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Research Article

Effect of salinity on the epidermal chloride cells of *Periophthalmus waltoni*: Immunohistochemistry characterization of Na^+-K^+-ATPase pump and Na^+-K^+-$2Cl^-$ cotransporter

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Abstract

The Na^+-K^+-ATPase (NKA) and Na^+-K^+-$2Cl^-$ cotransporter (NKCC) are the major ionic transporting proteins that regulate the secretion of chloride in marine bony fish. Their expression and activity are expected to increase with salinity. This study aimed to investigate the effects of salinity on the epidermal chloride cells of *Periophthalmus waltoni* to elucidate NKA and NKCC functions using immunohistochemistry method. For this purpose, 15 mudskippers (weight: 16.76±0.42 g, length: 16.62±1.10 cm, and water salinity: 38 g/L) that were collected from Mahshahr City were used. The fish were divided into three groups: FW group (with salinity of 1 ppt), BW group (15 ppt), and SW group (35 ppt) and treatment for two weeks. At the end of the experiment, the fish were collected, euthanized, and fixed in 4% paraformaldehyde for 24 h. Using specific antibodies, NKA and NKCC were localized in epidermal ion cells, and they were able to react to different salinity levels. The results of this study confirmed the model that states, NKA, and NKCC are responsible for the secretion of chloride from the chloride cells of bony fish.

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Introduction

The Walton's mudskipper, *Periophthalmus waltoni*, is one of the amphibians of the Persian Gulf and has a gray body and dark spots. Its average size in the rivers leading to the sea is reported to be 15 cm. This species is found in abundance in Iran and the surrounding areas on the banks of the rivers of the Persian Gulf and all over the coasts of the Persian Gulf. The banks of rivers that have a sandy beach with mud are a good place for this aquatic life, they prefer muddy waters. On the bank of the river, they distance themselves from the water to a distance of about five meters. The water temperature in the habitats of this fish reaches 38 ˚C and lives easily in a salinity of 36.6 to 40 g/L (Esfandiyari *et al*., 2022).

In fish skin, along with other organs such as kidneys and lungs, plays a key and vital role in regulating ionic exchange such as ammonium in various environmental conditions (Ostaszewska *et al*., 2016). Ionocytes commonly known as chloride cells or mitochondrial-rich cells functions regulate ionic exchange in the fish skin. These osmoregulatory cells are located more in the epidermis of marine fish, than the epidermis of freshwater fish (Hawkes, 1974; Glover *et al*., 2013). Under an optical microscope, the ioncytes 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 (Rakers *et al*., 2010).

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 ionocyte are connected to adjacent epithelial cells by blocked-taped connections. Various pumps and transporters have been identified that regulate exchanges of ions across ionocytes including $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and Na^+/K^+-ATPase (NKA) cotransporter (Sakuragui *et al*., 2003).

All bony fish maintain an almost constant internal osmotic pressure, regardless of whether they are in seawater or freshwater (Evans *et al*., 2005). The NKAs are present in the gills, kidneys, and skin cells of bony fish to absorb ions in freshwater species and secrete salt in marine species (Little *et al*., 2020). It is involved in the basolateral release of sodium from the chloride cell into the blood (Foskett and Scheffey, 1982).

The NKCC cotransporter has been immunohistochemically localized in the basolateral membrane of chloride cells in marine or species saltwater euryhaline fish (Wilson *et al*., 2000). It is essential for the secretion of Na^+Cl^- in saltwater fish (Pelis *et al*., 2001; Marshall, 2011).

The aim of this study was to investigate the effects of salinity on the epidermal chloride cells of *P. waltoni* to elucidate NKA and

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NKCC functions using immunohistochemistry method.

Materials and methods

Fifteen adult *P. waltoni* with an average weight of 16.76±0.42 g and an average length of 16.62±1.10 cm were used in this study. They were purchased from local fishermen in Mahshahr city located by the Persian Gulf (in the summer of 2014) and were subjected to research according to the ethics of research approved by the University of Tehran. physicochemical parameters of sampling location were salinity 37.2 psu; temperature 38˚C; dissolved oxygen 8.4 mg/L and pH 7.3. The fish were divided into 3 groups and acclimatized to a salinity of 1 ppt (parts per thousand) (freshwater group [FW]), 15 ppt (brackish water group [BW], and 35 ppt (seawater group [SW]) for 2 weeks. Each group included five fish. The treatment period was performed in the Faculty of Veterinary Medicine anatomy hall (Bu-Ali Sina University). The fish were kept in 30-liter aquariums. The characteristics of the water were checked every 24 hours, and if the physicochemical properties of the water changed, the water was replaced. Treatment period, the animals feed on the mud at the bottom of the aquarium. All procedures performed in this study involving animals were in accordance with the ethical standards and considerations of the veterinary faculty of Tehran University, Iran, at which the study was conducted.

At the end of the experiment, animals were euthanized and tissue samples were collected from dorsal, ventral, anterior, and posterior areas and fixed in 4% paraformaldehyde for 24 hours. Slides obtained from different parts of the skin and epidermis were histochemistry (alcian blue (AB) and periodic acid–schiff (PAS) stain) and immunohistochemically studied. For the immunohistochemical study, the slides were first washed in an acid and alcohol solutions with a concentration of 70% HCl in 1% EtOH at 60°C for 15 min using an Ultrasonic Cleaner machine. They were then washed in water for 15 min and exposed to distilled water for another 15 min. They were dried gradually at 37°C for 24 h. The slides were immersed 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, decreasing the concentration of alcohol, and finally in distilled water. The slides were then boiled in 0.05% citraconic anhydride solution for 30 min and were placed in distilled water for 10 min to cool down. Then, they were placed in an incubator at 37°C for an h. The slides were immersed first in SDS (sodium dodecyl sulfate) solution for five min and then, in TPBS (tween phosphate buffered saline) solution for 5 to 10 min, 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. Rabbit polyclonal antisera directed against 17 amino acids from a highly conserved region of the α-subunit of salmon Na^+/K^+-ATPase (diluted 1:500) was used for immunocytochemical detection of Na^+/K^+-ATPase. A mouse monoclonal antibody (T4) directed against

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the 310 amino acids at the carboxyl terminus of the human colonic $Na^+/K^+/2Cl–$ cotransporter (Wilson *et al*., 2000).

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. A 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 (humidified chamber) for an hour. After that, the samples were placed in TPBS for 5 min and then mixed DAPI (4′,6-diamidino-2-phenylindole) (60 mL of TPBS with 5 µL of DAPI), which was used for the molecular staining of the nucleus (Goncalves *et al*., 2016). SPSS V.21 Software was used for data analysis. The distribution of data was controlled by K-S test and since the distribution of all data was normal, parametric tests were used for data analyses. A one-way ANOVA test was used to compare two groups and a t-test to compare several groups with each other. The Tukey test was used followed by ANOVA test. Results were shown as average±SD (standard deviation) with a minimum significance $p<0.05$.

Results

There were no deaths during the study. The histological structure of *P. Waltoni's* skin using AB staining and PAS staining is shown in Figure 1.

Dorsal skin

At dorsal regions, of the skin included the hypodermis layer (Hypodermis), which was made up of loose connective tissue containing connective cells that connected the skin to the body's muscles. The dermis layer was visible in the middle part, which was distinguished by a positive PAS color. The upper layer was the epidermis, which was made up of epidermal cells. The epidermis had the outermost layer, the middle layer, and the inner layer called stratum germinativum. The mucosal cells in the outer layer were distinct because the mucus inside was purple and had reacted positively to both PAS-induced red and blue of Alsian-blue. The mucosal cells were squamous, unlike other aquatics which these cells are goblet shape in them. The middle layer of the epidermis contained swollen cells that shrank slightly in tissue sections due to cellular connections and became multifaceted. The swollen cells were free of mucus and did not respond positively to AB+PAS staining. The inner layer of the epidermis, stratum germinativum, contained basal cells and lymphocytes. The basal cells in this layer were a row of cuboidal cells that were arranged in a certain order on the basement membrane (Fig. 1A). In the histomorphometric study of the epidermal thickness of the dorsal skin, BW and SW groups had a significant decrease compared to the FW group ($p<0.05$). Also, examining the thickness of the middle layer showed that there was a significant decrease in BW group compared to the other groups

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(p<0.05). However, there was no significant difference in the thickness of the outermost layer (Table 1).

Table 1: *Periophthalmus waltoni* skin different part epidermis thickness adapted to FW, BW and SW.

Area	Groups	Epidermis thickness ±SD	Middle layer thickness ±SD	Outermost layer ±SD
Dorsal area	FW group; ppt = 1	84.03± 5.26^{a}	67.11± 4.98^{a}	17.10± 2.78
	BW group; ppt = 15	54.97± 7.98^{b}	37.96± 3.14^{b}	16.24± 2.96
	SW group; ppt = 35	60.06± 7.83^{b}	60.08± 5.62^{a}	21.02± 2.16
Ventral area	FW group; ppt = 1	66.02± 3.64^{a}	53.09± 3.64^{a}	15.04± 0.84^{a}
	BW group; ppt = 15	46.11± 3.08^{b}	35.18± 3.78^{b}	12.37± 0.64^{b}
	SW group; ppt = 35	56.13± 2.74^{c}	36.45± 4.09^{b}	13.08± 0.45^{b}
Anterior area	FW group; ppt = 1	46.21± 2.65^{a}	34.65± 1.47^{a}	12.30± 0.96^{a}
	BW group; ppt = 15	38.06±3.01^{b}	28.15± 1.56^{b}	9.05± 0.88^{b}
	SW group; ppt = 35	39.11± 2.28^{b}	30.61± 1.08^{b}	10.46± 0.57^{b}
Posterior area	FW group; ppt = 1	42.07± 2.36^{a}	28.46± 1.56^{a}	13.01± 0.84^{a}
	BW group; ppt = 15	34.19± 3.49^{b}	24.36± 0.9^{b}	9.70± 0.75^{b}
	SW group; ppt = 35	36.25± 2.06^{b}	26.78± 1.23^{b}	10.98± 1.03^{b}

Mismatched letters show a significant difference (p<0.05).

Ventral skin

At the ventral region, the dermis layer contained scales. These scales were not observed in some parts of the skin of *P. waltoni* (Fig. 1A and C). The epidermis had the outermost layer, the middle layer, and the inner layer named stratum germinativum. The basal cells in the stratum germinativum layer were sometimes present among scales and their divisions produced upper cells of the epidermis. The middle layer of the epidermis contained swollen cells. The mucosal cells with dark and elongated nuclei were squared in contact with the outside environment and more interestingly, large blood capillaries were seen in parts of the outer layer of the epidermis (Fig. 1B). In the histomorphometric study of the epidermal layer thickness in the ventral skin, the lowest amount was related to the BW group (p<0.05). Also, in the examination of the middle and outermost layer thickness, the BW and SW groups had a significant decrease compared to the FW group (p<0.05) (Table 1).

Anterior skin

At the anterior regions of the skin, the dermis layer contained scales. The inner layer of the epidermis, stratum germinativum, contained basal cells and small blood capillaries (double arrow), which were also found in the outer layer of the epidermis. The middle layer of the epidermis contained swollen cells that lacked mucus and did not respond positively to PAS staining. The outermost layer contained mucus-secreting cells, inside which the mucus reacted positively to PAS staining and was observed to be red (Fig. 1D). Also, the skin different part thicknesses adapted to FW, BW, and SW in

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this fish were inserted in Table 1. In the histomorphometric study of the epidermis, middle and outermost layers thickness in the anterior area skin, the BW and SW groups had a significant decrease compared to the FW group (p<0.05) (Table 1).

Posterior skin

At the tail regions of the skin, the extensive loose connective tissue (hypodermis layer) contained connective cells and blood vessels. These small blood vessels were also seen in the outer layer of the epidermis (double arrow). The dermis layer had no scales and its dense connective tissue was recognizable. The middle layer of the epidermis contained swollen cells. Basal cells in the SG layer consisted of a row of cuboidal cells and were located on the basement membrane. The mucosal cells with a heterochromatin and elongated nuclei were visible squamous (Fig. 1C). In the histomorphometric study of the epidermis, middle, and outermost layers thickness in the posterior area skin, the BW and SW groups had a significant decrease compared to the FW group (p<0.05) (Table 1).

Number and area of chloride cells

while mean the number of chloride cells counting in whole of the body skin, there was no significant difference between the groups, but the area of these cells showed a significant increase in the SW group (p<0.05) (Table 2).

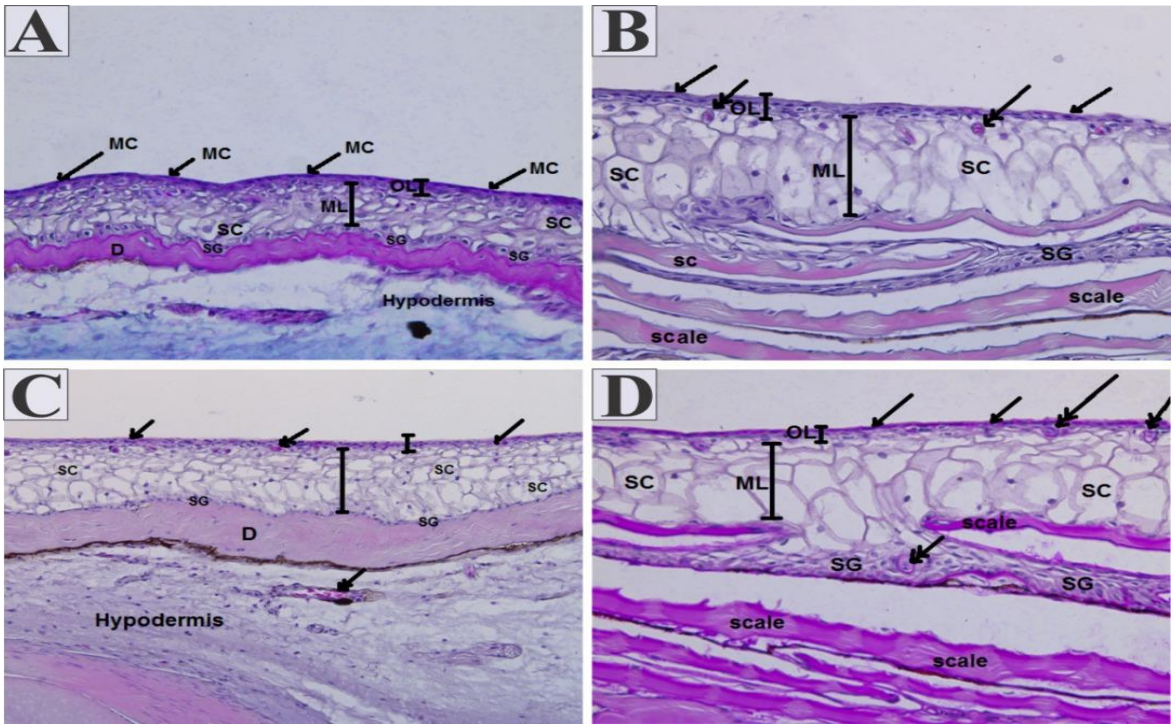

Figure 1: A. Skin of dorsal regions of *Periophthalmus waltoni*. The dermis layer (D), outermost layer (OL), the middle layer (ML), stratum germinativum (SG), mucusal cells (MC), swollen cells (SC), basal cells (BC) and lymphocytes (LM). AB+PAS staining, ×40. B: Skin of ventral regions of *P. waltoni*. The dermis layer (D), outermost layer (OL), the middle layer (ML), stratum germinativum (SG), basal cells (BC), swollen cells (SC), mucusal cells (arrow) and large blood capillaries (double arrow). H&E staining, ×40. C: Skin of tail regions of *Periophthalmus waltoni*. The blood vessels (double arrow), outer layer of the epidermis (double arrow), dermis layer (D), middle layer of the epidermis (ML) contained swollen cells (SC), Basal cells (BC), stratum germinativum (SG) and mucosal cells (arrow). H&E staining, ×40. D: Skin of anterior regions of *P. waltoni*. The dermis layer (D), stratum germinativum (SG), basal cells (BC), small blood capillaries (double arrow), middle layer of the epidermis (ML), swollen cells (SC) and outermost layer (OL). PAS staining, ×40.

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Table 2: Number and area of chloride cells.

Groups	Mean number of chloride cells per 100 µm length of different areas of epidermis ± standard deviation	Mean area of chloride cells (in cubic micrometers)±standard deviation
FW group; ppt = 1	1.83± 0.72	52.91± 6.55[a]
BW group; ppt = 15	2.08± 0.79	56.56± 9.47[a]
SW group; ppt = 35	2.50± 0.52	81.83± 5.43[b]

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

Immunohistochemistry results

The immunohistochemically results showed that chloride cells were present in the different areas of *P. Waltoni's* skin epidermis and participated in ion exchange. In different groups, the immune response of NKA increased with a slight increase in salinity, but no significant difference was observed in the BW Compared to FW. Chloride cells were multidimensionally stained for NKA in different layers of the epidermis and responded positively to this staining. The size, shape, and position of the cells that responded positively to NKA localization indicated that they were mitochondrial-rich chloride cells. The lack of NKA reaction was detectable in the nucleus and apical of the chloride cell. The staining pattern of the basolateral section was not changed by salinity for NKA. In all fishes, on different salinity, NKA was reported on the epidermis chloride cells. The number of positive NKA chloride cells in the epidermis increased with increasing salinity and was higher in SW than FW. The chloride cells of SW were larger than those of FW. Freshwater chloride cells were smoother and more elongated. However, the overall shape of chloride cells in the epidermis was not significantly altered by different salinity levels (Figs. 2 to 4). The staining pattern for NKCC was almost similar to that of NKA.

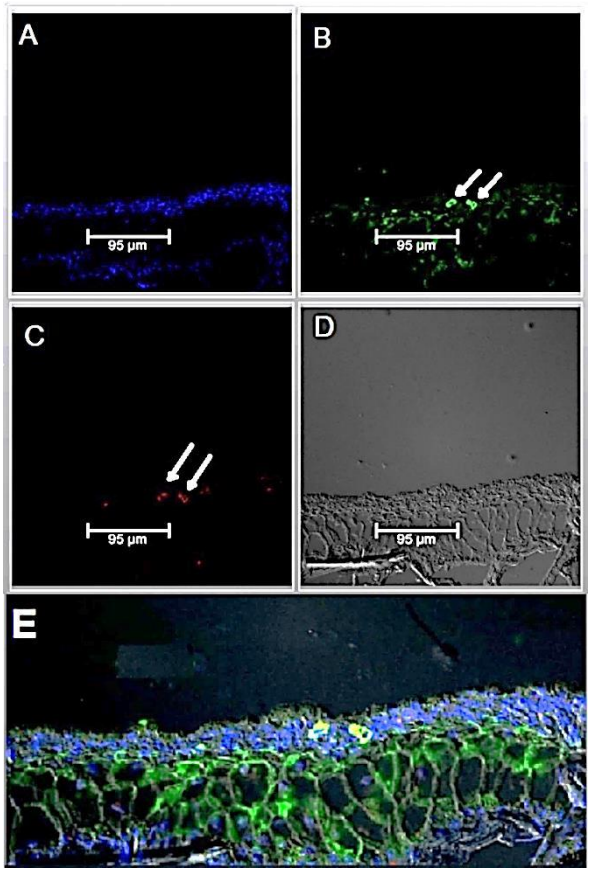

Figure 2: Immunohistochemical double labeling image of fish epidermis adapted to (FW) ppt = 1. A; the nucleus has blue staining, B; NKA has green staining, C; NKCC cotransporter has red staining, and D; epidermal background tissue that reacts negatively to immunohistochemical localization. Image E shows the merging of all 4 images in Figure 2.

All cells that were stained for NKA were simultaneously stained NKCC cotransporter, and the distribution of NKCC staining in chloride cells (staining throughout the cell except for the nucleus and most of the apical regions was similar to NKA staining.

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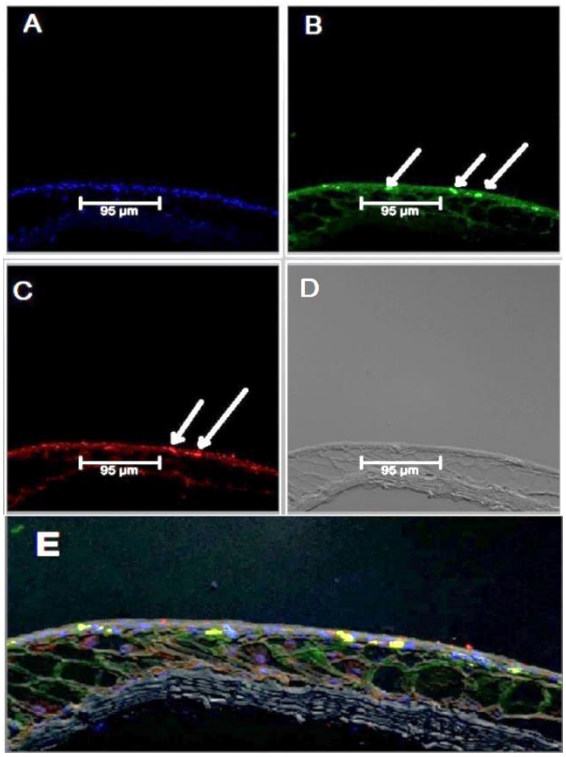

Figure 3: Immunohistochemical double labeling image of fish epidermis adapted to (BW) ppt = 15. Image A; the nucleus has blue staining, image B; NKA has green staining, image C; NKCC cotransporter has red staining, and image D; epidermal background tissue that reacts negatively to immunohistochemical localization. Image E shows the merging of all 4 images in Figure 3.

The NKCC stained areas of chloride cells were also equal to those in NKA. In other words, the number of NKCC-positive chloride cells in the epidermis increased with increasing salinity and also was more frequent compared to freshwater fish. In the epidermis, only chloride cells were stained for NKA and NKCC, while other cells in the epidermis only had background staining (Fig. 2E). The distribution of NKA and the NKCC was limited to the basolateral membrane of chloride cells. In addition, increasing salinity led to an increase in the number and area of chloride cells.

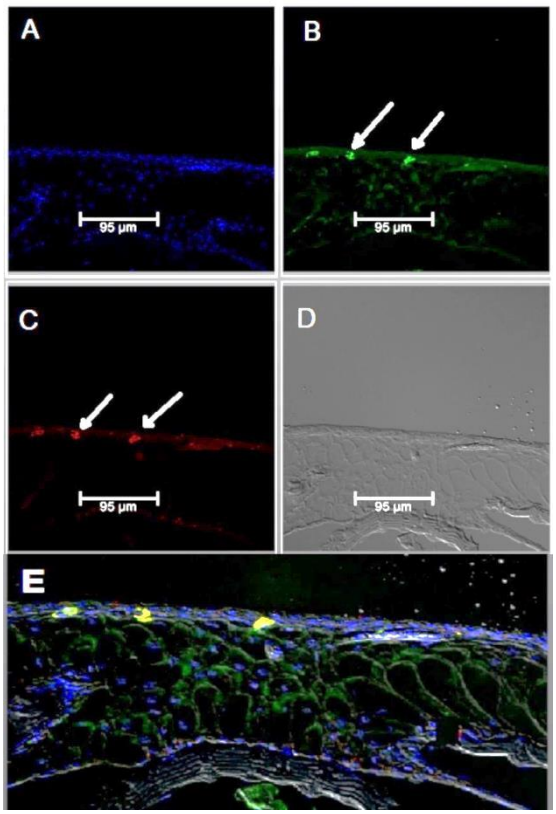

Figure 4: Immunohistochemical double labeling image of fish epidermis adapted to (SW) ppt = 35. Image A; the nucleus has blue staining, image B; NKA has green staining, image C; NKCC cotransporter has red staining, and image D; epidermal background tissue that reacts negatively to immunohistochemical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

Areas of the cell that responded positively to the simultaneous immunity of NKA and NKCC were calculated, which were positive for both proteins containing immunohistochemical stained ions (Table 2).

In the present study, the proposed model of chloride absorption and secretion in the chloride cells in the epidermis of mudskipper fish is presented in Figure 5.

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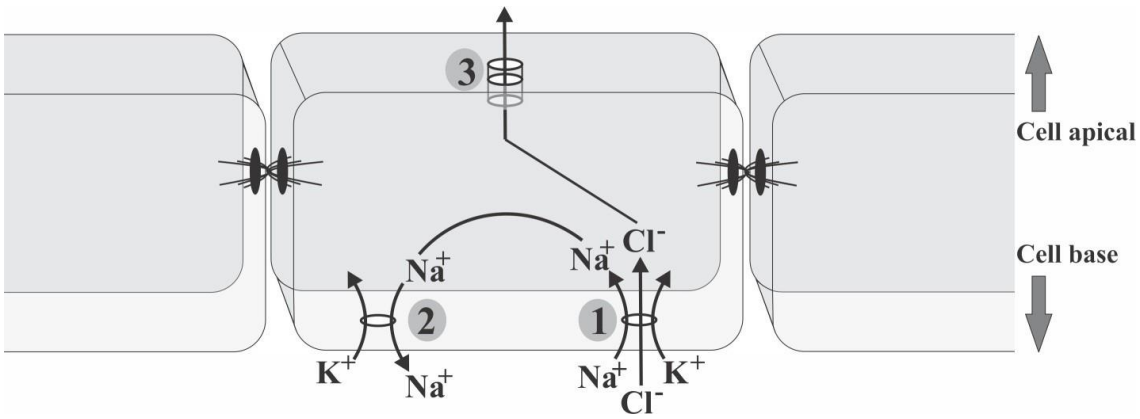

Figure 5: Proposed model of chloride absorption and secretion in epidermal chloride cells of mudskipper fish. 1. NKCC transporter pump - 2. NKATPase pump - 3. CFTR anion channel.

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 (Balment *et al*., 2006). In Anadromous and Catadromous fish, the function of these cells temporarily changes from secretion to absorption and vice versa (Hawkes, 1974). 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 (Balment *et al*., 2006; Esfandiyari *et al*., 2022). 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 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 (Goncalves *et al*., 2016).

Ionic movements in these cells are performed by various enzymes, the most important of which are NKCC and NKA cotransporter (Sakuragui *et al*., 2003; Evans *et al*., 2005). The distribution of NKCC protein in chloride cells of freshwater-adapted Atlantic salmon is completely overlapped with NKA protein in the basolateral membrane (Pelis *et al*., 2001).

The distribution of NKA and NKCC ion-transporting proteins in the gills of Hawaiian goby was the same, so except for the nucleus and apical of chloride cells, the rest of the chloride cells including basolateral membranes responded positively to the simultaneous localization of these proteins (McCormick *et al*., 2003).

NKCC is essential for the secretion of NaCl in saltwater fish, but its gene has not yet been cloned in bony fish. Previous studies have shown that NKA is widely present in the basolateral membrane but not in the most apical part of the cell based on electronic microscope images (Wilson *et al*., 2000; Mansourghanaei *et al*., 2022).

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Therefore, in the present study, a high level of NKA 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 and restricted to the basolateral membrane (Marshall *et al*., 2002). 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 (Pelis *et al*., 2001).

In most bony fish adapted to saltwater, chloride cells appear to contain NKCC and NKA 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. 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 (McCormick *et al*., 2003). The only exception to this general rule is the choroid plexus where both the NKCC1 and NKA are found in the apical membrane (Haas and Forbush, 1998).

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*. (2000) hypothesized that NKA and NKCC may be involved in ammonia excretion by the gills of mudskipper through the replacement of $NH4^+$ by K^+. In the model developed by Shigefumi Yokotu et al., for the secretion of Cl throughout the mudskipper skin, the presence of NKCC1 and NKA in the basolateral membrane and CFTR in the apical membrane of the skin chloride cells was implied (Glover and Goss, 2020).

Conclusions

It can be concluded that changes in water salinity can affect *P. waltoni* different skin layers thickness and the area of the chloride cells. The mitochondrial-rich cells are present in the different regions of the epidermis of *P. waltoni*. Results of the present study show that NKA and NKCC are attended in the mitochondrial-rich cells of *Periophthalmus waltoni*'s epidermis and participate in ion regulation. Number of the mitochondrial-rich cells, NKA, and NKCC are higher in fish adapted to seawater compared with lower salinities. Besides, NKA and NKCC occupy a larger area of the mitochondrial-rich cells in the high salinities.

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