

## Histochemical and biometric study of the gastrointestinal system of *Hyla orientalis* (Bedriaga, 1890) (Anura, Hylidae)

E. Akat, H. Arıkan, B. Göçmen

Department of Biology, Zoology Section,  
Faculty of Science, Ege University,  
Bornova-Izmir, Turkey

### Abstract

This study was carried out to assess the localization of hyaluronic acid (HA) and the distribution of glycoproteins in the gastrointestinal system of adult *Hyla orientalis*. Histochemical analysis of the gastrointestinal system in *H. orientalis* showed that mucous content included glycogene and/or oxidable dioxes [periodic acid/Schiff (PAS)+], neutral or acid-rich (PAS/AB pH 2.5+), sialic acid residues (KOH/PAS+) and acid sulphate [Aldehyde fuchsin (AF)+] glycoproteins. However the mucus content was not the same in stomach, small and large intestine. The mucus content of stomach included only glycogene and/or oxidable dioxes and sialic acid residues. Besides these histochemical methods, the localization of HA was detected using biotinylated hyaluronic acid binding protein labeled with streptavidin-fluorescein isothiocyanate (FITC). In the extracellular matrix of the submucosa, the reaction for HA was evident. Since HA was located in submucosa beneath the epithelial layer of gastrointestinal system, it has a significant role in hydric balance, and essential to provide the gastrointestinal system integrity and functionality. According to biometric results, there were statistical differences between small and large intestine in terms of the amount of material stained positive with PAS/AB, PAS, KOH/PAS and AF/AB. Additionally, number of goblet cells in the small and large intestine was significantly different.

### Introduction

The gastrointestinal system is one of the most metabolically active system and prominent at all life stages due to energy utilisation and perform digestion of the food, nutrient absorption and expelling waste products.<sup>1,2</sup> The occurrence of mucus is a common feature in digestive tract.<sup>3</sup> Mucus consists of approximately 95% water and 5% mucins.<sup>4</sup> Mucins are

high molecular weight glycoproteins produced by epithelial tissues.<sup>5</sup>

In vertebrates, glycoconjugates on the cell surface and in extracellular matrix exhibit many important functions including cell-cell and cell-matrix adhesion events, cell signaling and control of cell membrane permeability.<sup>6-8</sup> Hyaluronic acid (HA) is a dominant part of the matrix glycosaminoglycans in higher vertebrates. Although HA formerly known as a passive molecule, in recent years researchers have reported the new roles of HA such as promoting cell motility, regulating cell-cell and cell-matrix adhesion, proliferation, embryological development, repair and regeneration, cell interaction in cancer and vascular diseases.<sup>9-13</sup>

Amphibians have significant role as a study model in physiology, and this group is a standard model for the study of many biological processes.<sup>14</sup> However relatively few studies have been carried out on amphibian gastrointestinal tract. There are some reports investigating the gastrointestinal epithelial transformation from the larval to adult type and apoptosis.<sup>15,16</sup> Additionally, histological and histochemical changes in digestive tract of *Ceratophrys ornata* and *Xenopus laevis* during metamorphic climax were reported.<sup>17,18</sup> In general, studies related to digestive system were carried out on reptiles, birds and mammals. But the cell renewal system of adult epithelium in the amphibian stomach is similar to mammalian stomach, where cell proliferation is localized in the neck region of gastric glands. Therefore, the amphibian digestive tract serves as a model system for studying mammalian organ regeneration.<sup>19-22</sup>

The globally distributed Hylidae is one of the largest families of anurans comprising of more than 940 known species and 51 genera. The tree frog genus *Hyla* consists of 37 species found in North America, Central America, Europe and Asia.<sup>23,24</sup> The eastern tree frog *Hyla orientalis* is a small arboreal species. It goes to water only in the breeding season, preferring clean, deep, heavily-vegetated water.<sup>25</sup> *H. orientalis* was previously not distinguished from *H. arborea* (Linnaeus, 1758), but south-eastern European and western Anatolian *H. arborea* populations were considered a separate species.<sup>26</sup> *H. orientalis* (formerly *H. arborea*) is carnivore and has a broad dietary diversity which was expected as a consequence of exploiting the habitat both vertically and horizontally, possibly allowing access to a broader spectrum prey.<sup>27</sup> In addition, this species are easily captured and transported, easily maintained in the laboratory.

The aim of the current study was to analyze biometric and histochemical characterization, and the determination of HA in gastrointestinal system for a better understanding of the role of HA.

Correspondence: Dr. Esra Akat, Department of Biology, Zoology Section, Faculty of Science, Ege University, 35100 Bornova-Izmir, Turkey. Tel.+90.23.23112404 - Fax: +90.23.23881036. E-mail: esra.akat@ege.edu.tr

Key words: Gastrointestinal system, goblet cell, glycoproteins, hyaluronic acid, amphibian, *Hyla*.

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## Materials and Methods

### Histochemistry

The present study was carried out according to the Animal Ethical Committee of Ege University, Faculty of Medicine and approved by the Republic of Turkey Ministry of Forestry and Water Affairs (date: 24 January 2014, permit no: 72784983-488.04-20643). Eight specimens (four adult males and four adult females) of *H. orientalis* were captured from Menderes-Izmir, Turkey (N 38°15' and E 27°8') in April. After 48 h, animals were anaesthetized with ether, and euthanized by decapitation, and immediately afterwards the gastrointestinal systems were removed. Gastrointestinal tissue samples were fixed in 4% paraformaldehyde overnight at 4°C. Thereafter, the tissue samples were rinsed with 0.1 M phosphate-buffered saline (PBS), pH 7.2, and processed according to the standard histological protocols for paraffin embedding. Five-micrometer-thick sections were stained with Gill's hematoxylin-eosin (HE) to demonstrate the general morphology of the tissue. Histochemical techniques [Periodic acid/Schiff (PAS), PAS/Alcian Blue (AB) pH 2.5, KOH/PAS, Aldehyde fuchsin (AF) and AF/AB pH 2.5] were performed for the identification of glycoproteins.

The localization of HA was detected using biotinylated hyaluronic acid binding protein (B-HABP) (Benaroya Research Inst., Seattle, WA, USA). Sections were deparaffinized, and non-specific binding sites were blocked with

2% bovine serum albumin (BSA) in PBS, pH 7.2, and then incubated in B-HABP overnight at 4°C. After application of B-HABP which labeled with streptavidin-fluorescein isothiocyanate (FITC) (Sigma Chemical Co., St. Louis, MO, USA). All washes were performed using PBS. For negative control hyaluronidase digested sections were used. The specificity of the staining was controlled by digesting some of sections with Streptomyces hyaluronidase prior to the incubation with the probe. For positive control, sections were stained with B-HABP/HA. Sections were photographed using a Leica DM3000 microscope (Leica Microsystems, Wetzlar, Germany) that was equipped with a Leica digital camera (DFC290).

### Biometric analyses

Image J programme was used to calculate the number of goblet cells per unit of epithelial area (mm<sup>2</sup>). Biometric analyses were evaluated by measuring the amount of material stained positive with PAS/AB, PAS, KOH/PAS and AF/AB in small and large intestine in each frog. These measurements were taken directly from the sections using micrometric ocular. On each animal; the lengths (L) and widths (W) of one hundred randomly chosen goblet cells in ileum and large intestine were measured. The areas of positive staining cell (µm<sup>2</sup>) were calculated as  $LW\pi/4$ .<sup>28</sup> The differences were compared by 2-tailed *t*-test using SPSS 16.0. We set the significance level at  $P \leq 0.05$ .

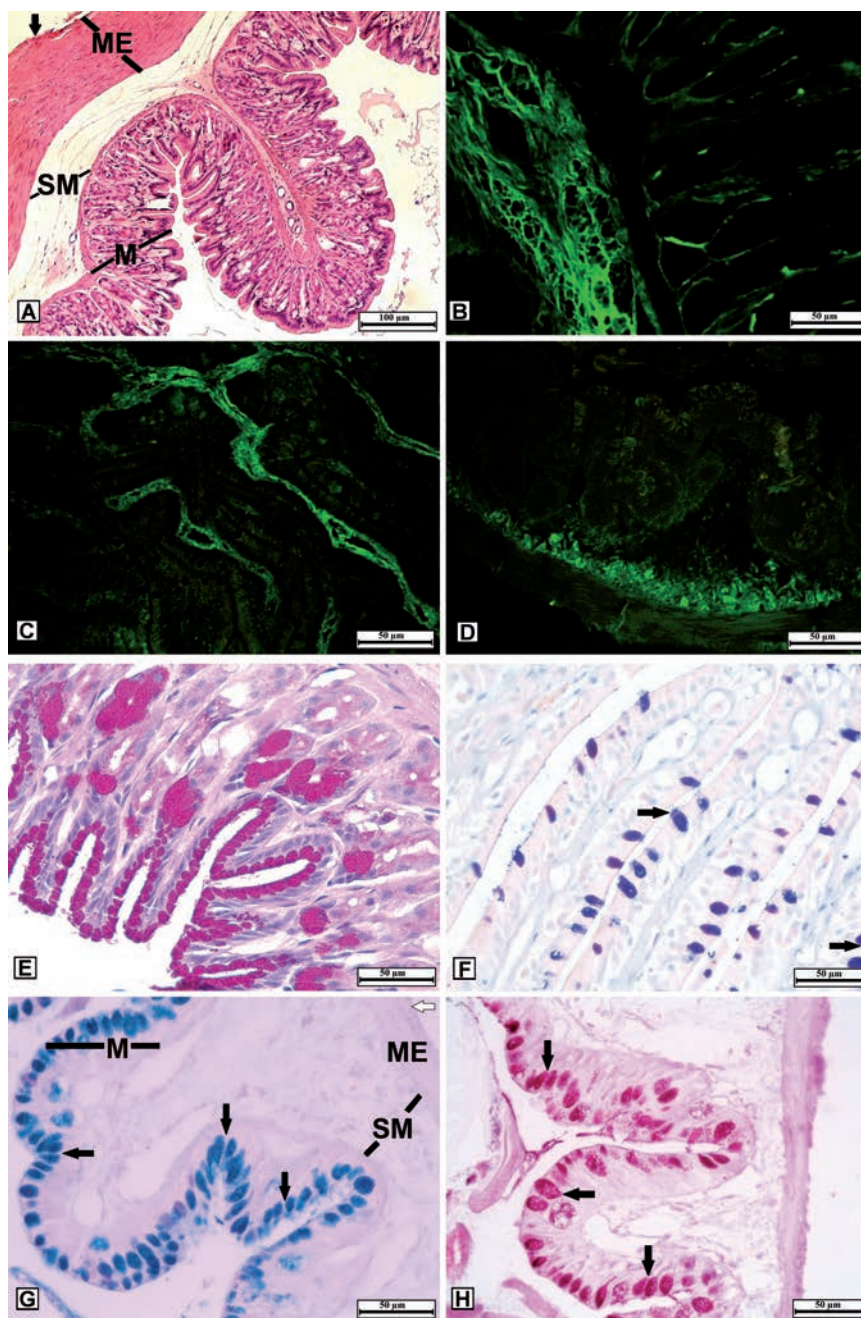
## Results

The gastrointestinal system of *H. orientalis* was composed of the typical layers as seen in higher vertebrates; mucosa, submucosa, *muscularis externa* and serosa (Figure 1A). The stomach of *H. orientalis* was subdivided into a wide corpus or fundus containing gastric glands and a short pyloric portion. The stomach was covered by serosa which was lined with a simple flat epithelium. The *muscularis externa* was composed of a thick layer of smooth muscle fibers. The submucosa was formed of loose connective tissue rich in blood vessels. The gastric mucosa was lined through its length by mucous secreting epithelial columnar cells with folds. Many gastric pits formed as a result of the mucosal layer into the *lamina propria* where gastric glands were located. The gastric glands were mostly of a simple tubular type. The gastric gland cells and apical portion of mucous cells showed PAS positive reaction (Figure 1 A,E) but did not react with AB and AF.

The mucosa of the small intestine was thrown into large numbers of high narrow lon-

gitudinal folds called intestinal villi. The mucosal epithelium of the small intestine was composed of two kinds of cells; absorptive cells and goblet cells (Figure 1F). The mucosal

epithelium of the small intestine showed complete absence of the intestinal glands and glandular crypts. The mucosal epithelium of the large intestine had fewer and shorter folds



**Figure 1.** A-D) Histological examination and immunofluorescence localization of Hyaluronic acid (HA) in the gastrointestinal system in *H. orientalis*. A) Layers of the stomach; M, mucosa; SM, submucosa; ME, *Muscularis externa*; black arrow, serosa. B) Stomach section. C) Small intestine section. D) Large intestine section; the localization of HA mainly in submucosa to maintain gastrointestinal system integrity and functionality. E-H) Histochemical evaluation of glycoproteins (GPs) in the gastrointestinal system of *H. orientalis*. E) Reacted positively to PAS in gastric gland cells and the apical portion of mucous cells. F) Strong sulphated GPs in goblet cells, AF/AB pH 2.5 (AB dominant). G) Neutral and/or acid rich GPs in goblet cells of large intestine; M, mucosa; SM, submucosa; ME, *Muscularis externa*; white arrow, serosa; PAS/AB pH 2.5 (AB dominant) H) GPs with sialic acid residues in goblet cells; KOH/PAS, goblet cells (black arrow).



containing simple columnar cells and numerous goblet cells. Glandular crypts were not observed in the mucosa of large intestine (Figure 1G). In both small and large intestine, the submucosa was formed of loose connective tissue containing a number of blood vessels. The next layer was the *muscularis externa* layer, composed of layers of smooth muscle. The outermost layer was the serosal layer, composed of a thin layer of epithelial tissue (Figure 1G).

By means of histochemical techniques the intestinal goblet cells with positive PAS reaction indicated that these cells produced mucus contains GPs with oxidizable vicinal diols and/or glycogen. The content of large intestine goblet cells showed positive reaction with PAS/AB pH 2.5 (AB dominant) (Figure 1G). The histochemical method (KOH/PAS) revealed the histochemical properties of mucous cells contained sialic acid residues in large intestine (Figure 1H). After aldehyde-fuchsin/alcian blue (AF/AB) staining method, the secretory contents of the mucous cells in both small (Figure 1F) and large intestine had a strong affinity to AB due to dominance of acidic glycoproteins. However, AF staining method showed that goblet cells of both small and large intestine also included GPs with sulphate.

Demonstration of HA immunoreactivity was carried out using an FITC labeled specific probe (B-HABP) in paraffinized sections by fluorescent microscopy. This reaction occurred with HA dispersed through the extracellular matrix. HA immunoreactivity indicated that it was located in submucosa beneath the epithelial layer of the gastrointestinal system. However, the HA immunoreactivity was not observed in smooth muscle of the gastrointestinal system (Figure 1 B,C,D).

According to our results, there were statistical differences between small and large intestine in terms of the amount of material stained positive with PAS/AB, PAS, KOH/PAS and AF/AB (Table 1). Additionally, number of goblet cells in the small and large intestine was significantly different (Table 2).

## Discussion

This is the first study that the demonstrate the HA presence in amphibian gastrointestinal tract. Histochemical methods proved to characterize the content of mucous cells which had neutral, acidic glycoproteins, glycoproteins with sialic acids and sulphated glycoproteins. The HA immunoreactivity was located in the submucosa beneath the epithelial layer of the gastrointestinal system.

Amphibian digestive tract is highly respon-

sive and sensitive to environmental cues.<sup>29</sup> Although, amphibians are a standard and classic model organism for the study of many physiological processes and serves as a good model system for studying organ regeneration of the digestive tract common to terrestrial vertebrates, there are a few reports related to amphibian digestive system. In recent years, some physiological studies were carried out for the effect of arousal and re-feeding on digestive tract morphology following aestivation or food restriction. In rats re-fed *ad libitum* after 11 days of total food restriction, small intestine mass increased by up to 75% within 24 h and by 200% within 3 days of re-feeding.<sup>30</sup> In the amphibians *Bufo alvarius*, *Ceratophrys ornata* and *Pyxicephalus adspersus*, re-feeding following 1 month of food deprivation during aestivation, resulted in an average increased in intestinal wet mass of over 200%.<sup>31</sup> Another study reported that the mass of the small intestine of burrowing frog *Cyclorana alboguttata* increased by over 450% within 36 h of re-feeding following 3 months of aestivation. Data from these studies and similar studies have demonstrated that the gastrointestinal tract of frogs is highly plastic and responds to variations in food availability both rapidly and highly reversibly.<sup>32</sup>

According to our results, the gastric mucin did not show AB or AF positive reaction, but reacted positively to PAS in gastric gland cells and the apical portion of mucous cells due to presence of neutral GPs. The same results were observed in some amphibians *Triturus*

*carnifex*,<sup>33</sup> *Bufo viridis*<sup>34</sup> and in some reptiles.<sup>35-37</sup> However, mucous cells of stomach in *Rana a. aurora*,<sup>38</sup> some species of reptiles,<sup>39</sup> many mammals<sup>40</sup> and some species of fish<sup>41</sup> produce neutral and acid mucins. Neutral GPs protect against mechanical injuries, pathogens and aggressive pepsin.<sup>38</sup> Additionally this type of mucin was poor in anions. This may prevent hydrogen ions and water molecules in the gastric juice being drawn into the protective mucus layer.<sup>42</sup> Based on this, because of stomach extract containing a high concentration of hydrogen ions, neutral GPs probably behave like a proton pump.

In *H. orientalis* the mucosa of intestine consisted of a single layer of columnar epithelial cells with goblet cells. There were statistical differences between small and large intestine in terms of the number of goblet cells. The increased density of goblet cells towards to the large intestine observed in this study. This situation probably related to an increased need of lubrication for the ejection of feces, and has been presented in some studies on vertebrate gastrointestinal tract.<sup>33,43-45</sup> The content of goblet cells in small and large intestine showed positive reaction with PAS/AB pH 2.5 (AB dominant). The AB sequence revealed acidic GPs that could be responsible for an increasing viscosity of the secretions.<sup>46</sup> Additionally, acidic GPs have been proposed to protect the intestinal epithelium against the degradative actions of glycosidases.<sup>47</sup> AF/AB pH 2.5 and AF staining methods showed that the goblet cells of *H. orientalis* contained acidic and sulphated

**Table 1. Mean, standard deviation, *t*-value and significance of the measured staining area ( $\mu\text{m}^2$ ).**

	Mean	SD	<i>t</i> -value	Sig. (2-tailed)
PAS/AB staining				
Small intestine	83.29	23.32	6.26	<0.05*
Large intestine	118.07	29.54	6.26	<0.05*
KOH/PAS staining				
Small intestine	77.08	27.98	6.50	<0.05*
Large intestine	115.07	28.48	6.50	<0.05*
PAS staining				
Small intestine	80.75	22.10	14.65	<0.05*
Large intestine	123.60	36.73	14.65	<0.05*
AF/AB staining				
Small intestine	85.31	25.57	6.12	<0.05*
Large intestine	124.66	36.94	6.12	<0.05*

**Table 2. Comparison of mean goblet cell numbers between small and large intestine.**

Goblet cell ( $\times 10^3$ )/mm <sup>2</sup>	Mean	SD	<i>t</i> -value	Sig. (2-tailed)
Small intestine	0.85	0.07	-8.45	<0.05*
Large intestine	2.3	0.14	-8.45	<0.05*

\*Significant at Student's *t*-test,  $P \leq 0.05$ .

glycoproteins. In this study most goblet cells showed AB positive material dominance like in most goblet cells of *P. antalyae*.<sup>48</sup> The presence of a large number of microorganisms is usually associated with the secretion of sulphated mucins.<sup>34</sup> Sulphated GPs are abundant in goblet cells of intestinal tracts, where protein digestion and trapping of bacteria (or other pathogens) occur, and may have a role in the stimulation of immunity there.<sup>43,49</sup> KOH/PAS staining method revealed the presence of sialic acid residues. Sialic acid residues and sulphated glycoproteins increase the secretion viscosity to play the main role of protection.<sup>50</sup> Additionally sialic acid residues together with sulphated groups cause the negative charge of the GPs and mask receptor sites for pathogen forms as bacteria, viruses and mycoplasma species.<sup>33,51</sup> To observe the effects of bacteria on intestinal goblet cell mucin production during post-hatch development, differences in the small intestine of conventionally reared and low bacterial load broiler chicks were examined. Mucin composition was influenced by bacterial colonization and an increased in sialylated mucin content was reported.<sup>52</sup>

According to our histochemical results, no differences were evident in glycoproteins secretion between the small and large intestine. However, the amount of material stained positive with PAS/AB, PAS, KOH/PAS and AF/AB in both the small and large intestine were significantly different. The greater number of goblet cells and staining area in large intestine compared with small intestine would suggest the large intestine may be a preferred region for bacterial colonization. HA was a major component of connective tissue matrices, where its function include promoting matrix assembly, viscosity of some fluids and tissue hydration because of its water binding property.<sup>53,54</sup> Based on this data point, HA acts as a water barrier, and it probably enables the transfer of water towards the epithelial layer of the gastrointestinal system. Therefore, HA and goblet cells work together to maintain tissue moisture, enable the gastrointestinal tract lubrication and reduce mechanical friction. HA is a fundamental constituent of the interstitial barrier. It provides to decrease tissue permeability, thereby increasing the viscosity of tissue. Catalyzing the hydrolysis of hyaluronic acid by hyaluronidase causes increased tissue permeability. Therefore it is used in medicine in conjunction with other drugs to speed their dispersion and delivery. Some bacteria, such as *Staphylococcus aureus*, *Streptococcus pyogenes* and *Clostridium perfringens* produce hyaluronidase to hydrolyse hyaluronic acid as means of increasing mobility through the body's tissues.<sup>55-61</sup> Based of these data points, HA with the GPs in goblet cells probably have a function as protection against chemicals and

bacteria or other pathogens. Therefore HA was widely localized in submucosa beneath the epithelial layer of the gastrointestinal system to act as chemical and biological defence barrier. It can be concluded that the contents of mucous cell were composed of neutral and acidic GPs; GPs with sialic acids and sulphated GPs. Considering the importance of glycoconjugates and HA, they act significant role including maintain tissue moisture, lubrication with reducing mechanical friction, and protection against chemicals and pathogens.

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