Original Paper

Thyroidal and osmoregulatory responses in tilapia (*Oreochromis mossambicus*) to the effluents of coconut husk retting

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Summary

The coconut husk retting in the backwaters of Kerala in Southern India, releases toxic effluents (CHRE), which pose a threat to the life of many inhabitants including fish. The indices of osmoregulatory activity and the levels of plasma triiodothyronine (T_3) and thyroxine (T_4) in the Mozambique tilapia *Oreochromis mossambicus* were quantified after exposing them to the effluents to understand the physiological mechanism of tolerance. The plasma glucose, an indicator of catecholamine secretion, remained unchanged in the tilapia exposed to CHRE. The plasma T_4 significantly increased in the tilapia kept in CHRE-rich water, though it declined in the fish kept for recovery in lake water. The plasma K^+ significantly decreased in the tilapia treated with CHRE, which returned to the basal levels in those kept for recovery. The Na^+, Ca^{2+} and PO_4^{3-} remained the same in both treated and untreated fish. The branchial Na^+, K^+-ATPase activity increased in the CHRE-exposed fish, and such an effect was not reversed in the recovery group. The renal Na^+, K^+-ATPase activity decreased in the lake water-exposed tilapia but not in the CHRE-treated fish. A reversal in the renal Na^+, K^+-ATPase activity was obtained in the tilapia kept for recovery in lake water. The intestinal Na^+, K^+-ATPase activity significantly declined in the CHRE-exposed tilapia but not in the recovery group. The data indicate that the presence of CHRE in lake water affects the osmoregulatory potential of tilapia without influencing their metabolic status. The up-regulated thyroid activity in the CHRE-exposed tilapia points to its involvement in the ion homeostasis during CHRE intoxication.

Key words: coconut husk retting, fish, metabolism, *Oreochromis mossambicus,* stress, thyroid hormones, tilapia

Introduction

Aquatic environment easily induces stress in their inhabitants due to the presence of various stressors including industrial effluents. Retting of coconut husk, an essential step in the coir production, in the saline stretches of backwaters of Kerala in southern India, poses a threat to the life of aquatic organisms due to the release of toxic effluents as by-products. The physico-chemical characteristics as well as the ecology of retting zone have been studied extensively (Aziz and Nair, 1986). The analysis of composite effluents of coconut-husk retting concentrate (CHRE) released during retting indicates high levels of toxic chemicals including sulphide and ammonia (Madhukumar and Anirudhan, 1996). A complete depletion of oxygen, leading to anoxia that lasts for several months, and persistence of a unique sulphide system are the other characteristics of the retting ground (Aziz and Nair, 1986).

In fishes, thyroid hormones (THs) influence several physiological processes including metabolism and osmoregulation (Gorbman et al., 1983; Leatherland, 1994; Peter et al., 2000; Gavlik et al., 2002; Oommen et al., 2007). Evidences have been presented that thyroxine (T_4) and triiodothyronine (T_3), the secretory products of thyroid gland, play a pivotal role in the regulation of the metabolic machinery of a number of fish species (Leatherland, 1988, 1994; Matty, 1985; Oommen and Matty, 1997). For example, the stimulatory actions of THs on intermediary metabolism have been reported in climbing perch, *Anabas testudineus* (Nair and Oommen, 1997). In addition, THs are reported to regulate mitochondrial oxidative (Peter and Oommen, 1989, 1993) and lipid metabolism (Varghese and

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Oommen, 1999) as well as the oxidative metabolism during exposure to biodegradable pesticide and fish poison (Peter and Oommen, 1991; Peter, 1996).

In teleosts, stressors evoke a complex neuro-endocrine response and it is generally accepted that fish depend on the release of catecholamines (Perry and Reid, 1993) and corticosteroids (Sumpter, 1997; Wendelaar Bonga, 1997) for coping with stressful challenges. It is generalized that hypothalamo-pituitary-thyroid axis exhibits down-regulation during its encounter to natural environmental variables in fish (Eales, 1985; Leatherland, 1988; Grau, 1988). Furthermore, it is known that stressors may influence the rate of energy utilization, thus affecting growth and metabolism (Wendelaar Bonga, 1997; Barton, 1997; Peter et al., 2004). The thyroidal control of stress response, especially on metabolic aspects of fish, has received little attention (Brown, 1993; Sumpter, 1997; Wendelaar Bonga, 1997; Peter et al., 2007). Studies in perch have demonstrated that exposure to stressors alters the metabolic and osmoregulatory homeostasis and the thyroid activity (Peter et al., 2004, 2007). Furthermore, alterations in energy metabolism, one of the main outputs of secondary stress responses (Barton, 1997), could be immediately beneficial to the fish under stress (Brown, 1993).

Mozambique tilapia is capable of tolerating a wide range of salinity (Kultz and Onken, 1993). Salinity tolerance in fish is dependent upon the appropriate physiological, biochemical and morphological adjustments to the given salinity (Sardella et al., 2004). Gills, being the primary site for maintaining water and mineral balance, are sensitive to the presence of pollutants. It is comprehensible that toxicants of various origins disturb osmoregulatory potential and the other physiological processes of fish (Wendelaar Bonga, 1997; Peter et al., 2004). Chemical stressors have frequently been shown to disrupt water and ion regulation in fishes (Engelhardt et al., 1981; Snell and Persoone, 1989). Toxicants reach piscine body through branchial and oral surfaces and impair metabolic and endocrine functions (Brown, 1993; Wendelaar Bonga, 1997; Peter et al., 2004).

Evidences have been presented in teleosts to the effect that chemical stressors influence the activity of thyroid and TH availability to various tissues. For example, exposure of fish to pesticides and metals, while down-regulating the level of plasma THs (Sinha et al., 1991; Gupta et al., 1997), others up-regulate their levels (Peter et al., 2007). In addition, it is known that the levels of plasma THs may be affected by varied forms of stressors (Bandeen and Leatherland, 1997). For instance, in cannulated brown trout, *Salmo trutta*, acid stress, depending on the availability of ambient Ca^{2+}, elevated plasma T_4 level without affecting plasma T_3 level (Brown et al., 1989).

It is likely that certain degree of compensatory and adaptive modifications may occur in respect of thyroidal and osmoregulatory functions of fish when exposed to the effluents of coconut husk retting. The osmoregulatory potential and the thyroidal effects in tilapia were examined after treating the fish with CHRE. Plasma glucose, plasma T_3 and T_4 levels together with Na^+, K^+-ATPase activity and the mineral contents were quantified in tilapia exposed to CHRE for 48 h with or without recovery for 96h in lake water.

Materials and Methods

Fish

The adult Mozambique tilapia, *Oreochromis mossambicus,* approximately 30-40g body weight, comprising both sexes were collected in large tanks and fed once a day with 1% body weight commercial fish feed. Before the commencement of experiment, fish were transferred to glass aquaria (45L) and kept for two weeks at a water temperature of 28 ± 1^0C and a photoperiod of 12 h L: D cycle. Feeding was stopped 24 h prior to sampling.

Protocol

The effect of a selected dose of 1:4 diluted effluents of coconut husk retting (CHRE) was tested in tilapia. Laboratory-acclimatted tilapia was divided into four groups of six each. Fish in group 1 were kept in fresh water and served as the control. Fish in group 2 were exposed to 1:4 diluted lake water (LW) and considered as lake water controls. Group 3 fish were exposed to 1:4 CHRE collected from acute retting zone of Paravur Lake. The group 4 fish were first exposed to CHRE for 48 h but were allowed to recover for 96 h in clean lake water.

Sampling and analysis

Fish in all groups were sampled on the same day and blood was drawn by caudal puncture using heparinized syringe. The blood was centrifuged at 5000 rpm for 5 min at 4ºC and plasma was separated and stored at -20^0C. The fish were sacrificed by spinal transsection and the gills, intestine and kidney were excised and kept in ice-cold 0.25M SEI buffer (pH 7.1) and stored at -20°C until further analysis.

Determination of plasma glucose, T_3, T_4 and minerals

Plasma glucose was determined colorimetrically using GOD/POD test kits (Span Diagnostics Ltd., New Delhi, India). Plasma T_3 and T_4 levels were measured by enzyme immunoassay (EIA) technique based on the magnetic solid phase separation (Serozyme, Guidonia Montecelio, Italy). The sensitivity of this method was checked by comparison of results from RIA based on competitive binding of ^{125}I-labelled T_3 or T_4 (Peter *et al*., 2000) with the EIA results (Peter *et al*., 2007). The plasma Na^+ and K^+ were measured using a flame photometric analyzer (Systronics 129, New Delhi) using standards (Remedix Diagnostics, Palakkad, India).

Na^+, K^+-ATPase specific activity

The ouabain-sensitive Na^+, K^+ dependent adenosine triphosphatase (Na^+, K^+-ATPase, E.C. 3.6.3.9) specific activity was measured in tissue homogenates as described elsewhere (Verbost *et al*., 1994; Peter *et al*., 2000). Saponin (0.2 mg.mg^{-1} protein) was routinely added to optimize substrate accessibility. About 100 mg each of gill filaments scrapped from the gill arch, kidney and anterior portion of intestine were separately homogenized in 2 ml 0.25 M SEI buffer (pH 7.1) and centrifuged at 700 x g for 10 min. The supernatant was used to measure the specific activity of Na^+, K^+-ATPase and 10 ml samples was added to all test tubes containing ATP mixture with or without ouabain (Sigma Chemical Co., St. Louis, MO, USA). The samples were incubated for 15 min at 37ºC in a medium containing 100 mmol. L^{-1} NaCl, 30 mmol L^{-1} imidazole, pH 7.4, 0.1 mmol L^{-1} EDTA, 5 mmol L^{-1} $MgCl_2$ and either 15 mmol L^{-1} KCl (medium A) or 1 mmol L^{-1} ouabain (medium E). Na_2ATP was added to a final concentration of 3 mmol L^{-1}. The reaction was stopped by adding 1.5 ml of ice-cold 8.6% TCA solution. The liberated inorganic phosphate, P*i*, was quantified spectrophotometrically (Systronics 2202, New Delhi). The specific activity of Na^+, K^+-ATPase was defined as the difference between the release of P*i* in medium A and in medium E, and was expressed as µmol P*i*$^{-1}$ h^{-1} mg protein^{-1}.

Statistics

Data from six fish in each group were statistically analyzed adopting one-way analysis of variance supplemented by SNK multiple comparison test using *Graphpad* software. Statistical significance was accepted at $P<0.05$. The values are depicted as mean ± SEM for six fish.

Results

Plasma glucose, T_3 and T_4

The plasma glucose in the lake water treated tilapia remained unaffected compared to the freshwater control (Fig. 1A). Neither CHRE exposure nor recovery from it altered the plasma glucose. The plasma T_3 did not respond to CHRE exposure or recovery (Fig. 1B). The plasma T_4 increased significantly ($P<0.001$) in tilapia following CHRE exposure and declined in the fish kept for recovery (Fig. 1C).

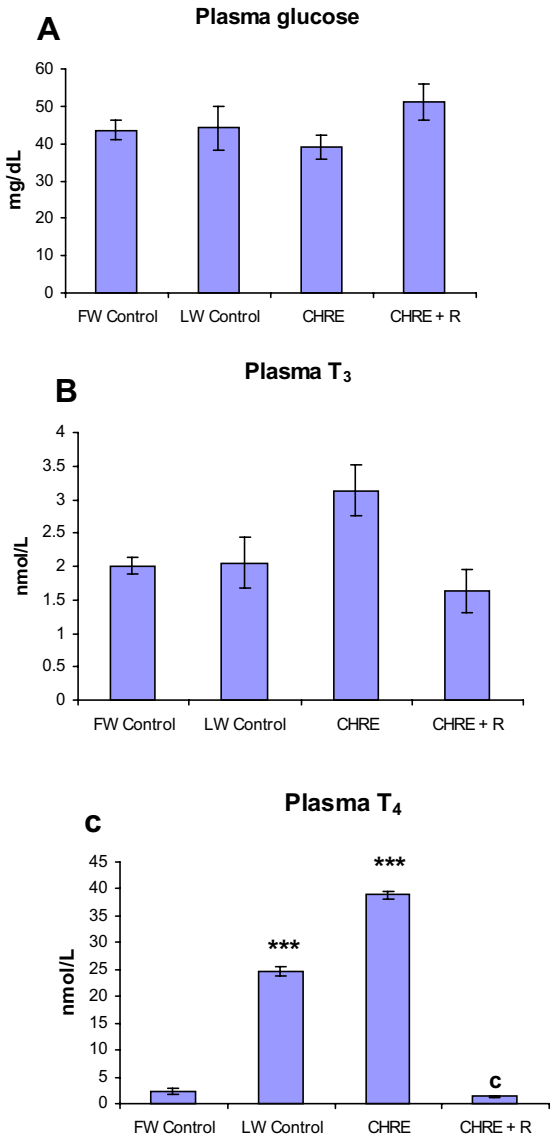

Fig.1. Levels of plasma glucose (A) and plasma T_3 and T_4 (B and C) in tilapia exposed to dilute lake water (LW; 1:4) rich in coconut husk retting effluent (CHRE) for 48h with or without recovery (R) for 96h in clean water. Each column is mean ± SEM for six fish. Statistical differences between fish groups were quoted after SNK test.
*** P<0.001 c: P<0.001, when compared to CHRE-exposed fish.

Na^+, K^+-ATPase activity

The branchial Na^+, K^+-ATPase activity increased significantly (P<0.05) in the CHRE-exposed tilapia and such an effect was not reversed completely in fish kept for recovery (Fig.2A). The renal Na^+, K^+-ATPase activity in the lake water tilapia decreased to significant levels (P<0.05) (Fig. 2B). The kidney of CHRE-exposed fish showed no change in the Na^+, K^+-ATPase activity while its activity declined (P<0.05) in fish kept for recovery (Fig. 2B). The intestinal Na^+, K^+-ATPase activity decreased significantly (P<0.05) in the tilapia exposed to CHRE but there was no change in fish in the recovery group (Fig. 2C).

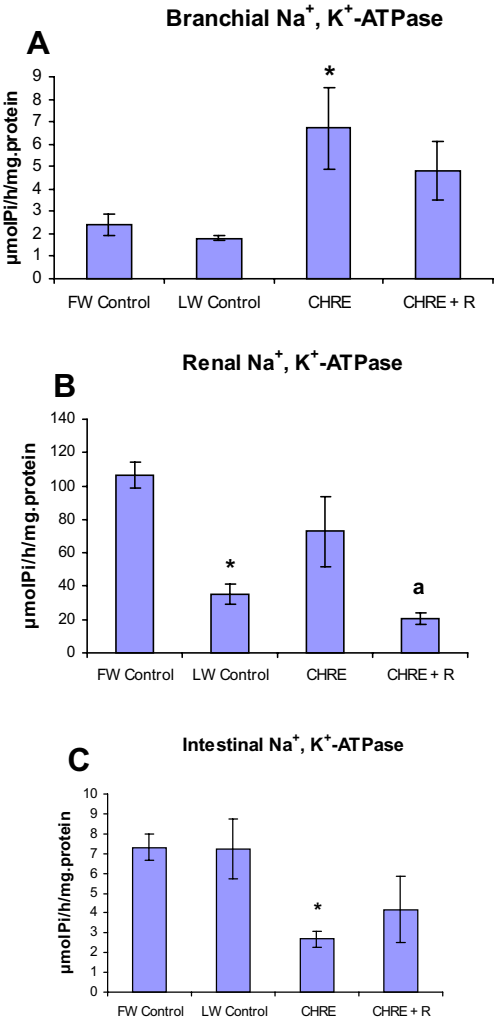

Fig. 2. Levels of Na^+, K^+-ATPase activity in the gills (A), kidney (B) and intestine (C) of tilapia exposed to dilute lake water (LW; 1:4) rich in coconut husk retting effluent (CHRE) for 48h with or without recovery (R) for 96h in clean water. Each column is mean ± SEM for six fish. Statistical differences between fish groups were quoted after SNK test.

* P<0.05 a: P<0.05, when compared to CHRE- exposed fish.

Minerals

The Na^+, Ca^{2+} and PO_4^{3-} in the plasma remained unaffected in tilapia either exposed to CHRE alone or with recovery (Table 1).

Status	Plasma ions (mmol/L)			
	Na^+	K^+	Ca^{2+}	PO_4^{3-}
FW Control	144.17±0.94	5.72±0.11	2.13±0.12	1.66±0.09
LW Control	142.00±3.34	6.86±0.63	2.07±0.08	1.75±0.12
CHRE	141.60±2.73	3.91±0.20*	2.21±0.09	1.64±0.11
CHRE+R	143.17±1.99	6.96 ±0.76	2.08±0.11	1.44±0.09

* P <0.05 compared to LW control

Discussion

The results indicate that the presence of CHRE in the lake water for 48 h causes activation of thyroid and modification of the osmoregulatory capacity in tilapia.

Plasma glucose, an index of stress condition in fish (Wendelaar Bonga, 1997), did not change after CHRE exposure. The absence of hyperglycemia clearly indicates a low level of stress status in CHRE-exposed tilapia. This, further, points to an inactivated endocrine stress axis in tilapia with restricted cortisol and adrenaline surge during CHRE exposure. On the contrarya hyperglycemic effect of stressors has been demonstrated in fish (Vijayan et al., 1997). It is known in fish that hyperglycemia, a metabolic response, occurs due to the activation of sympathetic-chromaffin or/and pituitary-interrenal axes resulting in the release of catecholamines and cortisol (Wendelaar Bonga, 1997). Furthermore, glycogenolysis and subsequent hyperglycemia are the well documented responses in fish to various pollutants, revealing the state of stress condition in fish (Fivelstad et al., 1993; Li, 1996; Peter et al., 2004, 2007).

Exposure of tilapia to CHRE increased the plasma T_4, highlighting a probable thyroidal involvement during chemical exposure. This further implies that a higher plasma T_4 may ensure the normal basal level of plasma T_3 in the CHRE-treated tilapia. Disturbances in the thyroid function have been shown in fish after xenobiotic exposure (Brucker-Davis, 1998). For example, exposure of catfish, *Heteropneustes fossilis* and *Clarias batrachus* to malathion and endosulfan caused changes in circulating thyroid hormones (Sinha et al., 1991; Yadav and Singh,1986). A decrease in T_3 has been reported in rainbow trout exposed

to acidic water (Brown et al., 1990) and starvation (Oommen and Matty, 1991). Thus, the activation of thyroid in tilapia clearly points to a role of THs during chemical stress. Many of the reported inconsistencies of thyroidal involvement in osmotic regulation are generally explained on the basis of their synergistic action with other hormones. In this context, the unaffected plasma T_3 in the CHRE-treated tilapia may be associated with the inactivation of stress axes including the interrenals. It appears that the unaffected T_3 could be due to the less utilization of this hormone by cortisol since cortisol is known for its clearance effects on plasma T_3 (Peter, 2007). The activation of thyroid in tilapia, as revealed from T_4 surge, may support an independent effect of TH on osmoregulation during CHRE exposure, though direct action of other osmoregulatory hormones on thyroid function cannot be ruled out (Leatherland, 1994).

Chloride cell and its Na^+, K^+-ATPase regulate Na^+ and K^+ distribution in fish during salinity acclimation (Karnaky, 1998; Evans et al., 2005). The elevated Na handling in the gills of tilapia exposed to CHRE may reflect an increased influx of Na^+ since many stressors have been shown to induce compensatory mechanisms in branchial ion transport (Arends et al., 1999). The elevated branchial Na^+, K^+-ATPase activity, thus, appears to be a part of general compensatory adaptive mechanism existing in the gills during toxicant exposure. It is evident that a number of stressors such as exposure to mercury Stagg et al., 1992), cadmium (Suresh et al., 1995) and changes in water temperature (Iger et al., 1995) decrease branchial Na^+, K^+-ATPase activity in freshwater fish. In addition, organochlorine pesticides and polychlorinated biphenyls have been shown to inhibit Na^+, K^+-ATPase activity in rainbow trout (Davis et al., 1972).

The increased branchial Na^+, K^+-ATPase activity after CHRE exposure also implies that these toxicants may interfere with permeability of branchial cell membrane necessitating an elevated Na^+, K^+-ATPase activity to restore the ion balance. This is consistent with the observation that water-borne toxicants enhance the permeability of membranes to ions (Wendelaar Bonga, 1997). Although CHRE exposure did not change the Na^+, K^+-ATPase activity in the kidney, a reversal in the activity was recorded in the tilapia kept for recovery. This suggests a major role of renal Na^+, K^+-ATPase in the compensatory mechanism of water and mineral balance in tilapia during chemical intoxication.

The osmotic and ionic physiology of intestine varies among freshwater and seawater fishes. The intestinal epithelia, which possess rich Na^+, K^+-ATPase activity, generally absorb Na^+ In the present study, significant reduction in the Na^+, K^+-ATPase activity was observed in CHRE-exposed tilapia, suggesting a decreased uptake of Na^+ from the intestinal lumen in freshwater and secrete Na^+ in seawater. This observation agrees with the pattern of activity of branchial and renal Na^+, K^+-ATPase after CHRE exposure. The results suggest that CHRE exposure, while maintaining the basal mineral contents in the plasma, demands modifications of Na^+ pump activity in the osmoregulatory epithelia of tilapia.

Na^+, K^+-ATPase activity also contributes to the secondary mechanism of Ca^{2+} balance and freshwater fish greatly depend on their gills as the primary site for Ca^{2+} uptake from water (Flik et al., 1985). In the present study, there is no change in Ca^{2+} and PO_4^{3-} levels after CHRE exposure, suggesting a tight regulation of Ca^{2+} and PO_4^{3-} balance in the CHRE-exposed fish. This is in contrast to the evidences reported on the hypocalcaemic effect of many toxicants (Flik et al., 1984), which are generally considered as a part of stress response in fishes (Li et al., 1998).

It is concluded that tilapia has an efficient osmoregulatory mechanism to tolerate the sublethal toxicity of CHRE where thyroid has a major role to play.

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