depending on the location of MC [44]. In turn, the acid nature of cytoplasmic granules was shown by pH 2.5 and 0.5 A B due to the presence of carboxylated and sulphated glycosaminoglycans, respectively, as reported by previous authors [5,12,33,43–45]. Both basic and acid natures of MCs granules were evidenced simultaneously in the same MC by using ferric

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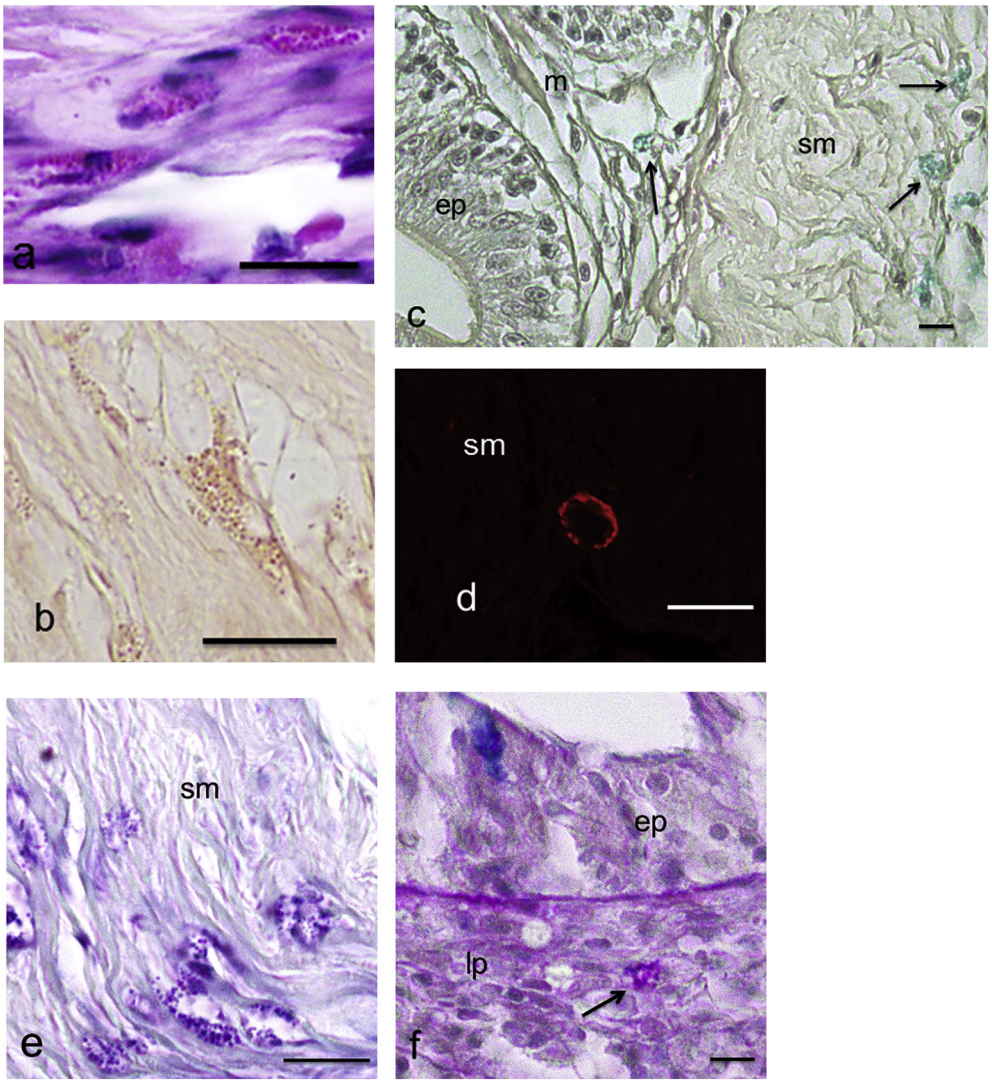

Fig. 1. Photomicrograph of Intestinal submucosa showing oblong-shaped MCs/EGC with oblong nucleus and cytoplasm containing spherical granules stained dark red by ferric hematoxilin-Congo red complex (a), red by Congo red (b), blue by 2.5 pH Alcian blue (arrow), mucosa (m), submucosa (sm) and epithelium (ep) (c) and in flame-red fluorescence by Orange acridine, submucosa (sm) (d). Metachromasia in mast cell granules when processed with toluidin blue, submucosa (sm) (e); simultaneous presence of glycoaminoglycans and neutral glycoprotein (arrow), lamina propria (lp), epithelium (ep), (f). Bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hematoxylin and Congo red. The acid nature of granule content has also been determined in fluorescence assay utilizing a cationic fluorochrome stain, acridine orange stain, which in the presence of acid substances emits a red fluorescence due to its interaction with glycosaminoglycans [46,47]. Fluorescent staining methods have been used by other researchers for identifying fish MCs with one report on *Hoplias malabaricus* attributing to heparin the fluorescent identification when appropriate fixative solutions are used [5].

Adding to the basic and acid properties above mentioned, the present study found that fat snook's MCs/EGCs bear a neutral character as determined through PAS staining. This neutral feature has also been reported in other fish species and is a finding in other classes of vertebrates [26,44,45,48].

In mammals, the metachromasia in cytoplasmic granules is one of the main features of mastocytes. It is due to sulfate glycosaminoglycans, such as heparina [2,41,45]. In fish, metachromasia has been observed in MCs of different species indicating a class similarity among vertebrates. In the present study metachromasia was also observed when using alcohol-based fixative solution, agreeing with previous reports [5,12,45], but differing from a study on *Sparus aurata's* MCs [49]. In zebra-fish, MCs metachromasia was observed with formalin fixative [26]. The metachromasia observed in intestine MCs from *Hoplias malabaricus* is believed to be due to sulfate glycosaminoglycans [5] while another research work in pike intestine MCs suggests that.the mast cells studied were true mammal mast cells [12]. In a similar fashion, the authors of the current study are led to conclude that fat snook's MCs are true mast cells since all staining findings are consistent with mammal mast cells.

4.2. Immunohistochemistry

The proteases tryptase and chymase are mediators in store kept together within secretory granules [1,50]. Tryptase is the main proteic constituent in human MCs, stored almost integrally within granules in its fully active form. Tryptase's main role involves epithelial reparation, tissue degradation, granulocytes recruitment and mast cells activation. Chymase is a vasoactive intestinal peptide involved in tissue remodeling degrading extracellular matrix

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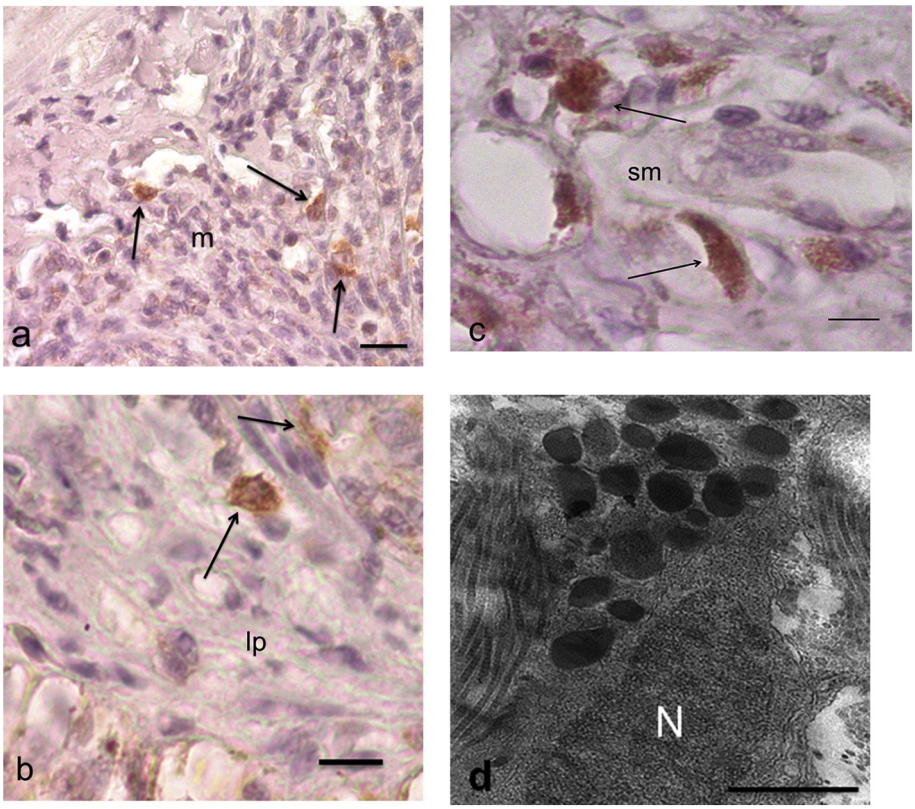

Fig. 2. Photomicrograph of intestinal mucosa (m) showing MCs/EGCs positive for tryptase (arrow), (a), positive for chymase (arrow) lamina própria (lp) (b), and positive for serotonin (arrow), submucosa (sm) (c). Bar = 10 μm. Ultrastructure showing MCs/EGCs with slightly indented euchromatic nucleus (n) and electron-dense spherical granules (d). Bar = 5 μm.

proteins including collagen precursor [1,29–31,51]. Studies in rats have shown that inadequate supply of mast cells as well as chymase are associated with decreased migration and epithelial permeability as well as with changes in the morphology of intestinal microvilli [52]. The activation of normal mast cells leads to degranulation and consequent secretion into the extracellular matrix of granule-stored mediators [31,50].

Mast cells heterogeny involves the expression of tryptase and chymase and accounts for the different term used by researchers. Mast cells occurring in the connective tissue and mucosa are referred to, as mast cells of the connective tissue (MCTC) and mast cells of the mucosa (MCM), respectively [25,27,50]. In humans, it is believed that the local environment is a factor associated with the different expression of these proteases by the two major subtypes of mast cells MCTC and MCM [53]. A study carried out in the small intestine has shown that the mucosa has a marked prevalence of mast cells secreting tryptase (T) and a small number of mast cells secreting tryptase and chymase (TC). Conversely, in the submucosa, (TC) mast cells were greatly prevalent while a small percentage of mast cells secreted tryptase (T) [27]. Interestingly, a whole culture population of skin mast cells was found to express both tryptase and chymase [54]. Even though mast cells are divided into different subtypes, their functioning differences and factors determining the development of these differences are poorly known [10,25,53]. Variations in the expression of these proteases have been reported in different mammal species. In fish, MCs also form subgroups characterized by the content of cytoplasmic granules, sensitivity to fixative solutions and their response to drugs [9].

The present study carried out in fat snook found that mast cells express both tryptase and chymase in the mucosa and submucosa. In both mucosa and submucosa layers, staining for chymase was intense whereas that for tryptase was only mild. Not many studies in fish report immunohistochemical analysis for these two proteases. However, a link can be established between two studies previously performed in zebra fish. In this animal, gill MCs were found positive for tryptase [26] and serum levels of this protease was reported to be higher in specimens infected with parasites [23], a sign similar to that found in mammals. The present research work evidencing the presence of the tryptase and chymase in the cytoplasmic granules of MCs/EGC was confined to the mucosa and submucosa of the small intestine of fat snook's MCs. Further studies on the secretion of proteases by MCs/EGC should be extended to other body sites and fish species, since the identification of the different types/subtypes of mast cells and the proteases that they express is crucial for a better understanding of the biological roles of these cells [1].

Another important inflammatory mediator produced and secreted by mast cells is serotonin [50], which was for a long time believed to be produced by rodent MCs only [8]. Presently, it is common knowledge that serotonin is synthesized by mast cells of humans and other vertebrates including fish [11], but until recently no study had identified the presence of serotonin in MCs/EGC [2]. Even currently, only a few immunohistochemical investigations have been carried out in fish, with one of them reporting serotonin in MCs from *D. truttae* intestine. The authors also reported increased number of MCs synthesizing serotonin in animals infected with parasites [11,55]. In the current study, immunohistochemical screening has also detected MCs/EGC containing serotonin in both mucosa and submucosa of the intestine of fat snook adding to the body of evidence regarding this inflammatory mediator in teleosts mast cells.

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4.3. Transmission electron microscopy

Mast cells are usually known to be associated with blood vessels. In the presented investigation, transmission electron microscopy imaging showed MCs/EGC in proximity to blood vessels, but in closely association with collagen fibers and cells of the connective tissue, as previously observed in different species of teleosts [18,56,57]. In general, these cells showed a spherical or slightly indented euchromatic nucleus and rarely visible a nucleolus. The granules in their cytoplasm were mostly spherical and varied in electron density as previously reported in other species of teleosts [18,26], and similarly observed in mammals [58]. It has been reported that transmission eléctron imaging can be used to identify mast cells secreting tryptase only and mast cells secreting both triptase and chymase since triptase appears as an electon-lucent área whereas chymase as an electron dense region in mammals [1]. Such a finding, however, was not reproduced in the present study.

5. Conclusion

The present study showed that fat snook's MCs/EGC have plasmatic granules with content exhibiting basic, acid and neutral character and that MCs/EGC shows a high degree of homology with mammal mast cells since metachromasia and presence of chemical mediators such as serotonin, tryptase and chymase were findings in the present research work. Futher studies, however, are needed to determine any existing heterogeneity among mast cells, thus defining MCs subtypes as reported in mammals and other teleosts. In this regard, immunohistochemical techniques that can identify simultaneously tryptase and chymase, for instance, seems to be of great help if one is to ascertain the single or simultaneous occurrence of mediators.

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