Evaluation of the Role of Na^+/K^+ ATPase Ion Transporters and $\text{Na}^+/\text{K}^+/\text{2Cl}$ and Na^+/H^+ Exchanger Cotransporters in Nephrons of *Periophthalmus Waltoni* Using Immunohistochemistry and Histology

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Abstract

Kidneys play an important role in regulating the balance of water and ions in freshwater and seawater fish. However, complex kidney structures impair a comprehensive understanding of kidney function. In this study, in addition to renal histology, $\text{Na}^+/\text{K}^+/\text{ATPase}$ ion transporter proteins and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ and NHE3 cotransporters were located in *Priophthalmus waltoni* kidney tissue to evaluate the ion regulation abilities of epithelial cells in various parts of nephrons. The renal tubules are composed of proximal tubules and distal tubules, followed by collecting tubes and finally collecting ducts. Light microscope immunohistochemistry was utilized to locate $\text{Na}^+/\text{K}^+/\text{ATPase}$ along renal tubules and collecting ducts. However, the distribution of the $\text{Na}^+/\text{K}^+/\text{ATPase}$ immune response varies in different sections. $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransporter positioning was reported only in collecting tubes and collecting ducts, and proximal tubes and distal tubes did not respond to $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransporter immunolocalization. Immunohistochemical response for NHE3 localization was detected only at the apex of epithelial cells of proximal tubules and collecting tubes. The distal tubes showed negative reaction and the collecting ducts showed a weak response to NHE3 safety immunolocalization.

Introduction:

Ion balance in freshwater fish is mediated by ion retention and ion acquisition mechanisms throughout the ion-regulating epithelium. The urinary system in fish consists of a renal-corpuses, proximal tubules, distal tubules, collecting tubules and collecting ducts. While the mechanism of ion retention restricts the leakage of ions across the epithelium, ion acquisition compensates for ions lost by the epithelium. Studies examining the ion regulation role of epithelium in maintaining hydromineral homeostasis in fish have generally focused on intracellular mechanisms including ion pathways through which active ion transport occurs. In contrast, less emphasis has been placed on ion retention mechanisms that limit ion loss by cells. When identifying intercellular pathways, multifaceted assemblies of ion pumps, transducers, and channels linked with the apical or basolateral membrane of the cell are detected (Loretz 1995; Marshall 2002; Evans et al. 2005). Intercellular pathways controlled by tight junctions between two cells are not yet well understood in aquatic vertebrates. Proteins involved in the tight junctions of adjacent epithelial cells communicate with each other and restrict the movement of solutes among cells (Cereijido and Anderson 2001).

Na transmission through renal and intestine epithelium as well as body surface is a major system by which Na homeostasis is regulated in vertebrates. Little is known about the Na carriers responsible for Na homeostasis in *osteichthyes* and mammals.

NHE3 is located in the brush border (apical) of the intestinal epithelial membrane (jejunum, ileum, and colon) and in the renal tubules (proximal tubule and distal straight tubule of Henle's loop). NHE3 is found in the apical membrane of ionocytes in the gills of both freshwater and seawater fish. Since the gills are the major site of ion regulation in fish (Choe et al. 2005), NHE3 may facilitate Na uptake and H secretion

in fish gills. However, NHE3 is known as a Na/H exchanger that can leak intracellular Na intermediates into fresh ambient water (Esaki et al. 2007; Lin et al. 2008).

NHEs catalyze the electrical and neutral exchange of Na and H (1:1 ratio) in the corresponding concentration gradient (Hayashi et al. 2002). NHE2 and NHE3 are expressed in the apical membrane of gastrointestinal and kidney cells, where they can participate in the systematic uptake of Na and H secretion. In the kidney, NHE2 are found in the thick ascending limb of the loop of Henle, macula densa, distal convoluted tubules and the collecting tubules (Chambrey et al. 1998). NHE3 is found mainly in the proximal convoluted tubule and to a lesser extent in the thick ascending limb (Biemesderfer et al. 1996). In mammalian proximal renal tubules, apical NHE3 interacts with Na/K-ATPase and Na/HCO₃ cotransporter channels in a mechanism responsible for reabsorption of sodium and HCO₃ in the kidney (Vallon et al. 2000).

Material And Methods:

Fifteen adult *Periophthalmus waltoni* with an average weight of 6.76 ± 0.42 g and a length of 16.62 ± 1.10 cm were used in this study. Live samples were purchased from local fishermen of Mahshahr city located by the Persian Gulf and were subjected to research according to the ethics of research practiced by University of Tehran. Samples were fixed in 4% paraformaldehyde for 24 hours. Slides obtained from different parts of the skin and epidermis were sent for immunohistochemical studies. For this study, the slides were first washed using an Ultrasonic Cleaner machine in acid and alcohol solution with a concentration of 70% HCl and 1% EtOH at 60 ° C for 15 minutes. They were then washed in water for 15 minutes and soaked in distilled water for another 15 minutes. The samples were then left to dry gradually at a temperature of 37 degrees for 1 day and night. After 2 days, the slides were immersed in a solution containing 245 ml of acetone and 5 ml of 3-aminoisoquinoline triethoxysilane. After placing tissue incisions on the coated slides, they were immersed in xylene and then in decreasing concentrations of alcohol ending up at 0% alcohol (distilled water). The samples were then boiled in 0.05% Citraconic Anhydride solution for 30 minutes before cooling in distilled water for 10 minutes and drying in an incubator at 37 ° C for 1 hour. The slides were then immersed in SDS solution for 5 minutes and then in TPBS solution for 5 to 10 minutes, after which 75 µl of blocking buffer was added to each section. The slides were then placed in a damp room. Two different primary antibodies were added to each section. Rabbit αR1 antibody and rat T4 antibody were used simultaneously on one section as the primary antibodies. After adding secondary antibodies to each section, the slides were kept at 4 ° C in a humidified chamber overnight. The next morning the slides were rehydrated in TPBS (Gonçalves et al. 2016).

Antibodies used in the current paper are as follows:

Primary Antibody	Target Enzyme	Animal Type	Dilution	Diluent	Secondary Antibody
α R1	$\text{Na}^+/\text{K}^+/\text{ATPase}$	Rabbit	1:500	Blocking buffer	Anti-Rabbit Secondary Antibody
α 5	$\text{Na}^+/\text{K}^+/\text{ATPase}$	Mouse	1:100	Blocking buffer	Anti- mouse Secondary Antibody
T4	$\text{Na}^+/\text{K}^+/\text{2Cl}$	Mouse	1:100	Blocking buffer	Anti- mouse Secondary Antibody
NHE3B	Na^+/H^+ Exchanger	Rabbit	1:200	Blocking buffer	Anti-Rabbit Secondary Antibody
Control Group					Anti-Rabbit Secondary Antibody

Secondary antibody (50 μl) was added to all sections including the control group. Blocking buffer was used to dilute the secondary antibody. For each 500 μl of solution containing secondary antibody, 1 μl of anti-rabbit secondary antibody and 1 μl of anti-mouse secondary antibody were used. After adding 50 μl of secondary antibody to all sections, they were incubated at 37 ° (humidified chamber) for 1 hour.

The samples were then placed in TPBS for 5 minutes. Then 60 ml of TPBS was mixed with 5 μl of DAPI and added to sections. DAPI, by binding to the genes, was used for molecular staining of the nucleus (Gonçalves et al. 2016).

Results:

Macroscopic studies showed that the kidney structures as two reddish-purple extraperitoneal tissues stretched below the spine and covering the roof of internal cavity. Loose connective tissue covered by a layer of mesothelial cells encapsulates the kidney. As we progress along the kidney from the head to the tail of fish, the number of malpighian bodies as well as proximal and distal convoluted tubules decreases while the number of collecting tubules and ducts increases. Each nephron is made up of the renal tubules and a malpighian body. The malpighian body and the Bowman's capsule are clearly visible. Bowman's capsule consists of a parietal layer which is a simple layer of simple squamous epithelium and a visceral layer of podocytes. Nucleated red blood cells can be identified in the renal sinusoids and hematopoietic parenchyma of the kidney. Proximal convoluted tubules, distal convoluted tubules, collecting tubules and collecting ducts can be respectively detected in nephron structure where in immunohistochemical studies each section shows different physiological properties regarding uptake and excretion of ions (Fig. 1).

Light microscope immunohistochemistry was utilized for locating $\text{Na}^+/\text{K}^+/\text{ATPase}$ along renal tubules and collecting tubules. However, the distribution of the $\text{Na}^+/\text{K}^+/\text{ATPase}$ immune response varies in different sections. Basal sections of proximal and distal convoluted tubules showed positive response to immunolocalization of $\text{Na}^+/\text{K}^+/\text{ATPase}$ while showing negative response in their apical sections. $\text{Na}^+/\text{K}^+/\text{ATPase}$

ATPase was present in all basolateral parts of collecting tubes and collecting ducts and reacted positively to Na⁺/K⁺-ATPase immunolocalization. Na⁺/K⁺-ATPase in the basal and basolateral portion of the epithelial cells in the renal tubule's exports 3 Na⁺ ions to the basal and interstitial tissue of the kidney while importing 2 K⁺ ions into the cell (Fig. 2).

The location of Na⁺/K⁺/2Cl⁻-cotransporter was recorded only in the basolateral part of epithelial cells with higher intensity in collecting tubules and lower intensity in collecting ducts (Fig. 3). This is while epithelial cells located in the proximal and distal tubules did not respond to the Na⁺/K⁺/2Cl⁻-cotransporter immunolocalization (Figure 4). This protein pumps Na⁺, K⁺ and Cl⁻ ions from the lumen into the epithelial cell (Fig. 4).

Immunohistochemical reaction for localization of Na⁺/H⁺/Exchanger was detected at the apex of epithelial cells of proximal tubules, collecting tubes and collecting ducts with very high, moderate and low intensities, respectively. Distal tubules did not respond to Na⁺/H⁺/Exchanger immunolocalization (Fig. 4). Na⁺/H⁺/Exchanger located at the apex of the cells mediates H⁺ export from cell to lumen in exchange for import of one Na⁺ ion into the epithelial cell.

Discussion:

Differential immunohistochemical staining of Na⁺/K⁺-ATPase have been carried out in different sections of Goldfish nephrons (Chasiotis et al. 2012). Differential Na⁺/K⁺-ATPase staining has also been carried out for different sections of nephrons of other fish species and basolateral localization of Na⁺/K⁺-ATPase has been reported for ion transports. In addition, specific patterns of distribution of Na⁺, K⁺-ATPase along the nephron have been reported for several other vertebrate groups (Piepenhagen et al. 1995; Kwon et al. 1998; Sturla et al. 2003). In Goldfish kidney, immunostaining response for Na⁺/K⁺-ATPase is primarily restricted to the basement membrane of renal epithelial cells in the proximal region of the nephron as well as the basolateral membrane of renal epithelial cells in the distal and collecting tubules (Chasiotis et al. 2012). In current study, expression of Na⁺/K⁺-ATPase was verified in all sections of nephron tubules.

Interestingly, in both *killifish* and *rainbow trout*, compatibility with freshwater or seawater environments does not cause immunohistochemical changes in the kidney at the microscopic level either for light or electron microscopy (Katoh et al. 2008). Two important NKCC isoforms, including the NKCC1 secretory form and the NKCC2 absorption form, can be found in vertebrates. In dogfish kidneys, however, the basolateral isoform (NKCC1) has been observed which is distributed in the proximal tubules while the NKCC2 isoform is observed apically from the proximal tubules to the collecting tubules (Biemesderfer et al. 1996). Secretory isoform (NKCC1) has also been found basolaterally in the large rectum of fish (Lytle et al. 1992). The anti-NKCC antibody used in the present study (T4) detects both secretory and absorption isoforms in a variety of animal tissues (Lytle et al. 1992). Therefore, in the present study, we cannot distinguish between these isoforms in different regions of the nephron. Katoh et al. Suggested that in the

rainbow trout kidney only NKCC2 is present and located apically, while in the *killifish* kidney NKCC2 isoform is present apically and the NKCC1 isoform is present basolaterally (Katoh et al. 2008).

The *killifish* kidney is divided into four regions in terms of ion transport: the first part of the proximal tubule, the second part of the proximal tubule, the distal tubule and the collecting tubule, each of which differs in terms of NKCC and Na^+/K^+ -ATPase function. NKCC is present basally in the first and second parts of the proximal tubule where they pump ions from interstitial tissue into epithelial cells, while in the distal and collecting tubules NKCC is present in the apical part of the cells and pumps ions from the lumen into the epithelial cells. Na^+/K^+ -ATPase is also present basolaterally in the second part of the proximal tubule as well as distal tubules and collecting tubules (Katoh et al. 2008). In the same study by Katoh et al. *rainbow trout* kidneys were examined for localization and function of NKCC and Na^+/K^+ -ATPase where Na^+/K^+ -ATPase was located in first and second part of proximal tubule, distal tubules and collecting tubules, although with higher intensities along the distal tubule compared to proximal tubule. NKCC was only reported apically in the distal and collecting tubules (Katoh et al. 2008). In the present study, detection of $\text{Na}^+/\text{K}^+/\text{2Cl}$ expression was limited to collecting tubules and collecting ducts with higher intensities in the latter.

NHE3 is located in the apical membrane ionocytes in the gills of freshwater fish and seawater fish (Choe et al. 2005). Immunohistochemically localization of NHE3 responded positively in the brush border of intestinal epithelium (jejunum, ileum, and colon) and in the renal tubules (proximal tubule and thick ascending limb) (Mahnensmith and Aronson 1985).

In a study carried out by Kato et al. on the expression of the third isoform of Na^+/H^+ exchanger in *Triakis scyllium*, the kidneys were divided into bundle and sinus regions from immunohistochemical viewpoint. In fixed sections of the *Triakis* kidney, NHE3-specific signals were detected in the apical membrane of the renal tubules in both the bundle and sinus regions (Li et al. 2013). The sinus region consists of second (proximal tubules and intermediate tubules) and a fourth (end of distal tubules) rings. Proximal tubules could be distinguished with their large diameter, long epithelium and the presence of a brush border that can be stained with anti- Na/K -ATPase antibody in the basolateral part of these cells. Distal tubes were distinguished with their lower diameter and thinner epithelium as well as the absence of brush border which leads to high staining response to anti- Na/K -ATPase antibody. In the sinus region of the *Triakis* kidney, specific signals for NHE3 were detected in part of the proximal as well as end of distal tubules.

In other words, NHE3 were both positive and observed in both tubules. NHE3-negative proximal tubes were often observed in the sinusoidal region near the bundle region. In the bundle region, the five tubular sections are placed side by side to conduct the flow and are separated by a sheath from the adjacent bundle region (Li et al. 2013). NHE3-specific signals were found in the apical membrane of one of the five tubes.

NHE3-positive tubes were detected in the bundle area with relatively large diameters consisting of long epithelial cells with no brush border and reacted strongly to Na/K -ATPase localization (Li et al. 2013).

These results clearly indicate that NHE3 is expressed in the first part of distal tubule (Li et al. 2013). In our current study, Na⁺/H⁺ Exchanger expression was detected only at the apex of epithelial cells of proximal tubules, collecting tubes and collecting ducts with high, moderate and low intensities, respectively, while distal tubules did not respond to Na⁺/H⁺ exchanger immunolocalization.

Abbreviations

SDS

Sodium dodecyl sulfate

TPBS

Tween phosphate buffered saline

DAPI

4',6-diamidino-2-phenylindole.

Declarations

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Consent for publication

Not applicable.

Availability of data and material

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.

Code availability

Not applicable.

Author contributions

KE designed the study and wrote introduction and part of the results. MB prepared the samples and did part of method and materials and provided the schematic figures. MAF contributed in preparing and revising draft. AKH prepared the samples and histological sections, also wrote part of results and

discussion and provided the schematic figures. HM prepared materials and revised the draft. All authors read and approved the final manuscript.

Ethics approval and consent to participate

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.

Conflict of interest

The authors declare that they have no conflict of interest

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Figures

Figure 1

Histological section of the anterior and posterior kidney in *Periophthalmus waltoni*. Stained with H & E, PAS and PAS-Alcian blue staining. H & E section staining shown that the urinary system in this fish consists of renal corpuscles, proximal tubules (PT), distal tubules (DT), collecting tubules (CT) and collecting ducts (CD). Positive reaction to PAS staining (in red) was observed in the basement membrane of all tubules and ducts and the apical part of the proximal tubules and relatively less in the distal

tubules. Positive reaction to PAS-Alcian blue staining (light blue) was observed in the apical part of the proximal tubules and collecting ducts.

Figure 2

Double labeling of Na⁺/K⁺-ATPase enzyme A) immunolocalization of Na⁺ K⁺-ATPase enzyme using 5a antibody (red) B) immunolocalization of Na⁺ K⁺-ATPase enzyme using R1α antibody (green) C) Nuclear staining of kidney tissue using DAPI (blue). D) the whole background of kidney tissue and E) All images merged. In Figures A and B, proximal and distal convoluted tubules have reacted positively to Na⁺/K⁺-ATPase localization in their basal/lateral portions and negatively in their apical portions. Na⁺/ K⁺-ATPase is present in all the basal and lateral portions of collecting tubules and collecting ducts. F) Schematic view of the immunolocalization of Na⁺ K⁺-ATPase enzyme of different parts of tubules and ducts.

Figure 3

Double labeling of Na⁺/K⁺-ATPase and Na⁺/K⁺/2Cl⁻ enzymes A) immunolocalization of Na⁺/K⁺/2Cl⁻ using T4 antibody (red) B) immunolocalization of Na⁺/K⁺-ATPase using R1α antibody (green) C) Nuclear staining of kidney tissue using DAPI (blue) D) the whole background of kidney tissue and E) All images merged. In Figure A, the Na⁺/K⁺/2Cl⁻ cotransporter is located only at the apex of the epithelial cells in the collecting tubules and to a lesser extent in the collecting ducts. This is while epithelial cells located in the proximal and distal tubules did not respond to the Na⁺/K⁺/2Cl⁻ cotransporter immunolocalization. F) Schematic view of the stained parts of tubule and ducts.

Figure 4

Double labeling of Na⁺/H exchanger and Na⁺/K⁺-ATPase enzymes A) immunolocalization of Na⁺/K⁺-ATPase enzyme using α5 antibody (red) B) immunolocalization of Na⁺/H exchanger enzyme using NHE3b antibody (green) C) Nuclear staining of kidney tissue using DAPI (blue) D) the whole background of kidney tissue and E) All images merged. Immunohistochemical activity for Na⁺/H/ Exchanger localization was detected only at the apex of epithelial cells of proximal tubules, collecting tubules and collecting ducts with high, moderate and low intensities, respectively. The distal tubules did not respond to Na⁺/H+ Exchanger immunolocalization. F) Schematic view of the stained of different parts of tubule and ducts.