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

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**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 mucus content included glycogen and/or oxidizable diols [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 glycogen and/or oxidizable diols 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. The occurrence of mucus is a common feature in digestive tract. Mucus consists of approximately 95% water and 5% mucins. Mucins are high molecular weight glycoproteins produced by epithelial tissues.

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. 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.

Amphibians have significant role as a study model in physiology, and this group is a standard model for the study of many biological processes. 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. Additionally, histological and histochemical changes in digestive tract of *Ceratophrys ornata* and *Xenopus laevis* during metamorphic climax were reported. 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.

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. The eastern tree frog *Hyla orientalis* is a small arboreal species. It goes to water only in the breeding season, preferring water only in the breeding season, preferring high molecular weight glycoproteins produced by epithelial tissues.

The localization of HA was detected using biotinylated hyaluronic acid binding protein (B-HABP) (Benaroya Research Inst., Seattle, WA, USA). Sections were deparaffinized, and stained with Gill's hematoxylin-eosin (HE) to demonstrate the general morphology of the tissue. Histochmical 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.

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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 (mm²). 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 (µm²) were calculated as LW/4.28. The differences were compared by 2-tailed t-test using SPSS 16.0. We set the significance level at P<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 1A,E) but did not react with AB and AF.

The mucosa of the small intestine was thrown into large numbers of high narrow longitudinal 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.
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. Positive PAS reaction occurred with HA dispersed through the extracellular matrix. HA immunoreactivity indicated that it was carried out using an FITC labeled specific antibody. This reaction occurred when HA was positive with PAS/AB, PAS, KOH/PAS and AF/AB staining methods showed that the 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 antibody in paraffinized sections by fluorescence 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 1B.C.D).

According to our results, the gastric mucin was positive with PAS/AB pH 2.5 (AB dominant) (Figure 1G). The histochemical method (KOHPAS) revealed the histochemical properties of mucous cells contained sialic acid residues in large intestine (Figure 1H). After aldehyde-fuchsin/alcan 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.

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, Bufo viridis and in some reptiles. However, mucous cells of stomach in Rana a. aurora, some species of reptiles, many mammals and some species of fish produce neutral and acid mucins. Neutral GPs protect against mechanical injuries, pathogens and aggressive pepsin. 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. 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 for lubrication for the ejection of feces, and has been presented in some studies on vertebrate gastrointestinal tract. 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. Additionally, acidic GPs have been proposed to protect the intestinal epithelium against the degradative actions of glycosidases. AF/AB pH 2.5 and AF staining methods showed that the goblet cells of H. orientalis contained acidic and sulphated neutral and acid mucins.

### Table 1. Mean, standard deviation, t-value and significance of the measured staining area (μm²).

|                          | Mean   | SD    | t-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          | 123.00 | 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*          |

*Significant at Student’s t-test, P<0.05. 

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

| Goblet cell (×10³)/mm² | Mean   | SD    | t-value | Sig. (2-tailed) |
|------------------------|--------|-------|---------|-----------------|
| Small intestine        | 0.85   | 0.07  | -8.45   | <0.05*          |
| Large intestine        | 2.3    | 0.14  | -8.45   | <0.05*          |

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 responsive and sensitive to environmental cues. 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. In the amphibians Bufo alvarus, 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%. 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.

According to our results, the gastric mucin with PAS/AB pH 2.5 (AB dominant) 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, Bufo viridis and in some reptiles. However, mucous cells of stomach in Rana a. aurora, some species of reptiles, many mammals and some species of fish produce neutral and acid mucins. Neutral GPs protect against mechanical injuries, pathogens and aggressive pepsin. 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. Based on this, because of stomach extract containing a high concentration of hydrogen ions, neutral GPs probably behave like a proton pump.

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glycoproteins. In this study most goblet cells showed AB positive material dominance like in most goblet cells of P. antalyae. The presence of a large number of microorganisms is usually associated with the secretion of sulfated mucins. 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. 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. 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. 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.

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 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 function include promoting matrix assembly and reducing mechanical friction. HA is a fundamental constituent of the interstitial barrier. It provides to decrease tissue permeability. Therefore HA was widely localized in submucosa beneath the epithelial layer of the gastrointestinal system to act as chemical and biological defense 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 bacteria or other pathogens. Therefore HA was present in submucosa beneath the epithelial layer of the gastrointestinal system to act as chemical and biological defense 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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