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RESEARCH ARTICLE

Ethotoxicological Role of Melatonin as an Anti-Stressor Agent in Heavy Metal Intoxicated Fish *Channa punctatus*

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Abstract Sublethal effects of arsenic trioxide, cadmium chloride, lead nitrate and mercuric chloride, were investigated on chromatophore morphology and behaviour of *Channa punctatus*. The protective role of melatonin was investigated. Changes were observed in the chromatophore pattern of fish scales and in fish behaviour after administration of the heavy metals. Heavy metal exposure caused restlessness in fishes and increased the dispersal of pigments in the chromatophores. Melatonin administration counteracted both these effects. The melatonin treatment did not modify metal concentration in scales, but it caused the aggregation of pigments in chromatophores of the fish. It also reduced the aggression observed in the fishes caused by the heavy metal administration. Toxicopathological alterations include statistically significant variations in the number, size and shape of the melanophores. The heavy metal-induced morphological changes in the melanophores indicate protective role against toxic insult of heavy metals.

Keywords *Channa punctatus* · Heavy metal · Melatonin · Chromatophore · Fish behavior

Introduction

Different species often provide different behavioural and physiological responses to stress and toxicant exposures. Melatonin, a key molecule of the vertebrate circadian system, can act as a modulator of these stress-induced responses. It is known that this hormone exerts an anorectic effect and reduces locomotor activity in some teleosts (Azpeleta et al. 2007). The objective of the present study is to analyze the stress levels in heavy metal intoxicated fish and the possible role of melatonin as a modulator of these stress responses.

A particular chromatophoric pattern in fish becomes visible when an inclination to maintain a specific environmental state is activated and, concomitantly, the fish is endangered or it falls into a 'perturbed state' (Wedemeyer and Mcleay 1981). Since the same physiological factors underlying the regulation of melanophores are involved in the regulation of stress too, this fact opens prospects towards ethological implications of stress. The present study, shows that, when fresh water fish, *Channa punctatus* is exposed to some major heavy metals like cadmium, lead, mercury and arsenic, the chromatophoric pattern of the fish changes in a specific manner. Fish chromatophores can be used as an indicator of arsenic toxicity (Allen et al. 2004). Fish chromatophores are capable of rapid pigment transport and possess a similar internal organization (Bikle et al. 1966; Green 1968; Murphy and Tilney 1974). These chromatophores are observed to cyclically aggregate and disperse their numerous pigment granules in a radial pattern from the cell center. It is therefore possible to watch the response of pigment granules and directly relate this behaviour to changes in the physiological conditions (Luby-Phelps and Porter 1982; Porter et al. 1983).

It has been observed that changes in the chromatophore pattern provide indices of the flux of circulating stress-relevant hormones. Different chromatophore patterns are seen in different situations from spontaneous exploration through agonistic behaviour. Differential regulation of the chromatophores can explain the variability of these chromatophoric patterns. Among the hormonal signals, melatonin is considered to be an internal "Zeitgeber" in

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vertebrates (Armstrong et al. 1989; Falcon and Collin 1989; Underwood and Hyde 1989). This seems to be related to the ability of the pineal organ to transform the environmental stimuli, mainly photoperiodic information, into nervous and hormonal signals. Melatonin is at elevated levels at night and basal levels during the day (Falcón 1999; Bromage et al. 2001). Melatonin has been reported to influence behavioural rhythms with varying results depending on the species. There have been several studies on the toxic effects of heavy metals on various animal systems; however less information is available about their effect on the behaviour of the subject observed. It is especially true, in sub-chronic heavy metal exposure, in the case of fishes. The present work outlines the heavy metal stress and effects of the hormone melatonin in *Channa punctatus*. An understanding of melatonin rhythmicity in heavy metal intoxicated fish may illustrate a solution to the problems of life in a temporally changing environment.

Materials and Methods

The teleost fish *Channa punctatus* (35 ± 5 g) were procured from fish farm and kept for acclimatization to laboratory conditions in glass aquaria for two weeks prior to their use in the experiments. During this period they were treated with the antibiotic, chloramphenicol (5 mg/l of water), as prophylactic agent. Water was changed every other day. Commercial food (Kijaro, Japan) containing wheat flour, rice bran, fish meal, vitamins, yeast, and calcium were offered to all the fish once a day. The food did not contain any heavy metal. Three experimental repeats were conducted. For all the experiments the fish were held in aquaria (23" × 46") with 20 liters of water. The water temperature was maintained at 25 ± 5 °C. In each experiment 50 fishes were divided into ten groups each. Fishes in group I–VIII were treated with heavy metal, while fishes of group IX received saline treatment and served as a parallel control; Fish of group X were reserved for melatonin (Sigma Chemical Company, St. Louis, MO, USA) treatment which was administered on 30th day of experiment. Prior to the commencement of the experiment the median lethal concentration of heavy metals for 96 h (96 h LC50 value) was calculated, following the 24 h renewal bioassay system and Trimmed Spearman–Karber method (Hamilton et al. 1977).

Fishes in group I and II were introduced and subjected to, a sub-lethal dose (0.8 mg/l, 10 % of 96 h LC50) of cadmium chloride (S. Merck, Bombay), fishes in group III and IV were subjected to a sub-lethal dose (2.8 mg/l, 10 % of 96 h LC50) of lead nitrate (S. Merck, Bombay), fishes in group V and VI were subjected to a sub-lethal dose (0.30 mg/l, 10 % of 96 h LC50) of mercuric chloride (S. Merck, Bombay) and fishes in group VII and VIII were subjected to a sub-lethal dose (1 mg/l, 10 % of 96 h LC50) of arsenic trioxide (S. Merck, Bombay) for 30 days. Treatment of heavy metals was given on every alternate day for 30 days and behavioural changes in the fish were observed by following the methods of Carpenter (1927), Wedemeyer and Mcleay (1981) and Pragatheswaram et al. (1989). The time, at which the fish loses its sense of balance and floats on its side or shows upside down movement, faster opercular activity, surfacing and gulping of air, erratic swimming with rapid jerky movements, hyper spiraling, convulsions and shows the tendency of escaping from aquaria, was noted following minute observations of these events.

On 30th day fish of group X were administered melatonin (10 μg/Kg body weight of fish) intramuscularly and fish of group IX were administered ethanol-saline at 10:00, 12:00, 14:00 and 16:00 h respectively, to observe the rhythm of change in behaviour of the fish. Fish behaviour was observed visually 10 min after the treatment and the observations were made for 15 min for all the experiments. Changes in fish behaviour frequency were more at 14.00 h. On the basis of this result, fishes of group II, IV, VI, VIII, and X were administered melatonin (10 μg/Kg) intramuscularly at 14 h on the 31st, 32nd and 33rd day of the experiment.

Scales were removed from the fishes, of the control group as well as of the treated groups, after periodic exposures to metals (30 days), and melatonin (14.00 h). Scales were stained with Borax Carmine and chromatophore patterns were examined under the microscope (Hogben and Slome 1931). For metal accumulation study, scales were digested in concentrated nitric acid and diluted by double-distilled Water. 1 g of scales was digested in 10 ml of concentrated nitric acid at 80 °C for 1 h. Metal concentration was estimated through Atomic Absorption Spectrophotometer (Perkin Elmer AA800). Fishes of the control group were monitored along with the toxicants at various concentrations to provide a reference for assessing any behavioral or morphological changes. Responses were recorded if they differed from the control and occurred in 10 % of the fish in each test tank.

Statistical Analysis

Students '*t*' test was used for statistical comparisons of both heavy metal accumulation in scales and for melanophore index, with the level of significance accepted as $p < 0.05$.

Results

Heavy metal exposure in our study increased stress in the fish resulting in their agitated behavior. Dispersion of

pigment in fish scale chromatophores was also observed. Melatonin administration counteracted both these effects. The melatonin treatment did not modify metal concentration in scales, but it reduced stress load as the restlessness in fishes reduced. Melatonin treatment also caused aggregation of pigment in the chromatophore of fish scales.

Metal Accumulation

Scales of fish can show bioaccumulation of metals. Our results (Table 1) show that concentration of cadmium chloride, lead nitrate, mercury chloride and arsenic trioxide did not show significant difference after Melatonin treatment and also there was no significant difference in metal concentration observed in the next two days.

Behavioral Symptoms Diagnosis

Marked changes in the behaviour of fishes were observed when exposed to various concentrations of the heavy metals for 30 days (Table 2). Heavy metal treated fishes initially showed rapid movement, faster opercular activity, surfacing and gulping of air. Arsenic treated fishes showed erratic swimming with rapid jerky movements, hyper-spiraling, convulsions and tendency of escaping from aquaria. Besides these, remarkable color change was observed and the loss of equilibrium was seen in the fishes. Mercury treated fishes struggled hard for aerial breathing with their restricted swimming movements. This was followed by loss of equilibrium and fishes slowly moved upward in a vertical direction. Cadmium treated fishes became progressively lethargic; swimming with widely spread fins and were often found on the surface of water. The increase in opercular movement and bottom to upward movement to overcome hypoxic condition was seen. Profuse mucus secretions and its coagulation all over the body of fishes were observed in lead treated fish. After melatonin treatment, the fishes swam less actively, than even the fishes kept as control. Melatonin treatment improved equilibrium in arsenic treated fish, however a very little improvement was seen in case of lead treated fish. Schooling which is the characteristic of this fish, was found to be weakened in the cadmium chloride and lead nitrate administered fishes, while after melatonin treatment it was restored once again.

Chromatophore Pattern

Microscopic examination, of the fish scales, administered with the various treatments was done to study the changes in the form and structure of chromatophores. On 31st, 32nd and 33rd day fishes were observed to show similar pattern of chromatophores. The pigment cells observed were smaller in size and more in number in heavy metal treated

Table 1 Accumulation of metals (μg/g) in scales of *Channa punctatus* after different exposure period

Exposure (days)	Control		Cadmium chloride (μg/g)		Lead nitrate (μg/g)		Mercuric chloride (μg/g)		Arsenic trioxide (μg/g)	
	Saline only Gp IX	Melatonin only Gp X	Gp I Cd	GP II Cd + Melatonin	Gp III Pb	Gp IV Pb melatonin	Gp V Hg	Gp VI Hg + melatonin	Gp VII As	Gp VIII As + melatonin
31	ND	ND	0.175 ± 0.012	0.174 ± 0.025	0.085 ± 0.022	0.083 ± 0.049	0.067 ± 0.046	0.062 ± 0.011	0.498 ± 0.042	0.485 ± 0.082
32	ND	ND	0.172 ± 0.034	0.175 ± 0.084	0.094 ± 0.052	0.085 ± 0.055	0.073 ± 0.018	0.065 ± 0.016	0.480 ± 0.034	0.490 ± 0.076
33	ND	ND	0.176 ± 0.019	0.173 ± 0.043	0.106 ± 0.012	0.096 ± 0.043	0.070 ± 0.064	0.065 ± 0.012	0.506 ± 0.017	0.488 ± 0.055

$n = 5$ in each exposure, values are represented as mean ± SE, $p < 0.05$, *ND* metal not detected

Table 2 Comparative behavioral responses given by freshwater fish *Channa punctatus* after exposure of different heavy metals

Metal treatment	Control		Cadmium chloride (µg/g)		Lead nitrate (µg/g)		Mercuric chloride (µg/g)		Arsenic trioxide (µg/g)	
Behavioral response	Saline only Gp IX	Melatonin only Gp X	Gp I Cd	GP II (Cd + melatonin)	Gp III Pb	Gp IV (Pb + melatonin)	Gp V Hg	Gp VI (Hg + melatonin)	Gp VII As	Gp VIII (As + melatonin)
Blinking of eye	–	****	*	***	*	***	*	***	*	****
Hyperactive	–	*	**	**	***	**	***	**	****	**
Hypoactive	–	****	–	****	–	****	–	****	–	****
Schooling	–	****	*	****	**	****	***	****	****	****
Un-coordinate swimming	–	–	****	**	****	*	****	**	****	**
Loss of equilibrium	–	–	****	**	****	***	****	**	****	*

"–" normal response, * abnormal response, ** mild increase response, *** moderate increase response, **** maximum increase response

fishes as compared to the normal fish melanophores. All melatonin treated fishes were observed to show punctuated pattern of chromatophores (Figs. 1, 2, 3, 4, 5; Table 3).

Fig. 1 Cadmium chloride + Melatonin: punctated pattern of chromatophores in scales of the fish treated with cadmium chloride for 30 days and melatonin treatment on 31st day

Fig. 2 Lead nitrate + Melatonin: punctated pattern of chromatophores in scales of the fish treated with Lead nitrate for 30 days and melatonin treatment on 31st day

Fig. 3 Mercuric chloride + Melatonin: punctated pattern of chromatophores in scales of the fish treated with Mercuric chloride for 30 days and melatonin treatment on 31st day

Fig. 4 Arsenic tri oxide + Melatonin punctated pattern of chromatophores in scales of the fish treated with arsenic trioxide for 30 days and melatonin treatment on 31st day

Table 3 Effect of cadmium, lead, mercury and arsenic on chromatophore pattern of fish

Metal treatment	Control		Cadmium chloride (μg/g)		Lead nitrate (μg/g)		Mercuric chloride (μg/g)		Arsenic trioxide (μg/g)	
Group	Saline only Gp IX	Melatonin only Gp X	Gp I Cd	GP II (Cd + melatonin)	Gp III Pb	Gp IV (Pb +melatonin)	Gp V Hg	Gp VI (Hg +melatonin)	Gp VII As	Gp VIII (As +melatonin)
Pattern of chromatophore	Stellate	Punctated	Reticulated	Punctated	Reticulated	Punctated	Reticulated	Punctated	Stellate	Punctated
Melanoph-ore index	45.56 ± 0.019	53.16 ± 0.110*	35.17 ± 0.024*	38.34 ± 0.162*, #	22.42 ± 0.120*	40.45 ± 0.067*, #	25.72 ± 0.067*	42.20 ± 0.063*, #	43.45 ± 0.107	50.20 ± 0.034

$n = 5$ in each group, values are represented as mean ± SE, $p < 0.05$, * denotes significant difference when compared to control (saline only) fish, # denotes significant difference when compared to control (melatonin only) fish

Fig. 5 Melatonin only: punctated pattern of chromatophores in fish treated with melatonin only

Fig. 6 Cadmium chloride: mixed pattern of chromatophores in scales of the fish treated with cadmium chloride for 30 days

Fig. 7 Mercuric chloride: reticulated pattern of chromatophores in scales of the fish treated with Mercuric chloride for 30 days

Fig. 8 Lead nitrate: reticulated pattern of chromatophores in scales of the fish treated with Lead nitrate for 30 days

A mixed pattern of chromatophores was observed in fishes treated with cadmium chloride on the, 30th, 31st and 33rd days (Fig. 6). Reticulated chromatophores were observed in fishes administered with mercuric chloride (Fig. 7), lead nitrate (Fig. 8) and arsenic trioxide treatment (Fig. 9) on the 30th, 31st and 33rd days.

Fig. 9 Arsenic tri oxide: reticulated pattern of chromatophores in scales of the fish treated with Arsenic tri oxide for 30 days

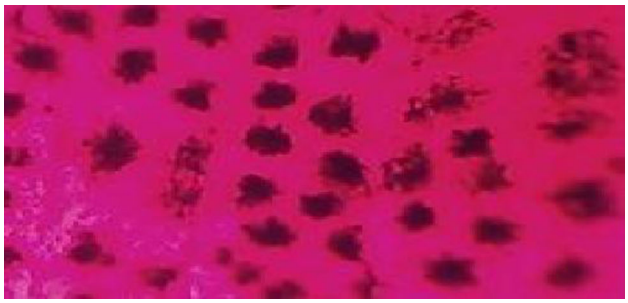

Fig. 10 Control: stellate pattern of chromatophores in fish treated with saline only

After the administration of melatonin, the central body and tentacle like processes of melanophores became less distinct as compared to control. The scales of fishes with, 30 days of heavy metal exposure were observed to show considerable increase in the degree of darkness, when compared with that of fishes, kept as control (Fig. 10), as well as, with those fishes which were treated with melatonin. However, observations for cadmium chloride treated fishes, showed a mixed pattern of chromatophores. The disintegration of many of the melanophores and the subsequent release of melanin into the surrounding tissues was also seen (Fig. 4). Even though many of the melanophores release their contents in the tissues, their astral arms and cell bodies were still observable.

Discussion

Fish skin, being directly exposed to the toxicants, is extensively used as a potent indicator of contaminated aquatic environment (Rajan and Banerjee 1991; Paul and Banejee 1996; Banerjee 1997). However, studies pertaining to the modulating effect of melatonin on fish behaviour and scale melanophores, in fishes administered with heavy metals are scanty. Therefore efforts have been made in the present study to investigate the toxic effects of sub-lethal concentrations of arsenic trioxide, cadmium chloride, lead nitrate and mercuric chloride, on the melanophores and on the behaviour of *Channa punctatus.*

Heavy metal induced changes differ from metal to metal, species to species and from one experimental condition to other. Determination of the toxicity is essential for determining sensitivity of animals to these toxicants. It is also useful for evaluating the degree of damage to the target organs and their resultant consequent physiological, biochemical and behavioural disorders (Vinodhini and Narayanan 2008).

The aim of the present study was to assess the modulating effect of melatonin against toxicity of heavy metals in the fresh water fish *Channa punctatus*. Behavioural changes have been established as a sensitive indicator of chemically induced stress in aquatic organisms (Suedel et al. 1997; Remyla et al. 2008). As evident by the results, behavioural alterations, like erratic swimming, restlessness and surfacing may be an avoidance reaction to heavy metal toxicity or to the change in sensitivity of chemo-receptors. Behaviour allows an organism to adjust to external and internal stimuli in order to best meet the challenges of survival in an ever changing environment (Atif et al. 2005). In this study, the resultant abnormal swimming behaviour and altered movements were considered to be the result of excessive elimination of skeletal minerals (Pragatheswaram et al. 1989). The results showed accumulation of heavy metals in the scales of the studied fishes. The higher concentration of metal, in the scales of these fishes may be because of adoptive capacity of fish to accumulate metals in the scales; however remarkable changes in fish behaviour were observed after melatonin treatment. Restlessness and erratic behaviour of the heavy metal treated fishes decreased when treated with melatonin. The possible role of melatonin as an anti-stress hormone has already been studied in fishes to some extent (Azpeleta et al. 2007). While the heavy exculpation of mucus over the body and body dispigmentation can be attributed to the dysfunction of endocrine system (Mishra and Pandey 1977), ecologically relevant behaviours affected by sublethal concentrations include: altered vigilance, startle response, schooling, feeding, prey conspicuousness, migration, and diurnal rhythmic behaviours (Little and Finger 1990; Zhou and Weiss 1998). Behavioural manifestations of acute toxicity in *Channa punctatus* were more or less similar to those reported in other fishes, exposed to heavy metals (Kasherwani et al. 2009). Behaviour provides a unique perspective linking the physiology and ecology of an organism and its environment (Little and Brewer 2001).

Remarkable color change was also observed in the heavy metal intoxicated fish. The skin of fishes act as the outer most defense barrier against the surrounding environmental stress and toxicant (Ojha 1997). Fish chromatophores can be considered as good biomarkers to access the health status of fresh water aquatic bodies in relation to metallic contaminants. The results of the present

study show that metals could induce chromatophores to disperse while melatonin treatment resulted as aggregation of their pigments.

Fujii (1961) recorded that melatonin effectively aggregated melanophore inclusions in the fish, *Chasmichthys gulosus*. Melatonin attenuates the acute stress response in a teleost fish (Azpeleta et al. 2007). The pineal gland produces melatonin at night only. Light inhibits AANAT (arylalkylamine *N*-acetyltransferase) activity and melatonin release in vivo or in vitro. At night, photoreceptor depolarization allows calcium (Ca^{2+}) entry (through voltage-gated Ca^{2+} channels) and cyclic AMP (cAMP) accumulation (Falcón 1999). Both contribute to increase of AANAT2 amount and activity through phosphorylation of the AANAT2 protein. This process is reversed by illumination, which sequentially induces photoreceptor hyper-polarization, dephosphorylation and degradation of AANAT2 through proteasomal proteolysis, resulting in the decrease of melatonin production (Falcón et al. 2001). In this study we injected melatonin in metal intoxicated fish during day time, when no intrinsic melatonin is supposed to be there in fish.

Since heavy metal toxicity actually evoked aggression in this species, it can be presumed that the action of the heavy metals is, not directly on the pattern of chromatophores, but it raised the stress load in the fish body. This increased the stress level in fishes and induced molecular responses for dispersion of melanophore. The present concept of melanophore motility is that, an increase or a decrease in the intracellular content of cyclic AMP (cyclic Y,5′-adenosine monophosphate) results in melanin dispersion or aggregation respectively (Novales 1971; Fujii and Miyashita 1976b). In *Channa punctatus* the melanosome dispersion in response to heavy metal stress is shown to be mediated by receptors in the same category. The results of the study are also consistent with this idea. Thus, the melanosome-aggregating action of melatonin may be exerted by the inactivation of adenylate-cyclase, which catalyzes the conversion of ATP into cyclic AMP. However, further studies are justly needed to clarify the mechanism by which melatonin interacts with its receptor molecules in the pigment cells. Altogether these data suggest that melatonin treatment can exert a possible anti-stress role in fresh water fish.

Conclusion

Behaviour is a selective response that is constantly adapting through direct interaction with physical, chemical, social, and physiological aspects of the environment. In the present study the chromatophore pattern was affected in different ways by heavy metal stress and melatonin. Different heavy metals caused a decrease in lightness and increase in dispersion of melanin, increased heavy metal load in scales, and increased restlessness in fish. Melatonin caused an increase in lightness and an aggregation of melanin and a decreased reflectivity of the aggression. It has no effect on metal load of scales and causes a modulating effect on fish activity. We can conclude that in an attempt to protect it self, from the toxic insult, the fish tries to disperse the pigment, this allows less light to enter into the fish's body and less illumination might result in more melatonin formation so that melatonin could act as an anti-stressor.

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