Korean J. Ichthyol. 12(1), 25~32, 2000

Structure and Cytochemistry of Skin in Spined Loach, *Iksookimia longicorpus* (Pisces, Cobitidae)

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The structure of skin was studied in *Iksookimia longicorpus* based on the micro-anatomical investigation of skin fragments taken from four regions. The epidermis was distinguished by two types of skin glands, a small mucous cell and a large club cell. The mucous cell was acid sulfomucins (some sialomucins) but the club cell did not give any histochemical tests for mucosubstances. The presence of a well defined lymphatic system with small lymphocytes was established in the stratum germinativum layer of the epidermis. A large number of blood capillaries run very close to each other just below the basement membrane, and a definite area giving AB and PAS positive was present between the basement membrane and scale.

Key words : skin, acidic sulfomucins, mucous cell, club cell, cutaneous respiration, *Iksookimia longicorpus*

Introduction

Respiration of fishes is performed by gill using the dissolved oxygen in water. However, some of fishes under condition as warm and stagnant reservoirs, swamps or environments undergoing periodic drought are subjected to be a reduction of the dissolved oxygen. To avoid these respiratory problem it has been known that they use water by their gill as well as air by other respiratory apparatus, a dual respiration. The respiratory apparatus is to hold or receive air with special organs as intestine, skin, the branchial chambers, the swim bladder, the labyrinthine organ (Liem, 1967; Johansen, 1970; Niva *et al.*, 1981; Munshi and Hughes, 1986; Moitra *et al.*, 1989; Itazawa and Hanyu, 1991; Ishimatsu *et al.*, 1998; Park and Kim, 1999). Among the cobitid fishes *M. fossilis* and *M. anguillicaudatus* of the genus *Misgurnus* have been known as air-breathing fishes, cutaneous respiration (Jakubowski, 1958; Park and Kim, 1999).

The present work was to study the structure of the skin of a freshwater loach *Iksookimia longicorpus*, and special emphasis was placed on the cytochemical nature of skin and skin glands of this species.

Material and Methods

The observed 10 specimens were collected from the Naktong River, Korea and ranged from 76.5 mm to 102.4 mm in standard length. The specimens were fixed in 10% neutral buffered formaldehyde. The skin fragments were taken from four regions; the top of the head, and the dorsal, the lateral and the abdominal region.

These fragments were dehydrated through a standard ethanol series to 100%, cleared in xylene and then embedded in wax (Paraplast, Oxford). 5 μm sections were deparaffinized and stained with Harris's hematoxylin, iron alum hematoxylin, counter-stained with eosin, and Masson trichrome stain (Gurr, 1956) for general histology. For blood cell giemsa method was used.

Mucin of gland were demonstrated by alcian blue solution (AB) at pH 1.0 and 2.5 (Steedman, 1950; Lev and Spicer, 1964), and periodic acid-

Schiff (PAS) method with or without prior digestion with diastase/saliva (Lilllie and Greco, 1947). In addition, PAS technique was employed in combination with AB and vice-versa for neutral and acid mucins. Acid mucin was shown by metachromatic reactions with toluidine blue (Tock and Pearse, 1965). Also, Aldehyde fucshin and with AB (Spicer and Meyer, 1960), and high iron diamine (HID) and with AB (Spicer, 1965) were used for nature of the acid mucins.

Acetylation and deacetylation for the confirmation of hydroxyl group was performed following Lillie (1954), Methylation and demethylation (Spicer, 1960) were done to confirm the acidic nature of the mucins.

Results

The integument of the *Iksookimia longicorpus* could be divided into three principal layers: the epidermis, the dermis and the subcutis (Fig. 1A). The epidermis and the dermis are separated by a thin basement membrane.

1. Epidermis

The average thickness of the epidermis was the thickest in the lateral region (approximately 175 μm), and the thinnest at the top of the head (approximately 75 μm). The thickness of the dorsal and the abdominal regions was 113 μm and 138 μm, respectively (Table 1).

1) Outermost layer

This layer was composed of polygonal cells and few flattened cells, arranged in one to six rows of cells (Fig. 1A, B). In between these cell, small spherical or flask-shaped unicellular mucous glands were discernible. The mucous cell have a large spherical body and a short narrow neck that opens on surface by a wide pore. They have a basal, spherical or oval nucleus with a thin rim of slightly basophilic cytoplasm, pushed at the periphery of the cell due to the heavy accumulation of its basophilic secretions (Fig. 1A, B, E, and G). Its secretory matter was highly vacuolated and basophilic. A few lymphocytes were present between epidermal cells and mucous cells (Fig. 1C).

Table 1. A relative thickness of four skin regions of *Iksookimia longicorpus*, ranged 76.5~102.5 mm SL (n = 10)

Skin regions	Epidermis (μm)			Dermis (μm)		
	Mean	Minimum	Maximum	Mean	Minimum	Maximum
Top of head	75	73	78.5	77	69.5	79.5
Dorsal region	113	97.5	113.5	148	139.2	175.5
Lateral region	175	167.5	178	225	195	275
Ventral region	138	130	138.5	198	185.5	210.5

2) Middle layer

This layer was mainly composed of voluminous club cells and a few flask or spherical mucous cells (Fig. 1A-D). In between these skin glands the elongated spindle shaped epidermal cells were found vertically. The cell boundary of these cells was usually not clear as it greatly expanded or stretched out due to the collateral pressure of these glands. The club cells arranged in three to four layers were oval or spherical in shape (Fig. 1A-C). The club cells were more numerous and larger than the mucous cell. They were usually uninucleate, having an oval nucleus, sometimes binucleate having two nuclei very close to each other (Fig. 1B). Their cytoplasms were finely granular or more or less homogeneous in nature and invariably showed some shrinkage due to fixations. Some of the club cells had a few vacuoles in their cytoplasm (Fig. 1B and C). The main thickness of the epidermis is due to the middle layer, skin glands, particularly.

3) Stratum germinativum

This layer was composed of a single layer of cuboidal cells on a thin basement membrane which is PAS positive, AB positive, took green color in Masson trichrome and gave γ-metachromasia with toluidine blue. Each basal cell has a prominent, lightly stained, centrally placed spherical or oval nucleus, and their cytoplasm was homogeneous (Fig. 1A-C). The average height and the width of the basal cells were approximately 5.5 μm and 3.5 μm, respectively. We found the small oval or round lymphatic spaces in between the cuboidal cells (Fig. 1B-C). One or two small lymphocytes had deeply stained nuclei, surrounded by small mounts of faintly stained cytoplasm were invariably found inside these space (Fig. 1C). They were stained purple in AB, blue in PAS and giemsa staining. The lymphocytes penetrated the middle layer, often reaching up to the outer cell layers of the epidermis.

The pit organs, single or in groups of two to three were distributed on the surface of the skin (Fig. 1G). Each pit organ is a pear-shaped structure, sunk below the epithelial cells and lie direc-

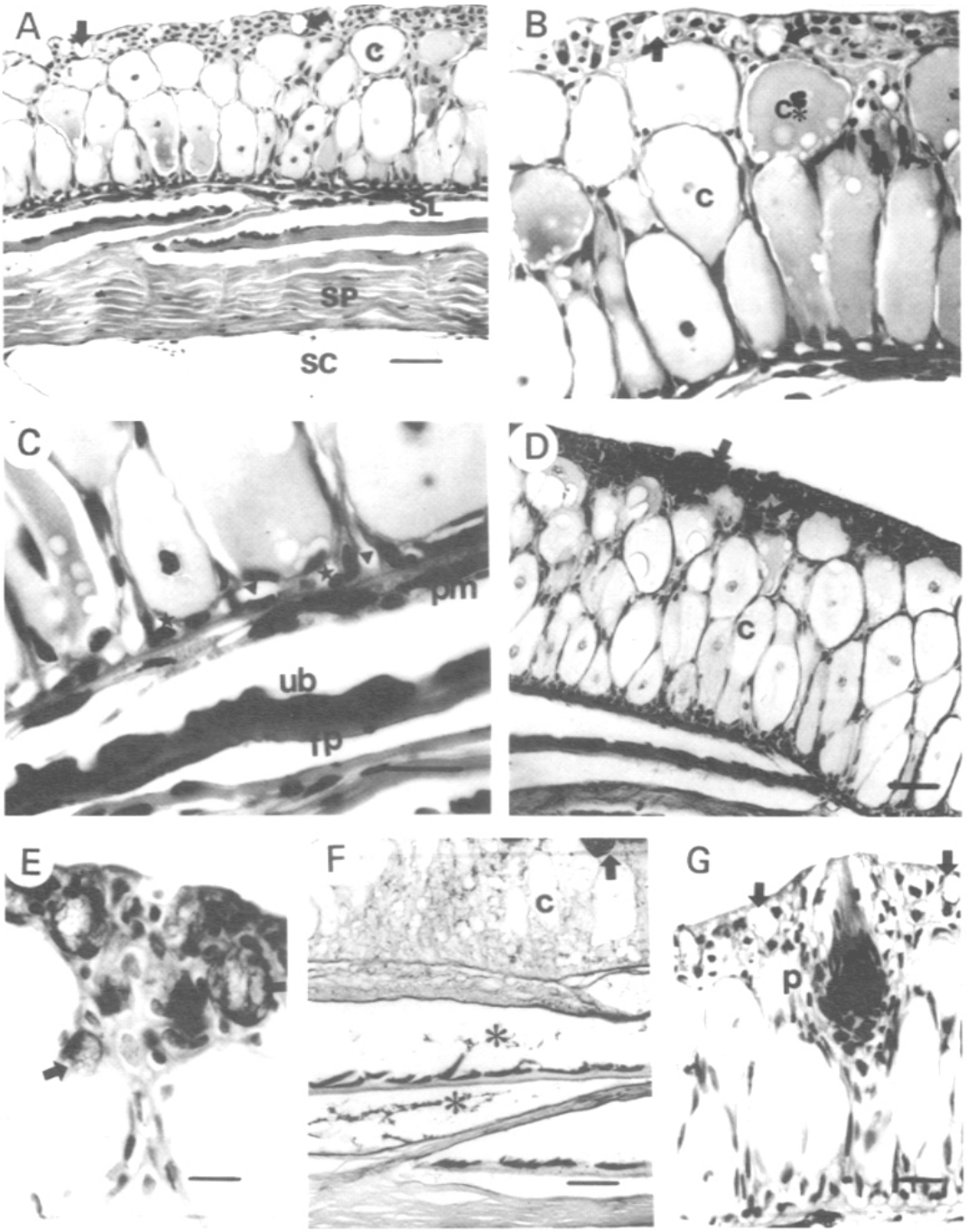

Fig. 1. Structure and cytochemistry of skin of *Iksookimia longicorpus*. (A) Cross section of dorsal skin showing the structure of the epidermis (arrows and c), dermis (SL and SP) and subcutis (SC) (Harris's hematoxylin). Bar = 25 μm. (B) Club cells of one (c) or two nuclei (c*), and flask or round shaped unicellular mucous cells (arrows) (Harris's hematoxylin). Bar = 25 μm. (C) Lympatic space (arrow heads) and lymphocytes (stars), structure of the scale (ub and fp) (Harris's hematoxylin). Bar = 5 μm. (D) Cytochemistry of mucous cells (arrows) and club cells (c) (AB-PAS reaction). Bar = 5 μm. E, Cytochemistry of mucous cells (arrows) (PAS technique). Bar = 10 μm. F, A definite area (*) (AB-PAS reaction). Bar = 25 μ. G, A pit organ (p) (Harris's hematoxylin). Bar = 10 μm. (**Abbreviations**: c, club cell; c*, binucleate club cell; fb, fibrillary plate (scale); p, pit organ; pm, pigment cells; sc, subcutis; SL, stratum laxum (dermis); SP, stratum compactum (dermis); ub, upper bony layer (scale); arrows, mucous cells; arrowheads, lymphatic spaces; stars (★), lymphocytes; asterisk (*), a definite areas).

tly on the basement membrane. In the intraepithelial layer there were blood vessels serving the sense organs of epidermis.

2. Dermis

The dermis consisted of a relatively thin upper layer of loose vascular connective tissue, stratum laxum, and a thick lower compact layer, stratum compactum (Fig. 1A)

1) Stratum laxum

This layer was well differentiated by the presence of a loose connective tissue in which blood capillaries abound and by the presence of a thin scale, which is lodged in pockets in the connective tissues (Fig. 1A-C). A loose connective tissue layer lying below the basement membrane was richly supplied with blood capillaries, which was connected with blood vessels of the stratum compactum and subcutis by capillaries running between the scale pockets. The loose connective tissue of the stratum laxum was mainly composed of a thin collagen bundle, stained green in Masson trichrome stained preparations. A layer of sparsely distributed pigment cells was found below the basement membranes, and also in the connective tissue pockets on the underside of the scales (Fig. 1A definite area was present between the basement membrane and the scales (Fig. 1F). The area was PAS positive and diastase resistant, AB positive, giemsa positive, and it gave γ-metachromasia with toluidine blue. With Masson trichrome stain they were negative. These materials were present in some of free space in which scales are lodged.

The scales of *I. longicorpus* consisted of two distinct layers-the upper bony layer and the inner fibrillary plate (Fig. 1C). However, the top of the head had no scale. The bony layer carried concentrically arranged ridges of striae on its free surface, while the lower fibrillary plate was mainly composed of parallel collagen fibers. The upper bony layers of the scale was basophilic in nature, and gave red colour with PAS technique, γ-metachromasia with toluidine blue, appeared blue in alcian blue and a deep green color in Masson trichrome stained preparations. The lower fibrillary plate was eosinophilic, weakly PAS positive, alcian blue negative and gave strong orthochromasia with toluidine blue. With the Masson trichrome stain preparations, it showed a green color.

2) Stratum compactum

This layer was characterized by the presence of bundles of coarse collagenous fiber arranged compactly in several layers (Fig. 1A). A few collagen fiber bundles ran vertically at intervals. This layer was weakly PAS positive and took a deep green color in Masson trichrome stained preparations for collagen. The thickness of the stratum compactum was approximately 88 μm. Between them there were a few pigment cells and blood capillaries. Pigment cells are distributed sparsely on the inner part of this layer

3. Subcutis

This was the innermost and thinnest layer of the skin and was situated in between the stratum compactum and the muscle (Fig. 1A). This layer was approximately average 135 μm. A large number of the nerves and blood vessels were found in this layer. In hematoxylin and eosin preparations this layer invariably shows numerous empty spaces which are occupied by fat cells (Fig. 1A).

4. Histochemistry of skin gland

1) Mucous cell

The flask-shaped or spherical unicellular glands were predominantly distributed in the outermost layer of the skin, but they were present sparsely in the middle layer. The mucous cells were various, reaching from 7~18 μm in height as described in the cells of the outermost layer. These cells gave a deep red color reaction with PAS technique, which is diastase resistant, γ-metachromasia with toluidine blue, blue with the AB at pH 1.0 and 2.5 (Table 2). The mucous cells, giving a red color with aldehyde fucshin and black color with high iron diamine, were likely to be sulfomucins. In mild methylation/AB and acetylation/PAS techniques they were negative, and in methylation /saponification-AB they were stained blue. Their nucleus were purple or red color with AB-PAS reaction and Masson trichrome stain.

2) Club cell

The club cells with oval or spherical shape were present only in the middle layer of the epidermis. Each club cell, showed various dimensions, was approximately 25~55 μm in height and was arranged in three to four rows of cells. With hamatoxylin/eosin stained preparations, the nuclei of club cells showed blue and the cyto-

Table 2. A summary of the histochemical tests performed to show the nature of skin glands in *Iksookimia longicorpus*

Types of glands	Haemato-xylin & eosin	Weigerts iron haematoxylin	Massons trich-rome	Alcian blue (pH 1.0)	Alcian blue (pH 2.5)	Toluidine blue	Periodic acid-Schiff (PAS)	Saliva /PAS	Diastase /PAS	AB-PAS	PAS-AB
Mucose cell	± B	+++ B	–	+ B	++ B	γ-meta	++ R	++ R	++ R	++ BR	++ RB
Club cell	+++ PN	–	++ G	–	–	ortho	–	–	–	–	–

Types of glands	Aldehyde fucshin	Aldehyde fucshin with AB	High iron diamine	High iron diamine with AB	Mild's Methylation followed by AB	Methylation followed by Saponification-AB	Acetylation followed by PAS
Mucose cell	+ R	++ BR	+ N	+++ BN	+ B	+ B	–
Club cell	–	–	–	–	–	–	–

B, blue; BN, bluish black; BR, bluish red; G, green; N, black; PN, pink; R, red; RB, redish blue; +, increasing intensity of reaction; ±, fairly present; –, absent.

plasm was stained light pink. However, they did not give any histochemical tests for mucosubstances (Fig. 1D-E; Table 2). The club cell took a greenish color with Masson trichrome staining and orthochromasia with toluidine blue.

Discussion

The cutaneous respiration has been known from several fishes as a air-breathing organ of dual breathing fishes (Jakubowski, 1958; Liem, 1967; Johansen, 1970; Mittal and Munshi, 1971; Mittal and Banerjee, 1974; Mittal *et al.*, 1980; Whitear, 1986; Park and Kim, 1999). In particular, the capacity of cutaneous respiration depends on the structure of the skin and the degree of its vascularization in relation to their habitats.

The skin of air breathing fishes has certain features such as a thicker epidermis due to a large glandular cell (*Misgurnus*, *Monopterus*), intraepithelial capillaries (*Periophthalmus*), and a well developed vascularization (*Heteropneustes*). These structures may be related with the amphibious habit of the fishes (Jakubowski, 1958; Liem, 1967; Johansen, 1970; Mittal and Munshi, 1971; Mittal and Banerjee, 1974; Whitear, 1986). The structure of skin of *Iksookimia longicorpus* was characterized by a thick epithelial layer having two types of glands, a small mucous cell and a large club cell. And it was a thin superficial layer consisting of 1 to 6 rows of cells, the localization of small scales inside the dermis, a well defined lymphatic system, a definite area giving AB and PAS positive, and a large number of blood capillaries just under the basement membrane. These results suggested that *Iksookimia longicorpus* are closely related to cutaneous respiration.

The epidermal thickness of *Iksookimia longicorpus* was thicker, approximately 75 to 175 μm, than other known air breathing fishes. In the thickness of the epidermis *M. fossilis* was 182 ~397 μm (Jakubowski, 1958), *M. anguillicaudatus* 169~255 μm, *Heteropneustes fossilis* 98 μm, *Mastacembelus pancalus* 44 μm, *Amphipnous cuchia* 119 μm (Mittal and Munshi, 1971), and *Monopterus albus* 75 μm (Liem, 1967) (Table 3).

The size and abundance of mucous cells plays an important role in supporting and maintaining the normal relationship of the cutaneous respiration. The stratum laxum layer of the dermis in *I. longicorpus* had a definite area with acid mucopolysaccharides. This area was found just under the basement membrane or present in some of free space in which scales were lodged. The presence of mucopolysaccharides in the stratum laxum was correlated with the semiterrestrial ecological habits of fish (Mittal and Munshi, 1971). Rogers (1961) reported that 1 g of mucopolysaccharides could bind or release 200 g or 500 g of water. Thus it was considered that the presence of mucopolysaccharides in the stratum laxum is adaptive modification for cutaneous respiration of *I. longicorpus*.

I. longicorpus had a vascularization system consisting of a pit organ and lymphatic space in the epidermis. A pit organ of a pear-shaped structure was present below the epithelial cells and lay directly on the basement membrane. Jakubowski (1958) described the pit organ as looplike vessels serving the sense organs of the epidermis in *M. fossilis* and considered that the vascularization of looplike vessels may be proof

Table 3. A summary of thickness of the dorsal epidermis in cutaneuous respiration fishes

Species	Epidermis (μm)			Literatures
	Average	Minimum	Maximum	
Misgurnus fossilis	182	–	–	Jakubowski (1958)
Misgurnus anguillicaudatus	162.8	–	–	Park and Kim (1999)
Monopterus albus	75	–	–	Liem (1967)
Notopterus notopteus	167	130	138.7	Mittal and Banerjee (1974)
Hereropneustes fossilis	98	64	130	Mittal and Munshi (1971)
Mastacembelus pancalus	44	7	39	Mittal and Munshi (1971)
Amphipnous cuchia	119	108	127	Mittal and Munshi (1971)
Iksookimia longicorpus	113	97.5	113.7	Present authors

–, no data

Table 4. A summary of histochemical nature and types of skin glands in air-breathing organ of dual breathing fishes

Species	Histochemical nature in Mucose cell	Literatures
Misgurnus anguillicaudatus	acid mucopolysaccharids	Park and Kim (1999)
Monopterus cuchia	strongly acidic sulphated mucopolysaccharides	Mittal *et al.* (1980)
Notopterus notopteus	acidic sulphated mucopolysaccharides	Mittal and Banerjee (1974)
Hereropneustes fossilis	weakly acidic sulphated mucopolysaccharides	Mittal and Munshi (1971)
Mastacembelus pancalus	strongly acidic sulphated mucopolysaccharides	Mittal and Munshi (1971)
Amphipnous cuchia	strongly acidic sulphated mucopolysaccharides	Mittal and Munshi (1971)
Iksookimia longicorpus	strongly acidic sulfomucins	Present authors

of air breathing. A well-defined lymphatic system with a series of lymph spaces containing small lymphocytes was present in the stratum germinativum layer of the epidermis. These lymphocytes penetrated intraepithelial layer. Such structures were not reported in *M. fossilis* (Jakubowski, 1958) but confirmed in *Misgurnus anguillicaudatus* (Park and Kim, 1999). The lymphatic system had functions of the supply of nutrition to the stratum germinativum for cell proliferation and to protect the epidermis from microorganisms or foreign proteins for protection of the epidermis (Mittal and Munshi, 1971).

As reported in *M. anguillicaudatus* by Park and Kim (1999), *I. longicorpus* had also small scales embedded in the superficial layer of the dermis. The small scales, rudimentary scales or absence of scales was found in a burrowing and a mud-dwelling fish (*Amphipnous*, *Monopterus*) and it was considered as an adaptation to its peculiar mode of life (Liem, 1967; Mittal and Munshi, 1971; Whitear, 1986).

The blood capillaries of skin are very important to air breathing fishes. Tamura *et al.* (1976) reported that *Periophthalmus cantonensis* have intraepithelial capillaries. In *M. fossilis*, *M. anguillicaudatus*, *Anguilla*, *Amphinous*, and *Monopterus* it was known that the blood vessels are present in the dermis (Jakubowski, 1958; Liem, 1967; Lethbridge and Potter, 1982; Park and Kim, 1999). In this case, diffusion of oxygen took place readily across the mucous coat of the epithelium though the blood vessels were situated at the deep dermis (Mittal and Munshi, 1971; Perry and McDonald, 1993).

The epidermis of *I. longicorpus* have two epidermal glands, the mucous cell and the club cell. As have been reported in other fishes, these skin glands contained a lot of water, and oxygen may easily penetrate them towards the deeper layers of the skin (Jakubowski, 1958; Mittal and Munshi, 1971). The Letterer (1959) and Rogers (1961) have reported that mucus substances had great ability to bind a large amount of water. Hora (1934) and Mittal *et al.* (1980) has shown that mucus has a remarkable power of precipitating mud held in suspension in water and that the mucus secreted by the skin in air breathing fishes may also be used in keeping the skin clear for respiration. As in Table 4, the nature of the mucous cell was acidic sulphated mucopolysaccharides (Mittal and Munshi, 1971; Mittal and Banerjee, 1974; Mittal *et al.*, 1980). The mucous cell of *M. anguillicaudatus* of family Cobitidae was acid mucopolysaccharides in nature (Park and Kim, 1999). In our histochemical observation

I. longicorpus was also acidic sulfomucins in nature of the mucous cell and the acidic mucosubstances seem to be characteristic of cutaneous respiration in dual breathing fishes.

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Received February 3, 2000
Accepted March 5, 2000

왕종개 *Iksookimia longicorpus* (Pisces, Cobitidae) 피부 구조와 세포화학적 특징

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왕종개 *Iksookimia longicorpus* 피부 및 피부샘(skin gland)의 구조와 그들의 세포화학적 특징을 조사하기 위해 등, 측면, 복부, 머리부분 등 4부분을 조사하였다. 왕종개의 표피(epidermis)는 작은 점액세포(mucous cell)와 커다란 club cell의 2 type의 gland가 분포하였다. 특히 mucous cell의 성분은 acidic sulfomucins이었으나 club cell은 점액물질(mucosubstance) test에 전혀 반응을 나타내지 않았다. 표피의 기저층(stratum germinativum)에는 림파구를 가지는 lymphocytic space가 잘 발달되어 있으며, 기저막(basement membrane) 바로 아래에는 많은 양의 모세혈관들이 서로 가깝게 분포하였다. 진피(dermis)의 stratum laxum에는 alcian blue와 PAS 반응에서 양성반응을 보이는 부분(a definite area)이 존재하였다.