The Effect of Different Salinity Levels on Chloride Cells of *Periophthalmus waltoni*'s Epidermis in Connection with $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ ion Transporter and $\text{Na}^+ / \text{K}^+ / 2\text{CL}$ Cotransporter using Immunohistochemistry Technique

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Research Article

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Abstract

Background: The $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ and $\text{Na}^+ / \text{K}^+ / 2\text{CL}$ cotransporter are two types of ions transporting proteins that are active in the secretion of chloride in bony fish. So, the level and activity of these transporting proteins are expected to increase in saline water. The aim of this study was to investigate the effect of different salinity levels on chloride cells of *Periophthalmus waltoni*'s epidermis in connection with $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ ion transporter and $\text{Na}^+ / \text{K}^+ / 2\text{CL}$ cotransporter using immunohistochemistry technique.

Results: Simultaneous localization of $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ and $\text{Na}^+ / \text{K}^+ / 2\text{CL}$ cotransporter showed that both were simultaneously present in epidermal ion cells and were able to react to different salinity levels.

Conclusion: The results of this study confirmed the model that states, $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ and $\text{Na}^+ / \text{K}^+ / 2\text{CL}$ cotransporters are responsible for the secretion of chloride from the chloride cells of bony fish.

Background

Fish's skin, along with other important organs such as kidneys and lungs, plays a key and vital role in regulating the entry and exit of ions such as ammonium in various environmental conditions [1]. Ion cells also play an important role in the regulation and entry / exit of ions in the fish skin. In bony fish, the ion cells are also commonly known as chloride cells or mitochondrial-rich cells. These cells are the main cells that regulate osmosis in fish, and are found more in the epidermis of marine fish, especially euryhaline species, than the epidermis of freshwater fish [2]. Under optical microscope, ion cells are larger than their adjacent epithelial cells and have an acidophilic cytoplasm with a nucleus located in the middle or near the base of the cell. These cells extend throughout the epidermis thickness, and the cell apical is in contact with the outside environment [3].

The cytoplasm of this cell has a high density of mitochondria. In saline-adapted fish, the apical plasma membrane is locally serrated and forms a small crypt filled with mucus, often broken into smaller units by blades or fine folds. Crypt size usually decreases in freshwater-adapted fish, but the fine folds may be more developed. The small tubes branching into the cytoplasm vaguely reach the blind terminals in the apical region, which contain numerous vesicles. Deep folds in the plasma membrane often appear in the basolateral region of the cell, communicating directly with the space inside the cell. The ion cells are connected to adjacent epithelial cells by blocked-taped connections. Ionic movements in these cells are performed by various enzymes, the most important of which are $\text{Na}^+ / \text{K}^+ / 2\text{CL}$ and $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ cotransporter [4].

All bony fish maintain an almost constant internal osmotic pressure, regardless of whether they are in sea water or fresh water [5]. NKA is present in the gills, kidneys and skin cells of bony fish. NKA in chloride cells has the highest known cell concentration, having more than 100 million molecules per cell whose

job is to absorb ions in freshwater and secrete salt in saline water [6]. NKA is most likely involved in the basolateral release of sodium from the chloride cell into the blood [7].

The transporting protein of NKCC cotransporter ion has been immunohistochemically located in the basolateral membrane of chloride cells of saltwater euryhaline fish [8]. The immunohistochemical localization of NKCC cotransporter in saltwater Salmon Smolts has also been investigated [9].

NKCC is essential for the secretion of NaCl in saltwater fish [10].

Results

There were no deaths during the study. The results showed that chloride cells were present in the different areas of *Priophthalmus Waltoni*'s skin epidermis and participated in ion exchange. In different groups, the immune response of $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ increased with a slight increase in salinity, but no significant difference was observed in the second group (BW). Chloride cells were multidimensionally stained for $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ in different layers of the epidermis and responded positively to this staining. The size, shape, and position of the cells that responded positively to $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ localization indicated that they were mitochondrial-rich chloride cells. Lack of $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ reaction was detectable in the nucleus and apical of the chloride cell. The staining pattern of basolateral section was not changed by salinity for $\text{Na}^+ / \text{K}^+ / \text{ATPase}$. Chloride cells of fish in all different salinity levels containing positive $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ were reported on the epidermis. The number of positive $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ chloride cells in the epidermis increased with increasing salinity and was higher compared to freshwater fish. The chloride cells of saltwater were larger than freshwater chloride cells (Figs. 1 and 3). Freshwater chloride cells were smoother and more elongated (Fig. 1). However, the overall shape of chloride cells in the epidermis was not significantly altered by different salinity levels (Figs. 1–3). The staining pattern for $\text{Na}^+ / \text{K}^+ / 2\text{CL}$ cotransporter was almost similar to that of $\text{Na}^+ / \text{K}^+ / \text{ATPase}$. All cells that were stained for $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ were simultaneously stained for $\text{Na}^+ / \text{K}^+ / 2\text{CL}$ cotransporter, and the distribution of $\text{Na}^+ / \text{K}^+ / 2\text{CL}$ cotransporter staining in chloride cells (staining throughout the cell except for the nucleus and most of the apical regions) was similar to $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ staining. The $\text{Na}^+ / \text{K}^+ / 2\text{CL}$ cotransporter stained areas of chloride cells was also equal to that of $\text{Na}^+ / \text{K}^+ / \text{ATPase}$. In other words, the number of $\text{Na}^+ / \text{K}^+ / 2\text{CL}$ - positive cotransporter chloride cells of the epidermis increased with increasing salinity, and also was more frequent compared to freshwater fish.

In the epidermis, only chloride cells were stained for $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ and NKCC, while other cells in the epidermis only had background staining (Fig. 1E). The distribution of $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ and the $\text{Na}^+ / \text{K}^+ / 2\text{CL}$ cotransporter was limited to basolateral membrane of chloride cells. In addition, increasing salinity led to an increase in the number and area of chloride cells. Areas of the cell that responded positively to the simultaneous immunity of $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ and $\text{Na}^+ / \text{K}^+ / 2\text{CL}$ cotransporter were calculated, which were positive for both proteins containing immunohistochemical stained ion (Table 1). In the

present study, the proposed model of chloride absorption and secretion in the chloride cells of the epidermis of mudskipper fish is presented in Fig. 4.

Table 1

Number and area of chloride cells; the third group of fish adapted to SW saline water show a significant difference in increasing the area of chloride cells. Mismatched letters show a significant difference ($p < 0.05$).

Groups	Mean number of chloride cells per 100 μm length of different areas of epidermis $\pm$ standard deviation	Mean area of chloride cells (in cubic micrometers) $\pm$ standard deviation
81.83 $\pm$ 5.43^b		
First group; PPT = 1 (FW)	1.83 $\pm$ 0.72	52.91 $\pm$ 6.55 ^a
Second group; PPT = 15 (BW)	2.08 $\pm$ 0.79	56.56 $\pm$ 9.47 ^a
Third group; PPT = 35 (SW)	2.50 $\pm$ 0.52	81.83 $\pm$ 5.43 ^b

The third group of fish adapted to SW saline water show a significant difference in increasing the area of chloride cells. Mismatched letters show a significant difference ($p < 0.05$).

Discussion

The functions of ion cells have been reviewed by various researchers. Usually, the function of ion cells in dehydration is to release ions into seawater, but in freshwater where fish are exposed to hydration from their surroundings, they are responsible for absorbing ions [11]. In Anadromous and Catadromous fish, the function of these cells temporarily changes from secretion to absorption and vice versa [2]. Ion cells have been shown to be responsible for the secretion of chlorine ions in seawater-adapted bony fish, whereas in freshwater bony fish, these cells are the place of calcium and chlorine ions flow into the epithelial tissue. Ion cells also play a separate role in the regulation of acid / alkali [11]. In Euryhaline species of bony fish, certain morphological changes occur in ionic cells during adaptation from freshwater to saline or seawater. In terms of properties, the number and size of these cells decrease during adaptation to seawater. Under special conditions where ion regulation in fresh water is imposed on fish, proliferation of these cells may also occur [12]. Ionic cells do not appear to be confined to bony fish, as even primitive fish, such as hagfish that have a highly permeable skin to water and as a result their blood plasma osmolarity is equivalent to that of seawater fish, have mitochondrial-rich cells in their gills that are morphologically similar to the ionic cells of bony fish. These cells have also been found in the gills and skin of lampreys that migrate between freshwater and marine environments, and the cartilaginous fish [11].

Ionic movements in these cells are performed by various enzymes, the most important of which are $\text{Na}^+ / \text{K}^+ / 2\text{Cl}^-$ and $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ cotransporter [4]. In other words, $\text{Na}^+ / \text{K}^+ / 2\text{Cl}^-$ and $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ cotransporter proteins are known as ion transporters [5].

The distribution of NKCC protein in chloride cells of freshwater-adapted Atlantic salmon is completely overlapped with $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ protein in the basolateral membrane [9].

These findings support the chloride secretion model by bone fish chloride cells, in which the presence of two ion transporting proteins of $\text{Na}^+ / \text{K}^+ / 2\text{Cl}^-$ and $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ cotransporter in the basolateral membrane of chloride cells as well as a CFTR chloride channel in the apical membrane of these cells, is shown.

The distribution of $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ and NKCC ion transporting proteins in the gills of Hawaiian goby was the same, so that except for the nucleus and apical of chloride cells, the rest of the chloride cells that include basolateral membranes responded positively to the simultaneous localization of these proteins [13].

NKCC is essential for the secretion of NaCl in saltwater fish, but its gene has not yet been cloned in bony fish.

Previous studies using electronic microscope have shown that $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ is widely present in the basolateral membrane but not in the most apical part of the cell [8].

Therefore, in the present study, high level of $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ and NKCC in chloride cells is likely to indicate their distribution in the basolateral membrane.

Marshall et al. (2002) recently found that NKCC immunity occurs in seawater-adopted fish throughout the chloride cell, but in freshwater-adopted fish it has a more limited distribution, which is restricted to the basolateral membrane [14].

Pelis et al. (2001) found that in seawater-adopted fish and freshwater-adopted fish, NKCC immunity occurred throughout the chloride cell, but in freshwater-adapted fish, the number of stained chloride cells and the NKCC immunity was lower [9].

In most bony fish adapted to saltwater, chloride cells appear to contain $\text{Na}^+ / \text{K}^+ / 2\text{Cl}^-$ and $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ ion transporting proteins in the basolateral membrane.

Cotransporter occurs in two important isoforms; secretory isoform (NKCC1) and adsorbent isoform (NKCC2). There is a tendency for NKCC immunity in the chloride cells of Hawaiian goby gills to be secretory [13]. In most tissues, the secretory form is found only in the basolateral membrane of chloride cells, while the absorbent form is found only in the apical membrane [13]. The only exception to this

general rule is the choroid plexus where both the NKCC1 and $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ are found in the apical membrane [15].

In the present study, the NKCC immunity throughout chloride cells indicates a basolateral distribution, which shows that this isoform is of secretory type. The large number of NKCCs in fish adapted to seawater indicates that the amount of this ion-carrying protein increases with the adaptation of fish to seawater, similar to the results found in other bony fish.

Wilson et al., hypothesized that $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ and NKCC may be involved in ammonia excretion by the gills of mudskipper through the replacement of NH_4^+ by K^+ [8].

In the model developed by Shigefumi Yokotu et al., for the secretion of Cl throughout the mudskipper skin, the presence of NKCC1 and $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ in the basolateral membrane and CFTR in the apical membrane of the skin chloride cells was implied [17].

Conclusions

The mitochondrial-rich cells are present in the different region of epidermis of *Periophthalmus waltoni*. Results of the present study show that $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ and $\text{Na}^+ / \text{K}^+ / 2\text{CL}$ cotransporters are attended in the mitochondrial-rich cells of *Periophthalmus waltoni*'s epidermis and participate in ion regulation. Number of the mitochondrial-rich cells, $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ and $\text{Na}^+ / \text{K}^+ / 2\text{CL}$ cotransporters are more in the fish adapted to sea water compared with lower salinities. Also, $\text{Na}^+ / \text{K}^+ / \text{ATPase}$ and $\text{Na}^+ / \text{K}^+ / 2\text{CL}$ cotransporters occupy a larger area of the mitochondrial-rich cells in the high salinities.

Methods

Fifteen adult fish with an average weight of 6.76 ± 0.42 g and an average length of 16.62 ± 1.10 cm were used in this study. Samples were obtained from the shores of Persian Gulf. Samples were purchased fresh and alive from local fishermen. After the usual measurements, the fish in 3 groups including group 1 with salinity of 1 PPT (FW group), group 2 with salinity of 15 PPT (BW group), and group 3 with salinity of 35 PPT (SW group) were adapted for 2 weeks. Samples were fixed in 4% paraformaldehyde for 24 hours. Slides obtained from different parts of the skin and epidermis were immunohistochemically studied. For immunohistochemical study, the slides were first washed in an acid and alcohol solution with a concentration of 70% HCl in 1% EtOH at 60° C for 15 minutes using an Ultrasonic Cleaner machine. They were then washed in water for 15 minutes and exposed to distilled water for another 15 minutes. They were dried gradually at 37° C for 24 hours and after 2 days, the slides were placed in a solution containing 245 ml of acetone and 5 ml of 3-aminoisoquinoline triethoxysilane. After placing the tissues on the coated slides, they were placed in xylol, and then in a decreasing concentration of alcohol, and finally in distilled water. The slides were then boiled in 0.05% Citraconic Anhydride solution for 30 minutes and were placed in distilled water for 10 minutes to cool down. Then, they were placed in an incubator at 37° C for 1 hour to dry. After that, the slides were immersed first in SDS solution for 5 minutes and then, in

TPBS solution for 5 to 10 minutes, after which 75 µl of buffer block was added to each section. Later on, the slides were placed in a damp room. Two different primary antibodies were added to each section. Rabbit αR1 antibody and mouse T4 antibody were used simultaneously on one section as the primary antibody. After doubling the primary antibody in each section, the slides were placed in a damp room and refrigerated overnight at 4° C. The next morning the slides were placed inside the TPBS.

Secondary antibody was added to all sections even in the control group. For this purpose, 50 µl of secondary antibody was added to each section. Blocking buffer was also used to dilute the secondary antibody. For every 500 µl of solution containing secondary antibody, 1 µl of Rabbit secondary antibody and 1 µl of mouse secondary antibody were used. After adding 50 µl of secondary antibody to all sections, they were incubated at 37° C (wet room) for 1 hour.

After that, the samples were placed in TPBS for 5 minutes, and then 60 ml of TPBS was mixed with 5 µl of DAPI and added to the sections. DAPI induced the molecular staining of nucleus, and was attached to genes within the nucleus.

Abbreviations

PPT: Parts per thousand; FW: Fresh water; BW: Brackish water; SW: Seawater; SDS: Sodium dodecyl sulfate; TPBS: Tween phosphate buffered saline; DAPI: 4',6-diamidino-2-phenylindole.

Declarations

Ethics approval and consent to participate

All procedures performed in this study involving animals were in accordance with the ethical standards and considerations of research ethics committee of Tehran university, Iran (Specific code: IR.UT.REC). Also the study was approved by the Ethics Committee with the approval number IR.UOZ.REC.1395.11 . Sampling of fishes was carried out with the permission and cooperation of the Khorramshahr University of Marine Sciences and Technology and the Veterinary Organization of Khozestan Province.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and analyzed during the current study are not publicly available due the confidentiality of their information but are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interest.

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Authors' contributions

H.M., A.K.H, M.B and K.E. designed the study. A.K.H and M.B analyzed and interpreted the data. H.M., M.A.F, and K.E. wrote and revised the paper.

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Figures

Figure 1

Immunohistochemical double labeling image of fish epidermis adapted to (FW) PPT = 1. A; the nucleus has blue staining, B; Na⁺ / K⁺ -ATPas has green staining, C; NKCC cotransporter has red staining, and D; epidermal background tissue that reacts negatively to immunohistochemical localization. Image E shows the merging of all 4 images in Figure 1.

Figure 2

Immunohistochemical double labeling image of fish epidermis adapted to (BW) PPT = 15. Image A; the nucleus has blue staining, image B; Na⁺ / K⁺ -ATPas has green staining, image C; NKCC cotransporter has red staining, and image D; epidermal background tissue that reacts negatively to immunohistochemical localization. Image E shows the merging of all 4 images in Figure 2.

Figure 3

Immunohistochemical double labeling image of fish epidermis adapted to (SW) PPT = 35. Image A; the nucleus has blue staining, image B; Na⁺ / K⁺ -ATPas has green staining, image C; NKCC cotransporter has red staining, and image D; epidermal background tissue that reacts negatively to immunohistochemical localization. Image E shows the merging of all 4 images in Figure 3.

Figure 4

Proposed model of chloride absorption and secretion in epidermal chloride cells of mudskipper fish. 1. NKCC transporter pump - 2. NkATPase pump - 3. CFTR anion channel.