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Cholinergic Activity in Mononuclear Cells of Nile Tilapia (Oreochromis niloticus) Fish

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Abstract. Presence of cholinergic components that can play an important role in the cellular homeostasis has been reported to be found in the immune system cells of mammals. The group of such components has been denominated non-neuronal cholinergic system. Nevertheless, up to this moment there are no reports of cholinergic components in lower vertebrates such as fishes, hence the objective of this study was to determine the presence and activity of cholinergic components in mononuclear cells in spleen of Nile tilapia (*O. niloticus*).The obtained results indicate that Acetylcholine (ACh) and Acetylcholinesterase (AChE) were found in mononuclear cells of fish. Such molecules can have an implication in important immunomodulation mechanisms. This type of studies can generate relevant information in order to understand neuroimmune communication in evolutionary terms.

Keywords: Acetylcholine, acetylcholinesterase, immune cells, nile tilapia, fish

INTRODUCTION

Acetylcholine (ACh) is a widely distributed neurotransmitter in the central and peripheral nervous system. It is synthesized from choline and acetyl-coenzyme A (acetyl-CoA) through the choline-acetyltransferase (ChAT) enzyme. Once synthesized, the neurotransmitter is stored in the presynaptic vesicle until neurons are activated. Once the ACh is released, it activates post-synaptic neurons through ionotropic nicotinic Acetylcholine receptor (nAChR) and metabotropic muscarinic Acetylcholine receptor (mAChR), both bonded to G protein. The stimulus induced by ACh is stopped when the neurotransmitter is degraded by cholinesterases (ChEs) enzymes [1, 2]. In this family of enzymes, acetylcholinesterase (AChE, E.C. 3.1.1.7), also called "true" cholinesterase, functions as an ACh hydrolyzer, producing acetate and choline, ending the nervous impulse [3]. This enzyme is mainly found in the brain, muscles, erythrocytes and cholinergic neurons [4]. On the other hand, butyrylcholinesterase (BChE; E.C.3.1.1.8) enzyme, also called "pseudo"-cholinesterase, is present in different types of tissue (intestine, liver, kidney, heart and lung); even though BChE has also the ability to hydrolyze ACh, its physiological function is not clear [5–7].

It is known that many components of the cholinergic system are present whether in bacteria or mammals, which makes the evolutionary and phylogenetic importance of this neurotransmitter evident [8]. In teleost fishes, such as zebra fish (*Danio rerio*), cholinergic neurons have been detected in practically every brain region [5]. Some physiological functions that have been related to this neurotransmitter are: visual and motor response, memory, olfactory mechanisms and neuromodulation on other physiological systems [10–13].

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In spite of the evolutionary, economic and ecological importance that fishes have (being the first vertebrates that appeared on the planet and currently represent more than half of vertebrates), up to this moment immunomodulation studies on these organisms are scarce [14]. It has been demonstrated that the cells of the immune system in mammals possess all the biochemical and molecular machinery to generate *de novo* ACh and metabolize this neurotransmitter, components that have been named cholinergic non-neuronal or cholinergic extra-neuronal system, which could play an important role in the regulation of immune response [8, 9, 15].

Therefore, the objective of this study was to determine the presence of cholinergic components (ACh and AChE) in mononuclear cells of Nile tilapia (*O. niloticus*), one of the species of teleost fish with major importance in tropical and subtropical countries around the world.

MATERIALS AND METHODS

Organisms

Nile tilapia male (273 ± 43 g y 20 ± 3 cm) were obtained from local fishery. The fish were maintained in a 400-L tank, during acclimatization period (4-week), they were fed with commercial food at a day-rate of 3% of fish body weight. Water temperature was maintained at $28 \pm 2^{\circ}$C and dissolved oxygen values were 6.0 mg/L. Each fish was sacrificed by ice bath and then weighed and measured. Later, spleen was excised and kept on ice and parameters were analyzed almost immediately.

Samples preparation

Spleen was obtained and laid in PBS buffer (pH 7.2). Then, the tissue was disrupted manually with gentle movements using a syringe plunger. The mononuclear cells were separated by density gradient using Histopaque-1077. Mononuclear cells were collected and washed at 3,500 rpm/30 min. The cell pellet was reconstructed in 500 μL of PBS and mechanically homogenized at 5,000 rpm/30 seg. Protein concentration was determined from the homogenate, according to the Bradford method (1976) [16], using bovine serum albumin as standard.

ACh determination

Determination of ACh was made using a fluorometric commercial kit (amplex Red Invitrogen). A standard curve was obtained (0.5–100 μM ACh) following the kit instructions. Shortly after that, a volume of the mononuclear cell homogenate (0.1 mg/mL of protein) was mixed with 100 μL of working solution (Horse Radish Peroxidase: 2 U/mL; choline oxidase: 0.2 U/mL; acetylcholinesterase: 1 U/mL).

The mixture (200 μL) was incubated for 60 min at room temperature and protected from light. The ACh concentration was determined by fluorometric microplate reader at 530 nm excitation and 590 nm emission. Background fluorescence was eliminated by subtracting the values derived from the non-acetylcholine control and the ACh values in the samples were determined through a standard curve.

Cholinesterase activities

In order to calculate the total activity of cholinesterase (ChEt), a volume of the homogenate of mononuclear cells was mixed (0.1, 0.2 or 0.3 mg of protein/mL) with different concentration of acetylcholin iodide (ASChI) (0.50 to 2.0 mM) and 10 nM 5,5-O-dithiobis(2-nitrobenzoicacid) (DTNB), which were used as substrate and indicator, respectively. The absorbance was determined at 405 nm. The enzymatic activity was calculated from the difference of absorbance (Abst2 - Abst1), were t1 = absorbance at the beginning of the reaction, while t2 = absorbance obtained every 5 min during 60 min. ChEt activity was expressed as mM/min/mg of protein [17]. To measure the AChE activity, previous to the addition of ASChI and DTNB, the homogenate of mononuclear cells was mixed with iso-OMPA (1.0 mM), a selective BChE inhibitor, and then incubated at room temperature during 30 min. Afterwards, AChE activity was determined as previously described. BChE activity was determined indirectly by calculating the difference: BChE = ChEt-AChE.

Statistical analysis

Mean ± SD was determined in each case. Data were analyzed using Sigma stat® (ver. 3.5. statistical software). One-way ANOVA and Tukey test were used. The statistical difference was determined with a level of $p < 0.05$.

RESULTS

Obtained results indicate that in spleen mononuclear cells presence of ACh was detected. Basal concentration of ACh in the homogenate of mononuclear cells was of 23.55 ± 6.03 μM (Table 1).

Table 1
Cholinergic components (ACh and AChE) in mononuclear cells of Nile tilapia fish

	Acetylcholine concentration (μM)
ACh	23.55 ± 6.03
	Cholinesterase activity (mM/min/mg protein)
ChEt	0.028 ± 0.011
AChE	0.019 ± 0.015
BChE	0.008 ± 0.007

On the other hand, cholinesterase activity (ChEt) was detected in mononuclear cells, which was more evident when the homogenate was used at a concentration of 1.0 mg/mL of protein, detecting a maximum activity between 20–35 min posteriors to the beginning of the reaction (0.064 ± 0.004 mM/min/mg protein). Meanwhile, ChEt activity diminished significantly ($p < 0.001$) from the 25 min by using 0.2 and 0.3 mg/mL of protein from the mononuclear cells homogenate, with an activity of 0.051 ± 0.002 mM/min/mg protein and 0.030 ± 0.001 mM/min/mg protein, respectively (Fig. 1).

In addition, ChEt activity in homogenized mononuclear cells was determined (0.1 mg/mL) by using different concentrations of ASChI (0.5–2.0 mM). Results indicated that most ChEt activity was detected at 20 minutes after the beginning of the reaction, by using 1.0 and 1.5 mM of substrate ($p < 0.05$) (Fig. 2).

In order to differentiate ChEt, AChE and BChE activities, reaction mixture was added with an inhibitor of BChE. Results indicated that from the activity of ChEt (0.028 ± 0.011 mM/min/mg protein), 68.6 % (0.019 ± 0.015 mM/min/mg protein) corresponds to AChE, while 31.4 % (0.008 ± 0.007 mM/min/mg protein) corresponds to BChE (Table 1 and Fig. 3).

DISCUSSION

The presence of cholinergic components that could be involved in the regulation of the immune response in mammal lymphocytes has been proved [1]. The extraneuronal cholinergic activity has been shown in both *in vitro* and *in vivo* models of humans and other mammals. Thus, Kawashima et. al., 1998 [18] determined the content of ACh in three T-cell lines of humans,

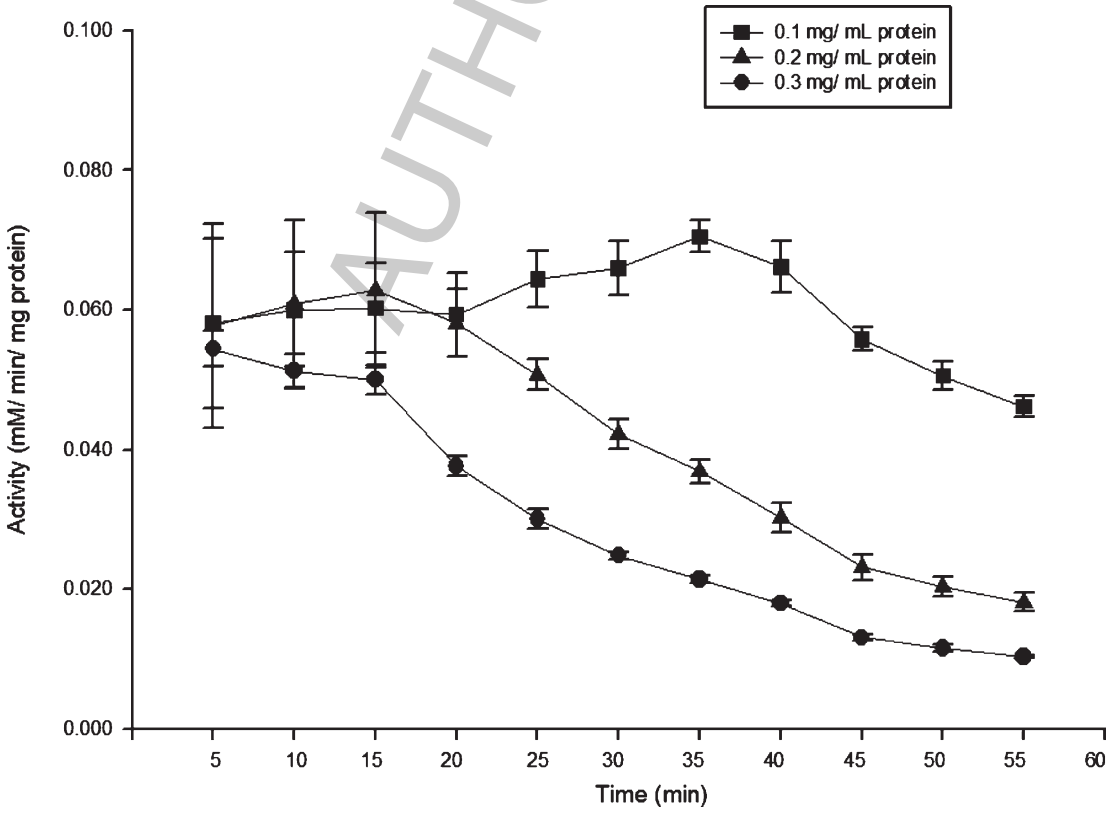

Fig. 1. ChEt activity in homogenized of mononuclear cells (0.1, 0.2, 0.3 mg/mL of protein). Enzyme reactions were performed using substrate 0.125 mM. Each point represents three independent experiments. Values are shown as mean ± SD.

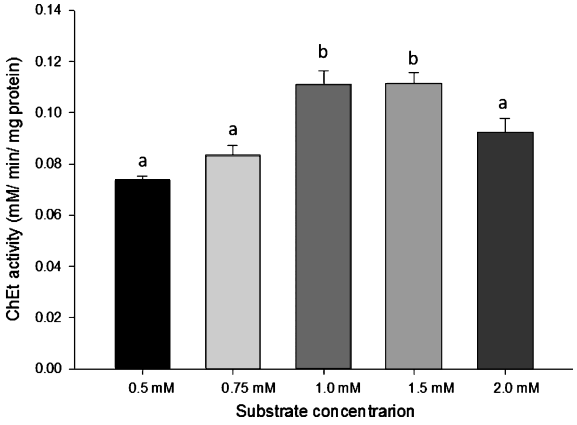

Fig. 2. ChEt activity in homogenized of mononuclear cells using substrate concentration (0.5–2.0 mM). Enzyme reactions were performed using homogenized at 0.1 mg/mL of protein. Readings were made at 20 after initiation of the reaction. Each bar represents three independent experiments. Values are shown as mean ± SD. Letters different indicate $p < 0.05$.

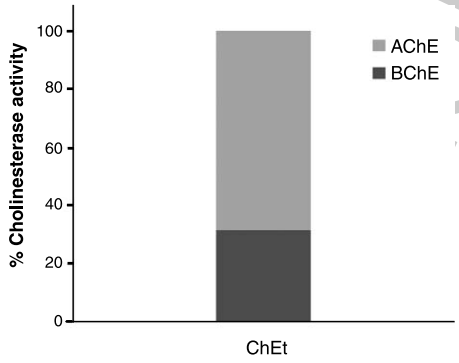

Fig. 3. Percent activity of AChE and BChE in mononuclear cell homogenate ($n = 6$). Calculations were performed based on enzymatic reactions using 0.1 mg/mL of protein homogenate and 0.125 mM substrate. BChE activity was determined indirectly through the use of Iso-OMPA an AChE inhibitor.

showing levels of 79.6 ± 8.9, 36.2 ± 3.5 and 9.1 ± 0.8 pmol/10^6 cells for MOLT-3, HSB-2 and CEM, respectively. In addition, Rinner et al. (1998) [19] also determined levels of ACh in lymphocytes from thymus (1521 ± 270 pg/10^6 cells), spleen (1340 ± 311 pg/10^6 cells) and Peripheral bloodlymphocytes (1148 ± 182 pg/10^6 cells) of rat, showing that the ACh content in lymphocytes is similar in each of the tissues. Studies made in order to compare levels of ACh present in different types of leucocytes indicate that in T lymphocytes the enzyme ChAT is expressed constitutively, while in the B-cell lines and monocytic cell lines low levels of ChAT expression were detected, in consequence, T lymphocytes seem to be the main cells of the immune system in charge of the synthesis of this neurotransmitter [15, 20].

Conversely, different studies have determined the concentration of ACh in blood and plasma of different animal species all along the evolutionary line [21]. However, in low vertebrates such as fishes, data regarding the extra-neuronal cholinergic system are scarce. From decades ago, the cholinergic innervation in lymphoid organs of fish was reported. Studies in cod (*Gadus morhua*) reveal that the spleen receives cholinergic nervous input through a branch of the anterior splenic nerve [22, 23].

There are no reports regarding basal levels of ACh in immune system cells of fishes. Obtained results in this study indicate that concentration of ACh in mononuclear spleen cells of Nile tilapia (*O. niloticus*) is 23 ± 6.03 μM, similar to the value reported previously in whole spleen (18.8 ± 9.57 μM) [24], which makes evident the great contribution of this neurotransmitter to the splenic microenvironment from lymphocytes.

Neurotransmitter ACh is implied in different cell functions such as homeostasis cell maintenance, mitosis, cytoskeleton organization and cell interactions [8, 2]. Studies made in rainbow trout (*O. mykiss*) have proved that cholinergic agonist carbacol significantly increases the number of cells that produce antibodies, such as ROS concentration in leucocytes [25, 26]. Obtained results in our research group shown that exposition of lymphocytes of Nile tilapia (*O. niloticus*) at 40–80 μM of ACh, significantly reduce the mitotic capacity of these cells [24]. In contrast, experiments in tenca fish (*Tinca tinca*) and atlantic cod (*Gadus morhua*) have shown that exposure to ACh (1.0 nM) induce a significant reduction of splenic tissue; while exposure to atropine reverse this effect [23].

The activity of ChE (AChE and BChE) enzymes is an important factor in the functioning of the cholinergic system. These types of enzymes are located in different extra-neuronal tissues [27]. In fish, AChE is predominantly found in the brain and muscle tissue, while BChE is found in liver and plasma [28]. Regarding the presence of these enzymes in the immune system cells of mammals, the expression AChE has been reported in lymphocytes and it seems that such activity increases significantly after acute infections [29]. When comparing the expression and activity of AChE among lymphocytes, it has been reported that in both T and B lymphocytes AChE is expressed. Nonetheless, the majority of AChE activity appears to reside in T-lymphocytes, and no AChE activity has been detected in B-lymphocytes [30].

In this study, the cholinesterase activity was determined in mononuclear cells of spleen of Nile tilapia. Obtained results indicate that from ChET activity, 68.6 % was due to AChE and 31.4 % to BChE. Contrasting results have been reported in plasma of cyprinid fish, where it was found that from the ChEt activity, 83–96 % corresponded to BChE, while 4–17 % to AChE [31]. It is reported then that both enzymes have the capacity to hydrolyze ACh, although such capacity is greater for AChE compared to other cholinesterase [32].

Obtained results in this study shown a ChEt activity dependent on the substrate concentration. These results agree with those from Alpuche-Gual [28], by characterizing the enzyme AChE in brain and muscle tissue of reef fish *Haemulon plumieri*, and those from Botté et. al. (2012) [33], who determined the ChEt activity in juvenile *Acanthochromis polyacanthus* muscle.

This is the first report regarding characterization of cholinergic elements in immune system cells of fishes. These components, just like in mammals, can constitute a no-neuronal cholinergic system of the immune system cells, implied in mechanisms of neuroinmunomodulation. Hence, such types of studies can generate relevant information to understand neuroimmune communication in evolutionary terms.

CONFLICT OF INTERESTS

There is no conflict of interests, and the authors declare that they have no direct relationship with the previously mentioned commercial entities or any other related.

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