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Original article

Verapamil – L type voltage gated calcium channel inhibitor diminishes aggressive behavior in male Siamese fighting fish

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Abstract

Verapamil is a L-type voltage gated calcium channels inhibitor (VGCCI), which is a highly prescribed drug used in the treatment of hypertension, *angina pectoris*, cardiac arrhythmia and cluster headaches. Its common use caused its appearance in water environment. VGCC inhibit epinephrine release and cause many neuro-hormonal changes influencing also fish behavior. Siamese fighting fish was chosen to study the influence of verapamil given to the water on the beginning of experiment in 3 different concentrations of 0 (control), 8 and 160 $\mu g \cdot L^{-1}$, on aggressive behavior in these fish. The experimental fish were placed in individual glass containers for 3 weeks and the mirror test was used. The highest concentration led to a significant modulation of fish behavior after 1 week and the lower dose caused statistically significant behavioral changes after 2 weeks of verapamil treatment. Siamese fighting fish males exposed to verapamil had longer latencies to the first chase – 12.6 s (8 $\mu g \cdot L^{-1}$ of verapamil) and 18.8 s (160 $\mu g \cdot L^{-1}$ of verapamil) compared to 5.6 s in the control group, decreased attack frequency and shorter duration of these attacks. The number of attacks within 10 min was decreased from 38.3 in the control group to 27.1 and 16.1, respectively. Also the total duration of these attacks decreased from 354.8 (control) to 326.4 (decrease statistically insignificant) and to 194.8 s in verapamil treated groups. It was shown, that even relatively low concentrations of verapamil in water may have adverse effects on fish and probably other living organisms.

Key words: verapamil, fish, aggressive behavior, L-type voltage gated calcium channels inhibitor

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Introduction

In recent years many pharmaceuticals have been detected in increasing concentration in effluent, sewage treatment plant as well as surface and ground water. Several studies have reported the presence and ecotoxicity of various pharmaceuticals in aquatic environment (Crane et al. 2006, Fent et al. 2006, Bergheim et al. 2012). These compounds may affect sensitive nontarget organisms. Le at el. (2011) have shown that one of such pharmaceuticals – verapamil, in concentration of 4.2 mg · L^{-1} of water reduced the expression level of Vtg gene in *Daphnia magna,* what may decrease their reproduction ability.

Verapamil belongs to the group of L-type VGCCI. L-type calcium currents typically require strong depolarization for their activation and are blocked by different antagonists including dihydropyridines, benzothiazepines and phenylalkylamines. By relaxing the smooth muscles tone this compound dilates blood vessels and because of this it is commonly used to treat hypertension, *angina pectoris*, and also cluster headaches (Leone et al. 2011). Since high concentration of VGCC are found in sino-athrial and atrio-ventricular nodes, verapamil decreases the impulse conduction through these nodes and is used as antiarrhythmic agent. The mode of action of verapamil similarly to diltiazem and nifeadipine, is based on binding to the largest subunit α_1 of Ca^{2+} channels. This subunit incorporates the conduction pore, voltage sensor, gating apparatus and several regulation sites e.g. by the second messengers, drugs and toxins. Verapamil inhibits Ca^{2+} ions influx to the cells. VGCCs are the main Ca^{2+} currents in muscle and endocrine cells initiating many activities such as muscle contraction (excitation-contraction coupling), hormone secretion, neurotransmitter release and neurons migration. Several data show antinociceptive effects of organic Ca^{2+} inhibitors of L-type VGCC (Kania et al. 2009). These inhibitors potentiate the analgesic action of κ-opioid receptor agonists (Gullapali and Ramarao 2002), as well as morphine by decreasing opioids; tolerance (Shimizu at al. 2004). Bongianni et al. (1986) have shown that VGCCI suppress not only metabolic but also behavioral expression of the morphine withdrawal syndrome. In experiments performed on mice it was shown that verapamil blocked amphetamine and also physostigmine induced foot-shock induced aggression (Srivastava et al. 1997). It was postulated by Michaluk et al. (1998) that VGCCI have antinociceptive properties but they also change territorial behavior in animals (Kavaliers 1987). Such effects were probably caused by inhibition of Ca^{2+} entry into neurons preventing appearance of synaptic vesicles in axon terminals and release of neurotransmitter into the synaptic cleft. It was shown that verapamil inhibits release of dopamine, histamine and corticotropin-releasing factor (CRF) (Filby et al. 2010), which may modulate fish behavior (aggressive display). Davis and Bauer (2012) have shown in experiments performed on rats, that activation of L-VGCCs is necessary for the long term retention of fear excitation.

Only in USA over 28 million of patients are under *angina pectoris* and hypertension treatment and verapamil is the main prescription drug for a large part of this group (FDA 1995). Such extensive use of this medicament is a reason for its appearance in drinking water. Many drugs and/or their active metabolites are capable to persist for a long time in the aquatic environment and are present in waterways. Even in small concentrations they can influence wildlife including fish. Li et al. (2011) have shown multiple responses in fish (rainbow trout) indicating that verapamil induced physiological stress. To assess potential effects of verapamil on aquatic organisms the changes in male Siamese fighting fish (*Betta splendens*) behavior was studied, by testing aggressive behavior in this fish used as a model species.

Materials and Methods

Thirty adult male Siamese fighting fish were purchased from a local pet supply store Zoomix (Warsaw, Poland). Each animal, average weight 1.66 ± 0.14 g, was placed in its own glass container. Each container was filled with 2 liters of fresh water that was dechlorinated. Fish in aquariums were kept in room temperature 22-24°C in 12/12h light cycle and fed once a day dry feed. Duration of the adaptation period was 1 week, and then in one week intervals fish were subjected to behavior tests for other 3 weeks. To monitor the reactions of the fish a 10 minutes mirror test was used. In one corner of the tank, a mirror (25.4 x 15 cm) was placed and the reaction of fish to its own image seen in the mirror was observed. After adaptation period, the fish were randomly divided into 3 groups. Ten fish served as controls and 20 animals were divided into 2 experimental groups. The fish the first experimental group were treated with verapamil in concentration of 8 μg · L^{-1} of water. The animals the second group were treated with 20 time's higher concentration of verapamil (160 μg · L^{-1}). Verapamil was added to the water once at the beginning of the experiment.

The observer sat in the front of the tank and recorded the occurrence of the display for each trial on a standard score sheet. Latency time to the first chase (expressed in seconds), attacks number and their

Table 1. The influence of verapamil concentration given to the water on parameters of aggressivenes in Siamese fighting fish. Summary obervations from the present study.

Verapamil concentration µg/L	Observation number n	Latencies time to the first chase s	Number of attacks within 10 min	Attacks duration within 10 min. s
0	60	5.6 ± 2.4[a]	38.3 ± 12.0[a]	354.8 ± 112.7[a]
8	30	12.6 ± 6.3[b]	27.1 ± 8.6[b]	326.4 ± 120.9[a]
160	30	18.8 ± 12.9[b]	16.1 ± 7.4[c]	194.8 ± 64.5[b]

Means ± SD
Means in columns not shearing the same superscript letter differ at p≤0.05

Table 2. The influence of different verapamil concentration in water on latencies to the first chase in Siamese fighting fish during 3 week experimental period. Week 0, n=30, weeks 1÷3, n=10.

Verapamil concentration µg/L	Time of observation (weeks)			
	0	1	2	3
0	6.03 ± 2.36[a]	5.5 ± 3.6[a]	4.5 ± 2.1[a]	5.4 ± 3.8[a]
8		9.6 ± 5.4[a,b]	11.5 ± 6.7[b]	14.6 ± 9.4[b]
160		10.8 ± 5.2[b]	20.5 ± 15.5[c]	25.1 ± 14.7[c]

Means ± SD
Means in columns not shearing the same superscript letter differ at p≤0.05

Table 3. The influence of different verapamil concentration in water on the number of attacks in Siamese fighting fish during 3 week experimental period. Observation time = 10 min. Week 0, n=30, weeks 1÷3, n=10.

Verapamil concentration µg/L	Time of observation (weeks)			
	0	1	2	3
0	40.2 ± 14.8[a]	31.9 ± 11.4[a]	39.6 ± 13.9[a]	37.4 ± 15.2[a]
8		33.0 ± 11.4[a]	30.3 ± 17.2[a]	18.1 ± 7.4[b]
160		18.0 ± 13.9[b]	16.9 ± 13.1[b]	14.5 ± 7.3[b]

Means ± SD
Means in columns not shearing the same superscript letter differ at p≤0.05

Table 4. The influence of different verapamil concentration in water on total attack duration (per 10 min) in Siamese fighting fish during 3 week experimental period. Observation time = 10 min. Week 0, n=30, weeks 1÷3, n=10.

Verapamil concentration µg/L	Time of observation (weeks)			
	0	1	2	3
0	347.5 ± 124.7[a]	318.5 ± 94.2[a]	384.4 ± 132.4[a]	383.6 ± 114.0[a]
8		320.4 ± 141.6[a]	292.8 ± 137.8[a,b]	265.9 ± 109.4[b]
160		212.7 ± 97.4[b]	201.1 ± 107.0[b]	170.5 ± 31.8[c]

Means ± SD
Means in columns not shearing the same superscript letter differ at p≤0.05

duration within 10 min following placing of the mirror were recorded.

The experiment was approved by the Local Ethics Commission for Care and Use of Laboratory Animals and Animal Welfare (N° 19/211).

Results

Table 1 presents average results (weeks 0-3) of aggressiveness parameters such as latencies to the first chase, number of attacks within 10 min and attacks

duration in Siamese fighting fish treated with different verapamil concentrations. Latency times to the first chase increased significantly in the verapamil treated fish from 5.6 (control) to 12.6 (8 $\mu g \cdot L^{-1}$) and 18.8 s (160 $\mu g \cdot L^{-1}$). Number of attacks and their duration were decreased significantly in verapamil treated fish. The number of attacks within 10 min was decreased from 38.3 in the control group to 27.1 and 16.1, respectively. Also the total duration of these attacks decreased from 354.8 (control) to 326.4 (decrease statistically not significant) and to 194.8 s in the verapamil treated groups. All the changes observed were time related (Tables 2, 3, 4). After one week of verapamil treatment, which was present in water at the lower concentration of 8 $\mu g \cdot L^{-1}$, only slight, statistically insignificant changes were observed. Three weeks of the experiment caused more pronounced effects and statistically significant prolongation of the latencies (Table 2) to the first attacks, as well as a drop in the number of attacks (Table 3) and their duration (Table 4). When verapamil concentration was higher (160 $\mu g \cdot L^{-1}$) the changes in behavior of Siamese fighting fish appeared earlier and after one week they were statistically significant (Tables 2, 3, 4).

Discussion

Ichihashi et al. (2004) have studied the development of agonistic behavior in the male Siamese fighting fish and revealed that this fish is an excellent subject to study aggressive behavior. This unconditioned aggressive display is elicited by fish own image seen in the mirror (or appearance of another male Siamese fighting fish) and is terminated by an attack. This fish has been found to be highly territorial and recognized as largely nonsocial species (Snekser et al. 2006). Another model to study aggressive behavior is the zebra fish (*Danio rerio*) which is a group-living species and aggression is used by dominant individuals to occupy territories over spawning sites and/or to protect high social status.

In our experiments in order to minimize additional stress connected with fish removal from their tanks to the test tanks, a Pearson method was used (Pearson 2005), in which all the fish are subjected to mirror test in their own tanks.

Despite of high variation of reaction time after the exposure of examined the fish to their mirror image, we found significant longer latencies to the first chase of Siamese fighting fish exposed to different concentrations of verapamil. In the same time we found a decreased number of attacks during 10 min period in fish exposed to verapamil. Also the total duration time of attacks was significantly lower in the verapamil treated fish.

In aerobic condition, verapamil in groundwater undergoes a bacterial inherent biotransformation but it is not readily biodegradable (<20%) (BASF Verapamil HCl, SDS). The half-life of verapamil has been estimated to be 10.2 ± 1.6 days (Steinbach et al. 2013). Little is known about the elimination of verapamil during sewage treatment. Verapamil main and final metabolite was found to be a compound known as D617 (Knoll nomenclature) (Trautwein et al. 2008).

In Safety Data Sheet of Verapail HCl it was recognized that LC50 for *Leuciscus idus* is 4.6÷10.0 $mg \cdot L^{-1}$. In a short-term exposure study performed on juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to verapamil LC_{50} was 2.72 $mg \cdot L^{-1}$ (Li et al. 2010).

Toxicity tests performed on fathead minnow during 28 days posthatching have revealed that in early life stages verapamil (in a dose of 600 $\mu g \cdot L^{-1}$) causes decrease of the growth in the treated fish, however, without any effect on the survival (Overturf et al. 2011). Also in experiments on early stage of carp life it was shown that concentration ≤ 463 $\mu g \cdot L^{-1}$ had no effect on the accumulated mortality, hatching, growth and ontogeny of the fish. However, in concentrations 46.3÷463.0 of verapamil higher occurrences of malformations and edemas were observed (Steinbach et al. 2013).

In long-term experiments (6 weeks) performed also on juvenile rainbow trout Li et al. (2012) used different doses of verapamil – 0.5, 27 and 270 $\mu g \cdot L^{-1}$. The authors have found no significant changes in parameters measured (morphological indices, hematological and antioxidant parameters) in trout exposed to environmental related concentrations of verapamil (0.5 $\mu g \cdot L^{-1}$) but have observed significant changes in physiological and biochemical responses in the fish exposed to higher concentrations of this drug.

Using the same concentrations of verapamil as Li and coworkers (2012), Burkina et al. (2012) did not found any changes of hepatic CYP 450 of rainbow trout exposed to verapamil. In aquatic organisms this L-type calcium blocker was recognized as p-glycoprotein-mediated multixenobiotic resistance mechanism inhibitor which may influence protein-related transport and reactivity in the intestine and liver (Doi et al. 2001). However, it was also indicated that this detoxification mechanism is not a sensitive indicator of xenobiotic concentration in the ecosystem (Damare et al. 2009).

In the present study (duration of 3 weeks) we used similar verapamil concentrations which were far below estimated LC_{50} (0.3% of LC_{50}) of this drug for

fish. Appearance of longer latencies to the first chase, decrease in the number of attacks and shorter duration of these attacks are evidences of anti-aggressive influence of verapamil. Inhibition of fish aggressive behavior might be connected with diminished transmission of substances such as: 5-HT, DA and histamine. The role of 5-HT is not quite clear in fish behavior but it was shown by Clotfelter et al. (2007) that it decreases aggression in Siamese fighting fish via 5-HT1A receptors.

Many extensively used drugs are released directly into the aquatic environment in a form of active substances or theirs metabolites. Appearance of these substances in aquatic environment often includes disposal of unused drugs. Many pharmaceuticals are released directly into the environment after passing through wastewater treatment processes, which are often not designed to remove such drugs from effluent. Previously it was found that verapamil concentration in freshwater ranged from 0.06 to 0.9 $\mu g \cdot L^{-1}$ (Khan and Ongerth 2004, Al-Rifai et al. 2007). However, Sedlak et al. (2001) based on US data have predicted the appearance of verapamil in wastewater on the level of 2.4 $\mu g \cdot L^{-1}$. Higher local concentration is also possible to appear e.g. in hospitals wastewater which are often assumed to be the most toxic to aquatic life. No clear proofs have been collected that such small concentrations of drugs at which they are often found in the water environment may have any adverse effects on animals living in local ecosystems. In case of verapamil it seems that even such relatively low concentration as 8 $\mu g \cdot L^{-1}$, which is a little bit higher than environmentally related concentrations of this drug, has a significant impact on male Siamese fighting fish behavior. Therefore, an environmental risk of this compound cannot be excluded.

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