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ORIGINAL PAPER

Toxicological impacts of a botanical pesticide, azadirachtin on corpuscles of Stannius of stinging catfish, *Heteropneustes fossilis*

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Abstract *Heteropneustes fossilis* were subjected to 41.89 mg L^{-1} of azadirachtin for short-term exposure (96 h) and 10.47 mg L^{-1} of azadirachtin for long-term exposure (28 days). The fish were killed on 24, 48, 72 and 96 h in short-term and 7, 14, 21 and 28 days in long-term experiment. Corpuscles of Stannius (CS) were fixed on these time intervals. CS remain unchanged till 72 h in short-term azadirachtin exposure. Accumulation of granules has been noticed in the aldehyde fuchsin (AF)-positive cells at 96 h. The nuclear volume of these cells remains unchanged. The AF-negative cells of CS of azadirachtin-exposed fish exhibit a slight increase in their nuclear volume after 96 h. In long-term exposure, the CS remain unchanged up to day 14. Increased granulation in the AF-positive cells has been noticed following 21-day exposure. Moreover, the nuclear volumes of these cells show a significant decrease. Heavy accumulation of AF-positive granules and few degenerating cells are also noticed at 28 day in azadirachtin-exposed *H. fossilis*.

Keywords Botanical pesticide · Corpuscles of Stannius · Calcium · *Heteropneustes fossilis*

Introduction

Botanical pesticides are the chemicals that have evolved in plants for their defense against phytophagous insects. For the control of insect pests, several plants have now been used (Komalamisra et al. 2005; Rahuman et al. 2008; Sileshi et al. 2009; Dubey et al. 2010). The seed, leaves and other parts of neem tree *Azadirachta indica* (family Meliaceae) contain an insecticidal active ingredient—azadirachtin ($C_{35}H_{44}O_{16}$). It is a naturally occurring substance related to an organic molecule class—tetranortriterpenoids (limonoids). Azadirachtin is now used for control of pests and other harmful animals (Punzo and Parker 2005; Mondal et al. 2007; Winkaler et al. 2007; Senthil Nathan et al. 2008; Shanmugasundaram et al. 2008). Inhibition of acetylcholinesterase activity in various fish tissues has been reported by exposure to neem seed pesticide—neemta (Parveen et al. 2004). It has been reported that azadirachtin significantly enhanced the antibody response and an inverse relationship was observed between the dose of azadirachtin and the degree of immunostimulation (Chitra et al. 2008). Azadirachtin is also used in fish farms for the control of fish parasites and fish fry predators (Winkaler et al. 2007).

In fish, toxicants present in aquatic environment disturb water and ion homeostasis. These physiological disturbances have been correlated with the structural damage of the gills caused by these toxicants as gills are supposed to be an important organ for the uptake, biotransformation and excretion of toxicants (Evans 1987; Wendelaar Bonga and Lock 1992; Agbozu et al. 2007). In spite of their great vital importance, gills are delicate structures which are affected by all kinds of environmental influences, such as physical changes of water, microorganisms and toxicants. Any amount of damage to the gills would result in immediate effect on ion homeostasis and will evoke compensatory osmoregulatory responses. Calcium, in vertebrates, plays a vital role in a variety of biological processes such as membrane permeability, muscle contraction, neuronal

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excitability, cell adhesion, fluctuations in acid–base balance and clotting of blood. It is also essential for ultimate initiation of many endocrine events. All vital processes depend on changes in osmotic concentration and ion composition of the body fluids. Any alteration in osmotic and ionic regulation would affect the normal physiological processes of the organisms and hence their survival in nature.

Corpuscles of Stannius (CS) are located on the kidney of holostean and teleostean fishes and considered to be unique to these groups of fishes. CS are superficially attached or partially/deeply embedded in the posterior two-third of kidney, located ventrally or ventrolaterally. They are oval, round or irregular in shape. The CS exhibit variation in number and are asymmetrically located. A thick connective tissue capsule envelops the gland and isolates it from the rest of the kidney tissue. From the capsule, connective tissue septa extend in the gland dividing the CS into several complete or incomplete cords or lobules. These cell cords contain epithelial cells possessing oval or rounded nuclei with sharp staining chromatin granules and often a small central nucleolus.

CS secrete the antihypercalcemic hormone stanniocalcin (STC) (Wendelaar Bonga and Pang 1991; Wagner 1994). Within the CS of few fish species, different cell types have been observed and were considered as type-1 (AF-positive) and type-2 cells (AF-negative) (Wendelaar Bonga and Pang 1991; Singh and Srivastav Ajai 1996; Srivastav Ajai et al. 2009, 2010; Mishra et al. 2009, 2010). The accumulation of secretory granules in the AF-positive cells can be considered as a good measurement for inactivity of these cells, which clearly indicates the inhibited release of granules thereby causing inhibited secretion of stanniocalcin. Presence of functional receptors for STC in non-piscine vertebrates has been suggested by few investigators on the basis of their experiments in which they observed CS extract-induced hypocalcemia in bird (Srivastav Ajai and Swarup 1982) and snake (Hasan and Das 1987). Ishibashi and Imai (2002) have suggested that STC homologs may also be present in tetrapods. Although no homologous structure of CS has been localized in higher vertebrates, immunocytochemically stanniocalcin (STC 1 and STC 2) has been identified in the kidney, ovary, pancreas (alpha cells) and bladder of human and rat (Ishibashi and Imai 2002; Song et al. 2006; Sazonova et al. 2008; Trindade et al. 2009). Recently, Roch and Sherwood (2010) have cloned three stanniocalcins from two invertebrates, the tunicate *Ciona intestinalis* and the amphioxus *Branchiostoma floridae*. Nowadays, STC1 is gaining more and more importance and has been shown to activate antioxidant pathways in endothelial cells and macrophages, thus displaying cytoprotective and anti-inflammatory actions (Sheikh-Hamad 2010).

Although several reports have shown that botanical pesticides can cause biochemical and histological changes in fishes (Chandra and Khuda-Bukhsh 2004, Winkaler et al. 2007) and mammals (Rahman et al. 1999, 2001; James et al. 2009), there is no study regarding their effect on endocrine regulation of calcium homeostasis in fish. The present study is the first report which aimed to access the effects of azadirachtin (trade name—Ozoneem Aza) on histological changes in the CS of stinging catfish, *Heteropneustes fossilis.*

Materials and methods

Collection and handling of fish

Stinging catfish (96 fish; body weight 23–29 g; total body length 14–17 cm) were collected locally (from Ramgarh Lake, Gorakhpur) and acclimatized for 15 days in plastic tanks (dimensions 48 inch × 40 inch × 22 inch; capacity 125 gallon) under laboratory conditions. The physico-chemical characteristics of the tap water used in the experiment were temperature 26.74 ± 2.11 C; pH 7.26 ± 0.09; hardness 135.25 ± 5.69 mg L^{-1} as $CaCO_3$; dissolved oxygen 7.85 ± 0.36 mg L^{-1} and no free chlorine. Water (entire volume) was renewed daily. The fish were fed daily with wheat flour pellets and ground-dried shrimps (prepared in the laboratory), 2–3 times per day (fed to saturation) during acclimatization period. The fish were not fed 24 h before and during the experimental period. The study was approved by the Animal Research Ethical Committee of DDU Gorakhpur University.

Procurement of extract and dose

Purified neem extract 'Ozoneem Aza' (containing azadirachtin A 23.78 % and azadirachtin B 3.59 %; Batch No. AZA-351, manufactured by Ozone Biotech, India) was used in the present study. The 96 h LC_{50} value of azadirachtin for *H. fossilis* is 52.35 mg L^{-1} (Kumar et al., 2010). The fish were subjected to 41.89 mg L^{-1} of azadirachtin (0.8 of 96 h LC_{50}) for 96 h in short-term exposure. In long-term exposure, the experiment was performed for 28 days by using 10.47 mg L^{-1} of azadirachtin (0.2 of 96 h LC_{50}). Concurrently, a control group was also run using the tap water containing alcohol due to that azadirachtin was firstly dissolved in alcohol and then added to tap water to obtain the desired concentration. No mortality was noticed during the experimental period.

Histological procedure

Six fish were killed (anesthetized with MS 222) on each time interval from both control and azadirachtin groups after 24, 48, 72 and 96 h in short-term exposure and after 7,

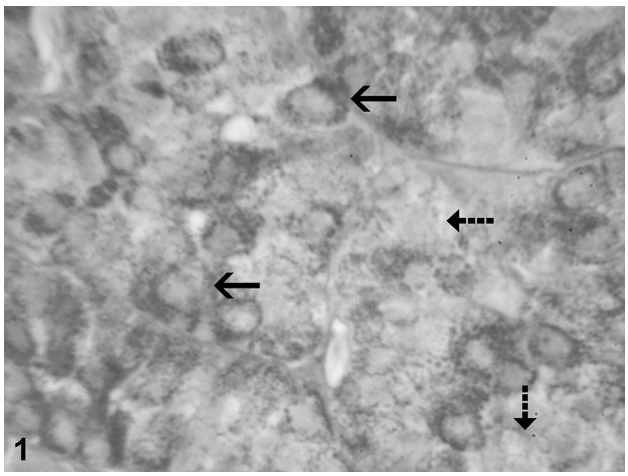

Fig. 1 Corpuscles of Stannius of control fish exhibiting AF-positive (*arrows*) and AF-negative cells (*broken arrows*). AF × 500

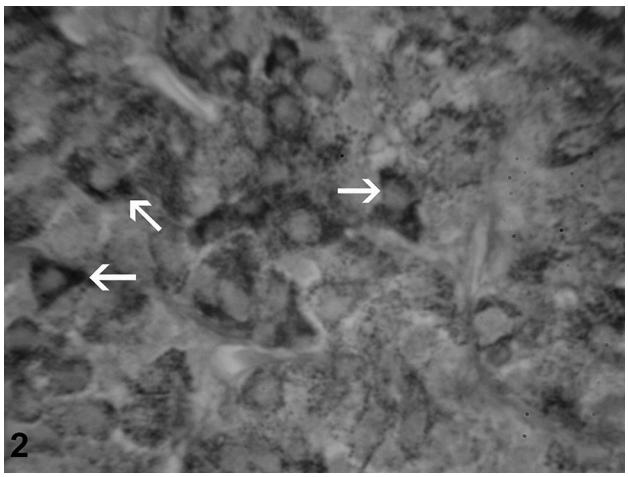

Fig. 2 Corpuscles of Stannius of 96 h azadirachtin-treated fish showing increased granulation (*arrows*) in AF-positive cells. AF × 500

14, 21 and 28 days in long-term experiment. The CS were fixed in aqueous Bouin's fluid. These fixed tissues were dehydrated in an ethanol gradient, treated with a clearing agent, infiltrated and embedded in paraffin, sectioned at 6 μm, floated on a heated water bath and mounted to glass slides. After drying overnight, paraffin was removed with a clearing agent, and tissue was rehydrated in an ethanol gradient and then stained with aldehyde fuchsin (AF) for light microscopic examination (Olympus CH 20i). Photomicrographs were taken with the aid of Olympus E 420 camera.

Statistical analysis

The nuclear indices (maximal length and maximal width) of CS were determined (fifty nuclei were measured per specimen, thus 300 nuclei were measured from six specimens) with the aid of an ocular micrometer and then the nuclear volume was calculated as:

$$\text{volume} = 4/3\,\pi\, ab^2$$

where '*a*' is the major semiaxis and '*b*' is the minor semiaxis.

Student's t test was used to analyze the statistical significance between the control and azadirachtin-treated fish. All data are presented as the mean ± SE of six specimens. The data were also subjected to two-way analysis of variance (ANOVA) by using treatment and time interval.

Results and discussion

In control fish, two cell types—AF-positive and AF-negative—have been noticed after AF staining (Fig. 1).

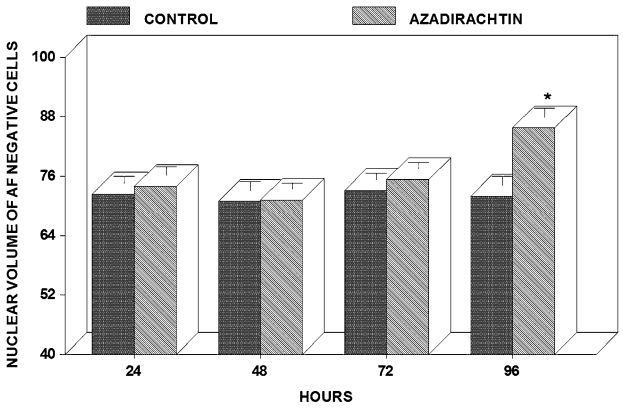

Fig. 3 Nuclear volume of AF-negative cells of *H. fossilis* treated with azadirachtin for short term. Each value represents mean ± S.E. of six specimens. *Asterisk* indicates significant differences ($P < 0.05$) from control

Corpuscles of Stannius remain unchanged till 72 h following short-term azadirachtin exposure. Increased granulation has been noticed in the AF-positive cells at 96 h (Fig. 2). The nuclear volume of these cells remains unchanged. The AF-negative cells of CS of azadirachtin-exposed fish exhibit a slight increase in their nuclear volume after 96 h (Fig. 3).

Analysis of variance indicated that in short-term experiment, the nuclear volume of AF-positive cells was not significant (among time intervals $F = 0.22$, ns; between treatments $F = 0.98$, ns), whereas for AF-negative cells, the values were significant (among time intervals, $F = 8.96$, $P < 0.0001$; between treatments $F = 16.24$, $P < 0.0001$).

Corpuscles of Stannius remain unchanged up to day 14 in long-term azadirachtin-exposed fish. Increased granulation in the AF-positive cells has been noticed following

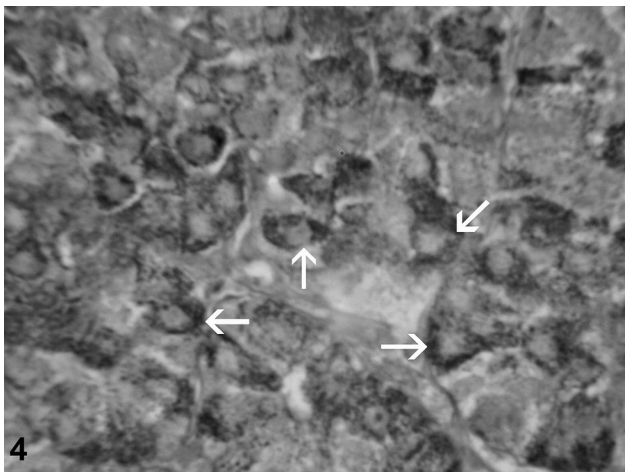

Fig. 4 Increased granulation (*arrows*) in the AF-positive cells of 21-day azadirachtin-exposed *H. fossilis*. AF × 500

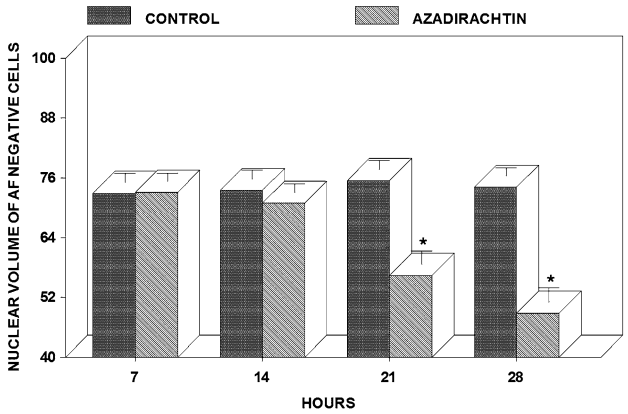

Fig. 5 Nuclear volume of AF-positive cells of long-term azadirachtin-exposed *H. fossilis*. Each value represents mean ± S.E. of six specimens. *Asterisk* indicates significant differences ($P < 0.05$) from control

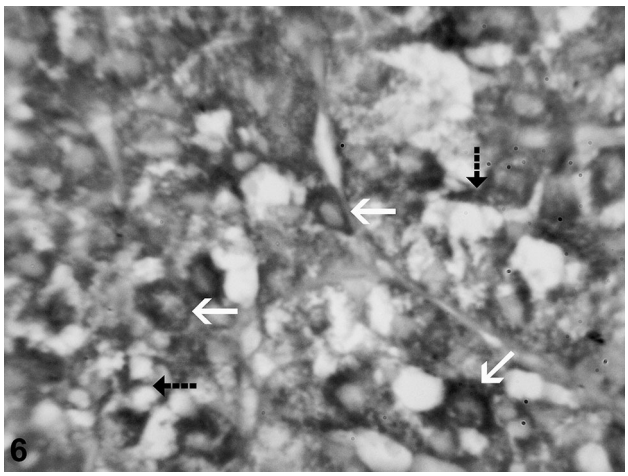

Fig. 6 Corpuscles of Stannius of 28-day azadirachtin-exposed fish showing heavy accumulation of secretory granules (*arrows*) in AF-positive cells. Also note degenerating AF-positive cells (*broken arrow*). AF × 500

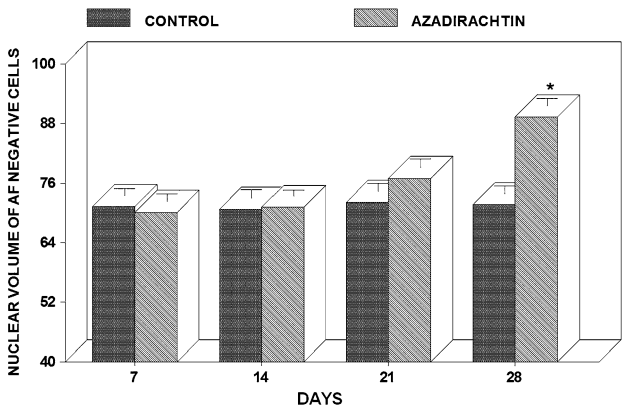

Fig. 7 Nuclear volume of AF-negative cells of long-term azadirachtin-exposed *H. fossilis*. Each value represents mean ± S.E. of six specimens. *Asterisk* indicates significant differences ($P < 0.05$) from control

21-day exposure with azadirachtin (Fig. 4). Moreover, the nuclear volume of these cells shows a significant decrease (Fig. 5). Heavy accumulation of AF-positive granules and few degenerating cells (Fig. 6) are also noticed at 28 day in azadirachtin-exposed fish. AF-negative cells of CS remain unchanged till 21 days of azadirachtin exposure. These cells show increase in their nuclear volume after 28 days following the treatment (Fig. 7).

For long-term experiment, ANOVA indicated that the nuclear volume of AF-positive (among time intervals, $F = 14.16$, $P < 0.0001$; between treatment, $F = 64.59$, $P < 0.0001$) and AF-negative (among time intervals, $F = 17.64$, $P < 0.0001$; between treatment, $F = 25.00$, $P < 0.0001$) cells was significantly different.

AF-positive cells of CS of fish responded to azadirachtin treatment by showing an increased accumulation of secretory granules and a decrease in nuclear volume. Prior to this study, responses of CS have not been studied after exposure to botanical pesticide. Accumulation of secretory granules and decreased nuclear volume of AF-positive cells have been recorded earlier by few investigators in response to exposure of fish to various toxicants (Srivastav Ajai et al. 2009, 2010; Mishra et al. 2009, 2010). AF-positive cells (type-1 cells) of the CS have been implicated with branchial calcium uptake in the fish through the secretion of a hypocalcemic hormone—stanniocalcin (Wendelaar Bonga and Pang 1991; Srivastav Ajai and Srivastav 1988; Tseng et al. 2009). The increased granulation in the AF-positive cells after azadirachtin exposure may be due to the prolonged hypocalcemia reported by Kumar et al. (2011) by similar treatment and can be explained on account of inhibition of the hormonal release and continued biosynthesis of STC. Accumulation of AF-

positive granules in CS has been noticed earlier in response to experimentally induced hypocalcemia in fishes kept in ambient acalcic freshwater (Tiwari 1993; Singh and Srivastav Ajai 1996). Similar accumulation of secretory granules in the calcitonin cells (responsible for the secretion of a hypocalcemic factor—CT) of mammals has also been noticed in response to hypocalcemia (Biddulph and Maibenco 1972; Swarup et al. 1980).

Conclusion

From the results of the present study, it is concluded that exposure of the fish to azadirachtin provoke disturbances in the blood calcium levels as well as in the histological structure of CS. As calcium is important for many vital functions including reproduction in the fish, any change in the blood calcium content would pose threat to these biological processes. Hence, near the fish ponds, azadirachtin should be used more carefully.

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