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HISTOCHEMICAL ANALYSIS OF GLYCOCONJUGATES IN THE BRANCHIAL MUCOUS CELLS OF *Apareiodon affinis* (STEINDACHNER, 1879) (CHARACIFORMES, PARADONTIDAE)

ANÁLISIS HISTOQUÍMICO DE LOS GLICOCONJUGADOS EN LAS CÉLULAS MUCOSAS DE LAS BRANQUIAS DE *Apareiodon affinis* (STEINDACHNER, 1879) (CHARACIFORMES, PARADONTIDAE)

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ABSTRACT. The histochemical characteristics of the mucous cells located in the gills of the fish *Apareiodon affinis* (Steindachner, 1879) (Characiformes, Paradontidae) were investigated. Several methods for the localization and characterization of glycoconjugates (GCs) with oxidizable vicinal diols, O -acyl sugars, O- sulphate esters and sialic acids residues without O-acyl substitution or with O-acyl substitution at C7, C8 or C9 were employed. Mucous cells were observed among the epithelial cells of the filament and the gill lamellae. No histochemical differences were detected between the mucous cells of primary and secondary lamellae. They contained high amounts of GCs with carboxyl groups and O-sulphate esters, together with moderate amounts of GCs with oxidizable vicinal diols, and low amounts of GCs with sialic acids. This work demonstrated the heterogeneity of the mucous cells GCs, which could be associated with different functions such as lubrication, protection and inhibition of the invasion and proliferation of pathogenic microorganisms and ionic regulation.
Key words: gills, glycoconjugates, histochemistry, mucous cells, teleost fish.

RESUMEN. Las características histoquímicas de las células mucosas de las branquias de *Apareiodon affinis* (Steindachner, 1879) (Characiformes, Paradontidae) fueron investigadas. Se utilizaron métodos para la localización y caracterización de glicoconjugados (GCs) con dioles vecinos oxidables, O-acil azúcares, ésteres orto-sulfatados y residuos de ácidos siálicos sin substitución o con substitución O-acilo en C7, C8 y/o C9. Se observaron células mucosas entre las células epiteliales del filamento y las laminillas secundarias. No se encontraron diferencias histoquímicas entre las células mucosas de las laminillas primarias y secundarias. Ellas poseen abundantes GCs con grupos carboxílicos y esteres O-sulfatados, junto con moderada proporción de GCs con dioles vecinos oxidables y baja cantidad de GCs con ácido siálico. Este trabajo demostró la heterogeneidad de los GCs de las células mucosas que podría asociarse con funciones diferentes como la lubricación, protección e inhibición de la invasión y proliferación de microorganismos patógenos y la regulación iónica.
Palabras clave: branquias, glicoconjugados, histoquímica, células mucosas, peces teleósteos.

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INTRODUCTION

The glycoconjugates (GCs) constitute the major component of the vertebrate mucosubstances. They are known to have a large variety of functions, from antimicrobial and antiviral to osmotic functions (1, 7, 8, 9). In fishes, mucosubstances also have an important role in ion regulation and diffusion (10).

The presence of mucous cells is a common character of teleost fish. Fish mucous cells elaborate and release different secretory components, mainly GCs. Mucous cells and the mucous composition they produce are influenced by the physicochemical conditions of the environment and its variations (5, 10).

The characid *Apareiodon affinis* (Steindachner, 1879) is a teleost belonging to the family Paradontidae, and it is geographically distributed by the rivers Paraguay, Paraná Medio and Bajo, Uruguay Medio and Bajo, and de la Plata, and western Brazil. It inhabits the muddy depths where it feeds on detritus (35). A. affinis is popularly known as "duro-duro", "canivete" or "charuto" in Brazil and as "virolitos", "piki" or other names in the rest of the Latin American countries where they occur (25). Variations in the chemical compounds present in all river water columns can alter the morphology and secretion of the gill mucous cells of fish (16). In this way, they are a useful model for studies on environmental impact (19).

The purpose of this study was to analyze the composition of carbohydrates in the mucous cells of the gills of A. affinis from the hydrographic basin of the River Uruguay Medio, in Uruguaiana, Rio Grande do Sul, Brazil.

This study will provide a more profound knowledge about the histochemical features of the mucous cells of A. affinis, and it represents the foundation upon which morphological comparisons at different environmental situations will be possible.

MATERIALS AND METHODS

Animals

Adult *A. affinis* specimens of both sexes were collected in São Marcos District, locality of Cantão, at the hydrographical basin of River Uruguay Medio , ("29º 30' 20.4" S / 56º 50' 41.9" W), in the Uruguaiana Comune, Rio Grande do Sul, Brazil (Fig. 1).

Fig. 1. Female specimen of *A. affinis* collected in São Marcos District.

Collections were done in the winter-spring 2005 and summer 2006 periods. Specimens were adults of both sexes. After collection the specimens were weighed and measured in site (13.3 ± 1.21cm length; 23.5 ± 6.8g weight). The gills were rapidly excised and fixed by immersion in 10% buffered formalin for light microscope studies.

Histological processing

Samples were routinely processed and embedded in paraffin. Four micrometer-thick histological sections were cut by microtome, prepared according to standard protocol and then stained using the following techniques: routine hematoxylin and eosin (H-E) stain, Masson trichrome stain for morphology and Mayer mucicarmin for mucin identification.

Histochemical processing

Sections of tissue were also treated with histochemical procedures to identify and differentiate GCs (Table 1). Sections were stained with: 1) PAS (periodic acid Schiff´s reagent) to demonstrate periodate reactive vicinal diols; 2) the acetylation before PAS technique to block the oxidation of the 1,2 glycol groups by the periodic acid; 3) the acetylation – saponification - PAS sequence to restore the 1,2 glycol groups which reacts with the periodic acid ; 4) -amylase

digestion before PAS reaction for a control of the presence of GCs with oxidizable vicinal diols; 5) PA*S (selective periodic acid Schiff reaction): oxidation for 1 h at 4°C with 0.4 mM periodic acid in approximately 1 M hydrochloric acid is used as a specific reagent for the selective visualization of sialic acids in the PAS procedure. The selectivity of the reaction is the result of an increase in the rate of the oxidation of the sialic acid residues together with a decrease in the rate of oxidation of neutral sugars; 6) KOH / PA*S (saponification-selective periodic acid Schiff reaction) to allow the characterization of total sialic acids. The saponification with 0.5% potassium hydroxide in 70% ethanol for 30 min at room temperature was performed to deacetylate sialic acid residues and was followed by PA*S; 7) KOH / PA*/ Bh / PAS (saponification-selective periodic acid-borohydride reduction-periodic acid Schiff reaction) for the characterization of neutral sugars; 8) PA / Bh / KOH / PAS (periodic acid-borohydride reduction-saponification-periodic acid Schiff reaction): this method was carried out using a 2 h oxidation at room temperature with 1% periodic acid. The aldehydes generated by the initial oxidation were reduced to Schiff-unreactive primary alcohols with sodium borohydride (PA-Bh). Following saponification (KOH), sialic acids with O-acyl substituents at C7, C8 or C9 (or which had two or three side-chains O-acyl substituents) and O- acyl sugars are PAS positive; 9) AB pH 2.5 (Alcian Blue 8GX pH 2.5): to demonstrate GCs with carboxyl groups (sialic acid or uronic acid) and / or with O-sulphate esters; 10) AB pH 1.0 and pH 0.5 (Alcian Blue 8GX pH 1.0 and pH 0.5) to demonstrate GCs with O-sulphate esters and very sulphated GCs respectively.

Procedures	Interpretation of staining reactions	References
PAS	GCs with oxidizable vicinal diols and/or glycogen	McManus (1948)
Acetylation-PAS	GCs with oxidizable vicinal diols and/or glycogen	Lillie & Fullmer (1976)
Acetylation-Saponification-PAS	GCs with oxidizable vicinal diols and/or Glycogen	Lillie & Fullmer (1976)
α-amylase- PAS	GCs with oxidizable vicinal diols	Pearse, 1985
PA*S	Sialic acid and some of their chain variants (C7 and/or C9)	Volz *et al*. (1987)
KOH/PA*S	GCs with sialic acid residues	Culling *et al*. (1976)
KOH/PA*/Bh/PAS	Neutral GCs with oxidizable vicinal diols	Volz *et al*. (1987)
PA/Bh/KOH/PAS	Sialic acid residues with O-acyl substitution at C7, C8 or C9 and O-acyl sugars	Reid *et al*. (1973)
AB pH 2.5	GCs with carboxyl groups (sialic acid or uronic acid) and/or with O-sulphate esters	Lev & Spicer (1964)
AB pH 1.0	GCs with O- sulphate esters	Lev & Spicer (1964)
AB pH 0.5	Very sulphated GCs	Lev & Spicer (1964)

Table 1. Histochemical reactions for carbohydrate moieties in mucous cells of A. affinis gills.

RESULTS

The gill arch structure of A. affinis is similar to that of other teleosts. Two epithelial types are clearly identified in the gills of A. affinis: primary or filamentary and secondary or lamellar. The former is stratified with epithelial cells, mitochondria-rich cells and mucous cells spread among them; the latter is a two-cell-epithelium morphologically adapted to gas exchange.

Mucous cells are detected among the epithelial cells of the primary and secondary gill lamellae. They appear depressed in the surface of the epithelial cells which cover them almost completely. Mucous cells are large, with abundant secretion granules that displace the nucleus to the basal margin of the cells. Mucus discharge is performed by exocytosis. Hematoxylin-eosin or trichrome colored preparations showed no color in the mucous cell content.

The histochemical procedures for visualizing and identifying GCs in the mucous cells of primary and secondary lamellae are summarized in Table 2.

Procedures	Reaction shades
PAS	2M
Acetylation-PAS	0
Acetylation-Saponification-PAS	2M
α-amylase- PAS	2M
PA*S	1-2M
KOH/PA*S	1-2M
KOH/PA*/Bh/PAS	2M
PA/Bh/KOH/PAS	3M
AB pH 2.5	3T
AB pH 1.0	2-3T
AB pH 0.5	2-3T

Table 2. Histochemical staining properties of GCs in mucous cells of *A. affinis* gills
(M, magenta; T, turquoise. Staining intensity: 0, negative; 1, weak; 2, moderate; 3, strong)

No histochemical differences were detected between the mucous cells of primary and secondary lamellae. Analyses of reactions which combined histochemical methods and their respective controls indicated that a single type of mucous cell appeared. The mucous cell contained high amounts of GCs with carboxyl groups and O-sulphate esters, together with moderate amounts of GCs with oxidizable vicinal diols, and low amounts of GCs with sialic acids.

The reaction with the PAS method indicated that moderate amounts of GCs with oxidizable vicinal diols were present (Fig. 2). The coloration disappeared after acetylation and recovered after saponification confirming the presence of GCs with oxidizable vicinal diols. Control sections subjected to α-amylase were positive to the PAS reaction after this treatment, so they must have contained neutral hexoses. The mucous cells were weakly positive to PA*S and KOH / PA*S reaction, thus indicating that GCs with sialic acids and some of their chain variants were scanty (Figs. 3 and 4). Therefore the strong reaction with the PA / Bh / KOH / PAS method indicated mainly the presence of O-acyl sugars (Fig. 5). Neutral GCs with oxidizable vicinal diols were revealed using the KOH / PA* / Bh / PAS procedure (Fig. 6).

Sequences of reactions utilizing Alcian blue at different pH levels showed the presence of GCs with carboxylic and O-sulphate esters (weak and strongly ionizated) (Figs.7-9).

DISCUSSION

In histological terms, the gills of *A. affinis* are basically similar to that of other teleost fish (7, 8, 11, 13, 14). Thus, we described two types of epithelia in *A. affinis*: that of the filament and that of the respiratory lamellae. Mucous cells were present in both epithelia. They were located sunk among the epithelial cells of the filaments and the secondary lamellae that covered them almost completely, as classically described in other teleosts (2, 5, 7, 8).

Histochemical methods have proved to be valuable tools for localizing and characterizing

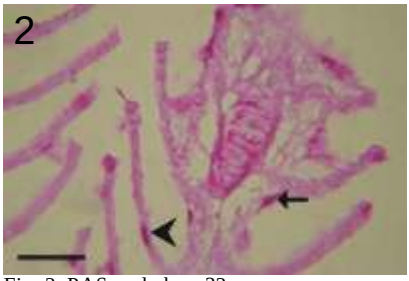

Fig. 2. PAS scale bar: 32 µm.

Fig. 3. PA*S scale bar: 28 µm

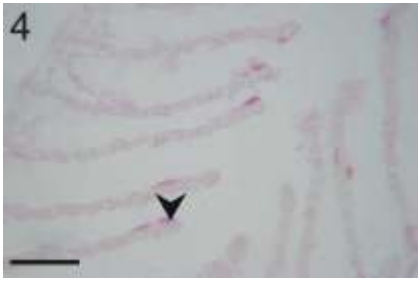

Fig. 4. KOH / PA*S scale bar: 40 µm

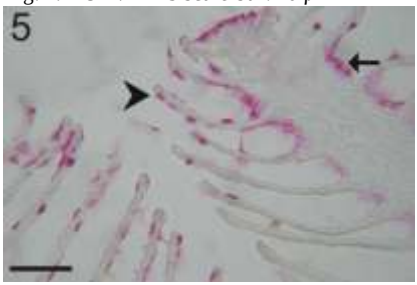

Fig. 5. PA / Bh / KOH / PAS scale bar: 56

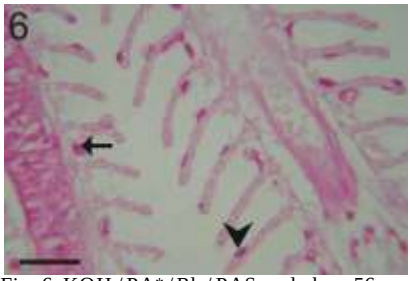

Fig. 6. KOH / PA* / Bh / PAS scale bar: 56 µm

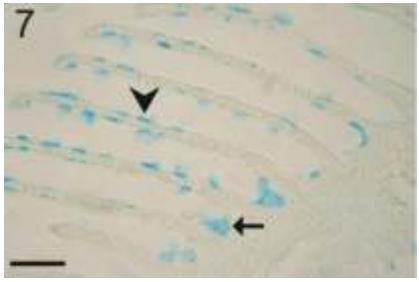

Fig. 7. AB pH 2.5 scale bar: 35 µm.

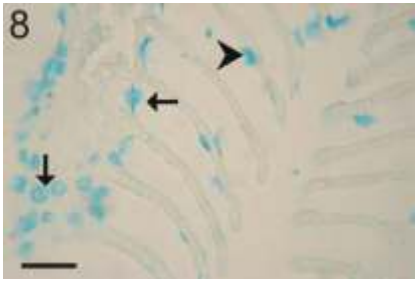

Fig. 8. AB pH 1.0 scale bar: 35 µm.

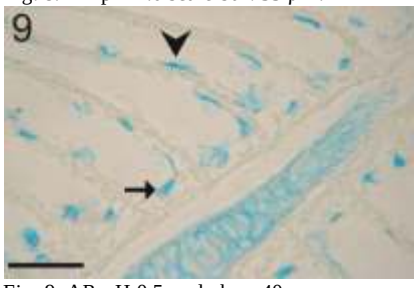

Fig. 9. AB pH 0.5 scale bar: 40 µm.

gill cells (7, 8). The histochemical methods used allowed us to characterize the mucous cells. The main components of mucus are high molecular weight GCs with numerous carbohydrate chains O-glycosidically linked to a protein core (3).

The contents of mucous cells from the primary and secondary lamellae from the gills of *A. affinis* were mostly neutral GCs, carboxylated and sulphated GCs, and scarce GCs with sialic acids and some of their side chain variants.

The different types of GCs detected in the mucous cells proved a high level of histochemical complexity, related to the diverse functions that the mucosusbstances display in freshwater fishes.

In the present study we have identified a single type of mucous cell in the gills of A. affinis. Mucous cells of A. affinis gills were generally similar to those described for the gills of *Odontesthes bonariensis* (6) and for the epithelium of the operculum of *Lepidocephalichthys guntea* (24).

As in *Solea senegalensis* (2, 30) and *Cynoscion guatucupa* (8) it was found that mucous cells of A. affinis gills secreted both neutral and acidic carboxylated GCs. These components were detected altogether in the same cell.

Acid GCs have been shown to coincide with increased mucus viscosity in the alimentary tract of fish (32), in air way epithelia of mammals (15) and in corals (21). The elaboration of sulphated GCs by mucous cells in A. affinis gills could be related to an increased viscosity of the mucus and to a lubrication of the surface of the fish gills. According to Mittal *et al.* (2002, 2004), the sulphated GCs could play a vital role in providing protection against mechanical damage to which these fishes are highly vulnerable because of their habitat, and also while moving. Furthermore, it has been postulated that sulphated GCs prevent the proliferation of pathogenic micro-organisms in freshwater fish which are more likely to become infected in this type of environment (22, 24, 27, 34). Thus, high amounts of sulphated GCs in the mucous cell secretions of the gills of A. affinis may also assign a significant resistance against pathogens and it would protect the fish.

Tibbets (1997) has described neutral GCs as less viscous than the acid GCs elaborated by mucous cells in the alimentary tract of *Arrhamphus sclerolepis krefftii*. The histochemical composition of the mucous secretion in the mucous cell of *A. affinis* gills has also revealed the presence of neutral GCs. The neutral GCs would thus lower the viscosity of mucus in this region. According to Mittal *et al.* (2002, 2004) mucus with lower viscosity is considered to be fairly easy to wash away with the respiratory water current. This fact would facilitate the respiratory process.

According to the scheme proposed by Harrison *et al.* (1987), the biosynthesis of GCs includes modifications of the secretory protein, and different stainings can represent the different cell stages. The synthesis of mucin GCs includes at least two modifications of the secretory protein: glycosylation of the protein followed by modifications of the sugar moiety. As a result, PAS negative mucous cells initially contain only proteins. PAS positivity could be related to the production of glycoproteins. The Alcian blue staining coincides with the carboxylation stage, and the presence of sulphated glycoproteins with the conjugation with sulphated groups (2, 8).

Care must be taken with comparisons concerning histochemical analyses of fish because in some cases, identical species under different conditions have shown differences in the type of GCs produced (29). Moreover, in some freshwater fish (*Monopterus cuchia* and *Pungitius pungitius*), the epidermal mucous cell sulphated proteins predominate in the mucus composition, whereas in marine fish (*Blennius tentacularis* and *B. sanguinolentus*), GCs with sialic acid prevail (34). Likewise, in the freshwater fish *O. bonariensis* (6), sulphated GCs predominate in their gills whereas in the gills of the marine fishes *Micropogonias furnieri* (5, 7) and *Cynoscion guatucupa* (8) many GCs with sialic acid are also found.

On the other hand, by means of histochemical techniques (29) four types of mucous cells in the branchial epithelium of the *Poecilia vivipara* were identified, and only one type is described in Solea senegalensis (2, 30). Then, the mucous cells of the gill epithelium of *A. affinis* synthesize different mucosubtances. The combination of GCs possibly enables the gills to respond quickly to changes in the environmental condition. The components of GCs found in the mucous cells of *A. affinis gills* may be related to the gills having the general osmoregulatory role of regulating the transfer of ions and fluids.

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