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Morphological, histochemical and immunohistochemical study of the gill epithelium in the abyssal teleost fish *Coelorhynchus coelorhynchus*

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Abstract: Histochemical and immunohistochemical study was carried out on nitrinergic innervation and neuroendocrine system in the gill epithelium of the abyssal fish *Coelorhynchus coelorhynchus*. The results showed that nNOS-positive nerve fibers, originating from the branchial arch were present in the subepithelial tissue of branchial primary filament. nNOS-positive neuroendocrine cells were also present in the primary filaments and secondary lamellae. Numerous mucous cells in the gill epithelium were AB/PAS-positive, while sialic acid was absent as confirmed by neuraminidase reaction and WGA lectin histochemistry. The mucus compounds in abyssal teleost fish are different from those found in pelagic species, being related to their living conditions. In abyssal species, greater numbers of chloride and neuroendocrine cells are involved in the movement of water and electrolytes. Neuroendocrine cells possess oxygen receptors which mediate the cardiovascular and ventilatory response to oxygen deficiency, as reported in teleost species. Besides, NO contributes through nervous stimulation to the regulation of vascular tone and blood circulation in the gill.

Key words: Gill - nNOS - Glycoconjugates - Lectins - Abyssal fish

Introduction

The mucus of fish gill epithelia is involved in many biological activities, such as swimming and defence against pathogenic microorganisms, parasites and pollutants [8]. The mucus contains sialic acid responsible for reduction of the pH [10], glycoconjugates [6, 9, 14] and many other compounds. The demonstration of nNOS in the invertebrate epithelial cells [12, 13] and in the gill epithelia of the catfish [34, 35], has suggested a regulatory role of NO in the secretory function. NO controls smooth muscle relaxation [18], the water-salt balance [15] and is involved in a diffuse chemoreceptor system sensitive to hypoxia, as shown in marine and freshwater teleost fishes [1-3, 7, 11, 25-31]. In the fish gills, the subepithelial nNOS- and VIP-positive nerve fibers act as non-adrenergic non-cholinergic (NANC) transmitters in the neuronal inhibitory regulation of gill respiration [16]. They also play a similar role in invertebrates with cutaneous respiration [12]. In the gill epithelia nNOS-positive neuroendocrine cells are involved, by paracrine mechanism, also in the modulation of epithelial integrity, secretion of mucus [21] and water and salt balance [5, 30-35]. An increase in the number of neuroendocrine cells has been shown in recent studies on fish gill in the environment with oxygen deficiency [17].

The aim of the present study was to investigate the glycoconjugate distribution pattern and nitrinergic innervation of the gill epithelium in the abyssal fish *Coelorhynchus coelorhynchus* in order to clear the involvement of NO in the neuronal regulation of the branchial epithelia function in environments with oxygen deficiency.

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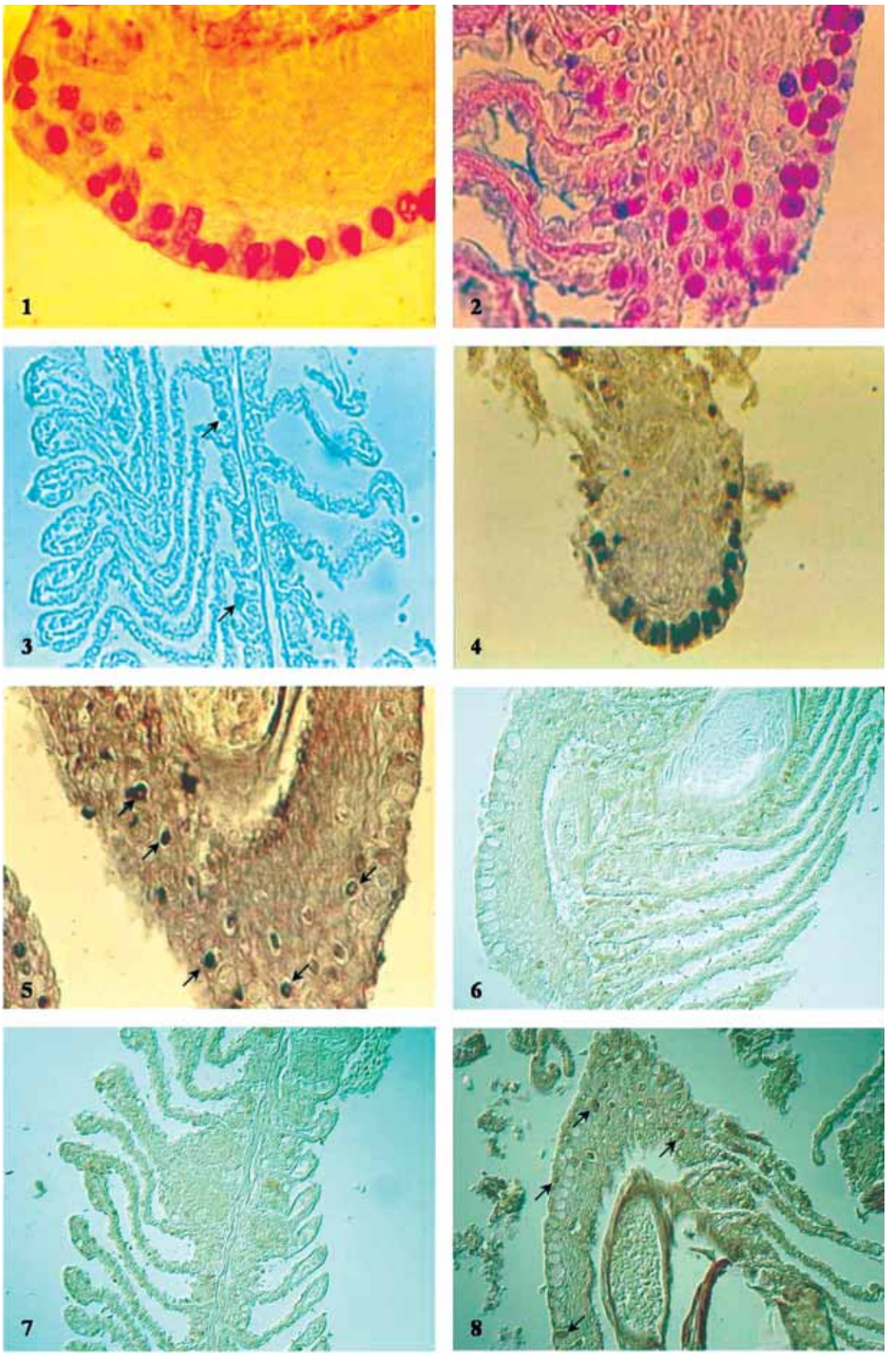
Materials and methods

Specimens of *Coelorhynchus coelorhynchus*, caught in the Straits of Messina (Italy), were transferred to our laboratory where dissection of gills was carried out. The dissected tissue samples were fixed in Bouin or Carnoy fluids. For histochemical and immunohistochemical study, tissue samples were fixed in 4% paraformaldehyde buffered with 0.01 M sodium phosphate (PBS), pH 7.4, at 4˚C for 2 h and embedded in paraplast.

Serial sections (5 μm thick) were stained with Mallory mod. Ignesti (Carazzi hemalum, 1% acid fuchsin, Mallory's solution) and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Galgano I (Mayer's hemalum, 0.1% acid fuchsin, 1% phosphomolybdic acid, Mallory's solution) methods.

Neuraminidase enzymatic digestion was carried out by *Vibrio cholerae* neuraminidase (500U/ml) diluted in acetate buffer, pH 5.5, for 16-24 h at 39-41˚C. The conventional histochemical methods used in this study are reported in Table 1.

The following biotinylated lectins (SIGMA, St. Louis MO, USA) were tested: ConA, WPA, WGA, SBA, PHA-L. The lectins and their sugar specifities are shown in Table 2. Sections were incubated with the corresponding inhibitory sugar in control experiments to test the specificity of lectin binding.

To reveal nNOS, tissue sections were incubated overnight at 4˚C in a humid chamber with the polyclonal anti-human brain nNOS antibodies, diluted 1:200 (rabbit anti-nNOS type I antibodies, code 606-259-1550; Biomol, Hamburg, Germany). Afterwards, sections were washed in PBS and incubated for 1 h with a goat anti-rabbit IgG-peroxidase conjugate (1:100; Sigma Munich, Germany). Peroxidase activity was visualized by incubation of the sections for 5-12 min at room temperature in a solution of 0.015% 3,3'-diaminobenzidine in 0.01 M Tris buffer, pH 7.6, containing 0.005% H_2O_2. Negative controls included omission of the primary antibody or its substitution with non-immune rabbit serum.

Table 1. Conventional histochemical methods for visualization and identification of glycoconjugates (GCs)

Procedures	GCs revealed
PAS	Glycoproteins
AB pH 1	Proteoglycans with sulphate group
AB pH 2.5	Proteoglycans with carboxyl groups
AB pH 2.5/PAS	Proteoglycans with carboxyl groups and glycoproteins
Neuraminidase digestion	Sialic acid
AB (C.E.C.)	Sialic acid

Results

The gills of *Coelorhynchus coelorhynchus* are formed by four holobranchs, each consisting of two hemibranchs with primary filaments and secondary lamellae.

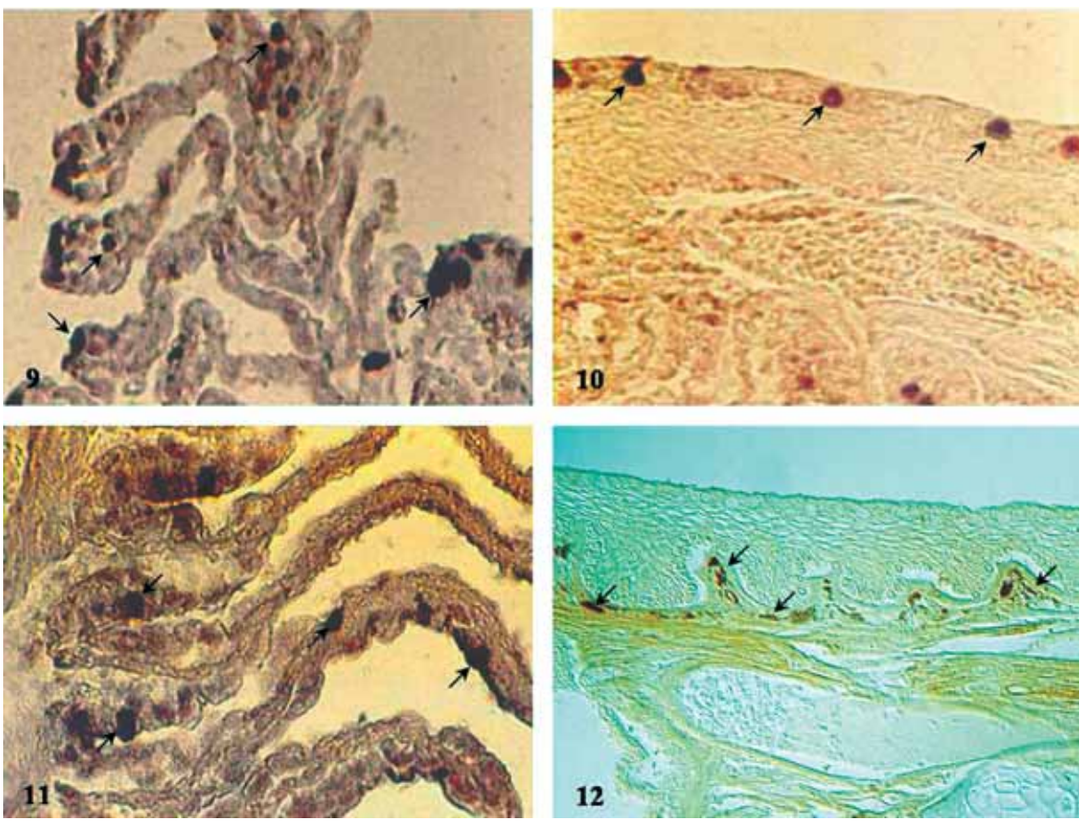

Fig. 1. PAS-positive mucous cells in primary filament of the abyssal fish *C. coelorhynchus*. × 1400. **Fig. 2.** AB/PAS pH 1-stained mucous cells in the primary filament of the branchial epithelia. × 1400. **Fig. 3.** Some AB-stained cells (arrows), after neuraminidase digestion, showing absence of sialic acid in the gill epithelia. × 900. **Figs. 4, 5.** Mucous cells in the epithelium of secondary lamellae positive to PHA-L **(Fig. 4)** and SBA **(Fig. 5)** lectins. Fig. 4: × 900; Fig. 5: × 1400. **Figs. 6, 7.** No labelling for ConA **(Fig. 6)** and WGA **(Fig. 7)** in the gill epithelia. × 750. **Fig. 8.** Some nNOS-positive neuroendocrine cells (arrows) in the gill epithelium. × 750. **Fig. 9.** In the epithelium of primary filament and secondary lamellae, nNOS-positive neuroendocrine cells (arrows) are seen. × 900. **Fig. 10.** nNOS-positive neuroendocrine cells (arrows) in the outer layer of gill epithelium. × 1400. **Fig. 11.** nNOS-positive neuroendocrine cells (arrows) at the base of secondary lamellae and in the primary filaments. × 1400. **Fig. 12.** nNOS-positive nerve fibers (arrows) innervating the gill epithelium can be seen in the connective tissue. × 1400.

Table 2. Lectins employed and their major binding specificities

Lectins	Origin	Carbohydrate specificity	Inhibitor sugar
ConA	*Canavalia ensiformis*	α-D-mannose, α-D-glucose	D-Mann
PHA-L	*Phaseolus vulgaris*	oligosaccharides	D-GalNAc
SBA	*Glycine max*	N-acetyl-D-galactosamine> D-galactose	D-galNAc
WPA	*Lotus tetragonolobus*	α-L fucose	L-fucose
WGA	*Triticum vulgaris*	N-acetyl-D-glucosamine> sialic acid	D-GlcNac

The gill filaments are vascularized by afferent and efferent arteries. The filament and lamellae epithelium contain pillar, chloride and mucous cells. After Mallory (mod. Ignesti) and Galgano I staining the cytoplasm of the pillar and chloride cells was red-orange. Chloride cells were found only in the primary filaments and in interlamellar zones. In the secondary lamellae, numerous capillaries, deriving from the afferent and efferent branchial arteries, were beneath the epithelium consisting of two layers of cells.

Mucous cells in the primary filament and secondary lamellae were PAS- (Fig. 1) and AB at pH 1-PAS-positive (Fig. 2). After C.E.C. staining at pH 5.8 and 0.2 M of $MgCl_2$, the alcianophylia decreased, confirming the presence of acid proteoglycans with carboxyl groups. Sialomucins were absent after acid hydrolysis according to Quintarelli *et al.* [19] and after neuraminidase digestion (Fig. 3), with and without saponification, followed by staining with AB at pH 2.5.

By lectin method, the mucous cells in the primary filaments and secondary lamellae were positive for PHA-L (Fig. 4) and SBA (Fig. 5) and negative for ConA (Fig. 6) and WPA. The cells that showed no staining with WGA indicated the absence of GlcNac and sialic acid (Fig. 7).

At the base of interlamellar space and in the epithelium of secondary lamellae, nNOS-positive neuroendocrine cells were found (Figs 8-10). In the subepithelial tissue of primary filaments, nNOS-positive nerve fibers originating from the branchial arch, were observed (Fig. 11). Controls for nNOS, after omission of the primary antibody, were negative.

Discussion

Our results have shown in the gill epithelium of *Coelorhynchus coelorhynchus* numerous glandular mucous cells with different chemical composition. In fact, the mucous cells showed glycoproteins and acid proteoglycans with sulphate or carboxyl groups. This is in accordance with results reported in *Anguilla anguilla* [4], in *Mugil cephalus* and in *Anoptichthys jordani* [22, 23]. Sialic acid absence was confirmed by neuraminidase digestion and WGA staining. The absence of glucose and mannose residues as well as sialic acid does not agree with previous observations regarding the chemical composition of mucus in the gills of pelagic fishes [9]. The mucus compounds differ in various teleost species according to their different living conditions. The different chemical composition of the mucus in the gills could be correlated with the abyssal life of C. *coelorhynchus.* The absence of sugar residues involved in oxygen reception, revealed by negative WPA and WGA results, does not support the oxygen diffusion.

In the freshwater fish, *Barbus filamentosus,* adapted to sea water, the excretion of NaCl was related to an increase in the number of chloride cells with Mg-dependent ATPase activity in the primary filaments [24]. In *C. coelorhynchus,* chloride cells are also present in clusters, as an excretory gland, in the primary filament. The NaCl excretion occurs through the vascular network in the subepithelial connective tissue of the primary filaments, as shown in other species [11]. With respect to pelagic fishes, osmoregulation in this abyssal species requires a greater abundance of cells involved in that activity. This was confirmed by the presence of nNOS-positive neuroendocrine cells in the primary filaments; such cells are involved in the movement of water and electrolytes as shown in other teleost species [30-32, 34, 35]. Some cells of the diffuse neuroendocrine system seem also to possess receptors for oxygen that mediate the cardiovascular and ventilatory response to oxygen deficiency, as shown in the catfish *Heteropneustes fossilis* [16]. In conditions of low oxygen exchange in fishes, the release of NO contributes, through nervous stimulation, to the regulation of vascular tone in the gills. As shown in higher vertebrates, vasoactive substances like NO control the pulmonary blood circulation [20].

In the subepidermal connective tissue, nNOS-positive nerve fibers were observed in proximity to the afferent arterial ramifications and in proximity to the neuroendocrine cells. In *H. fossilis* [15], nNOS-positive nerve fibers were associated with ramifications of the efferent arterioles and located in proximity to neuroendocrine cells at the base of the epithelium. The bioactive substances produced by cells of the diffuse neuroendocrine system (DNES) have important paracrine activities in osmoregulation and chemoreception of chemical agents. Neuroendocrine cells, in response to neuronal or

environmental stimuli, release NO by a paracrine mechanism for local regulation of muscle tone, as already suggested in the gills *of H. fossilis* [15].

The most important result of the present study is the demonstration of numerous neuroendocrine cells in the primary filaments and secondary lamellae and of chloride cells only in the primary filaments, which could be related to the abyssal habitat of this species. In fact, the DNES is involved in oxygen reception and the released NO regulates several functional activities including those of chloride cells, such as the excretion of salts.

This is the first data about the nitrinergic innervation and the DNES in the gills of an abyssal species. Further studies are necessary to clarify the role of NO and neuroendocrine cells and their involvement in the oxygen reception in abyssal fish.

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