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Original Article

Physiological responses of African catfish (*Clarias gariepinus*) to water-borne ferric iron: Effects on thyroidal, metabolic and hydromineral regulations

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Summary

With a view to understand the mechanism associated with the tolerance of excess water-borne iron, thyroidal, hydromineral and metabolic responses were studied in the freshwater African catfish *Clarias gariepinus* after exposing them to nominated concentrations (6.2 and 62 μ*M*) of ferric iron [Fe(III)] for 48 hr. Plasma triiodothyronine (T_3) and thyroxine (T_4) concentrations and the indices of metabolic and hydromineral regulations were analyzed in the iron-treated fish. Plasma T_4 ($P<0.001$) and T_3 ($P<0.05$) decreased in catfish after Fe(III) treatment. On the contrary, an elevated ($P<0.001$) plasma T_4 occurred in the fish kept for recovery in clean freshwater after iron-treatment. A significant ($P<0.01$) hyperglycemia was observed in 62 μ*M* Fe(III)-treated fish whereas plasma urea concentration remained unchanged. Ouabain-dependent Na^+, K^+- ATPase activity increased ($P<0.05$) in the branchial tissue of 6.2 μ*M* iron-treated fish but decreased in the renal ($P<0.05$), intestinal ($P<0.01$) and hepatic ($P<0.01$) tissues. Plasma Na^+ and PO_4^{2-} decreased ($P<0.05$), whereas plasma K^+ and Ca^{2-} increased ($P<0.05$) after Fe(III) treatment. Our results indicate that despite the modification in the metabolic and hydromineral regulation, water-borne Fe(III) suppresses the thyroid activity, and withdrawal of Fe(III) activates the thyroid function in African catfish, thus supporting the hypothesis that thyroid hormones are involved in iron handling in fish.

Key words: Catfish, *Clarias gariepinus*, ferric iron, osmoregulation, thyroid, fish

Introduction

Iron plays many fundamental roles in cellular metabolism and is an essential nutrient to almost all organisms including fishes. Iron homeostasis is tightly controlled *via* its uptake since there is no known regulated excretory mechanism for iron. In fish, iron uptake from the water *via* gills is probably negligible (Andersen, 1997), although recent evidences suggest a role for gills in iron acquisition (Peter et al., 2007; Cooper et al., 2007). Iron can also vary its redox state and can be rapidly oxidised from Fe^{2+} to Fe^{3+} (ferrous to ferric iron) in the presence of oxygen-generating superoxide anion through a series of redox reactions (De Silva et al., 1996; Aisen et al., 2001). Thus, iron can both be toxic and beneficial to organisms and its status in the body must be carefully regulated to provide sufficient iron for biological functions, whilst avoiding excess Fe since it induces oxidative stress (Baker et al., 1997).

In fish, acquisition of iron relies on many physiological mechanisms, which depends on both extrinsic and intrinsic factors. For example, fish with low iron status (i.e., low haematocrit) had enhanced ferrous iron uptake (Bury et al., 2001). On the other hand, epithelial mucus secretion, which makes an appropriate microclimate for metal solubility and transport (Glover and Hogstrand, 2002), provides a suitable environment for gills to absorb ions (Powell et al., 1999a, b). Surface waters have a small concentration of iron, as iron is usually oxidized to insoluble ferrous hydroxide and precipitates. The direct toxicity of iron is generally low but the lethal effects have been noted on exposure of fish to ferrous iron on poorly buffered low pH water (O'Neil, 1993). The insolubility of iron in the aquatic environment makes it unavailable for uptake by fish through gills rendering them derive daily iron requirements from the diet (Davis and Gatlin, 1991; Andersen, 1997; Watanabe et al., 1997; Bury et al., 2001).

The physiological response of fish to water-borne iron is not well understood. To elucidate the mechanisms associated with the tolerance of excess iron, thyroidal,

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metabolic and hydromineral responses of African catfish *Clarias gariepinus* to water-borne ferric iron were studied, and plasma T_3 and T_4, Na^+, K^+-ATPases activity, plasma metabolites and minerals were analyzed.

Materials and Methods

Fish

African catfish *Clarias gariepinus* weighing 51 ± 3 g were collected and kept in large glass tanks. They were acclimated in well water at 28 ± 1°C under natural photoperiod (12 L/12 D) for three weeks prior to experiment. Fish were fed with fish feed at a ration of 1.5% of body mass per day and food was withdrawn for 24h prior to sacrifice to ensure optimum experimental conditions.

Protocol

Two weeks prior to experiment, fish were divided into four groups of six each and kept in 60 L glass tanks. Fish groups II and III were exposed to 6.2 µ*M* and 62 µ*M* Fe(III) (as $FeCl_3$) respectively for 48 h. Fish in group IV were exposed to 62 µ*M* Fe(III) for 48 h and then kept in clean well water for 96 h. Fish in group 1 served as control.

Sampling and analysis

Fish were anaesthetized in 0.1% 2-phenoxyethanol (Sigma, St. Louis, MO, USA). Blood was collected from the caudal vessels using heparinized syringe. The blood was centrifuged at 5000 x g for 5 min at 4ºC and the plasma was separated and stored at -20°C until analysis. The fish were sacrificed by spinal transection and gills, kidney, liver and intestinal tissues were excised, kept in ice cold 0.25 *M* SEI buffer (pH 7.1) and stored at -20ºC.

Determination of plasma T_3 and T_4

Plasma T_3 and T_4 levels were determined by enzyme immunoassay (EIA) technique based on the magnetic solid phase separation (Serozyme, Guidonia Montecelio, Italy). The sensitivity of this method was checked by comparing the EIA results with the RIA based on competitive binding of ^{125}I-labelled T_3 or T_4 and the plasma hormones (Peter et al., 2000).

Plasma metabolites and minerals

Plasma glucose and urea concentrations were quantified colorimetrically using standard method of GOD/POD test kit (SPAN Diagnostics, India, and DAM kit, (SPAN Diagnostics, India) with a Spectrophotometer 2202 (Systronics, New Delhi, india). Plasma Na^+ and K^+ were measured with a flame photometric autoanalyser (Systronics, New Delhi, India) and plasma Ca^{2+} and PO_4^{2-} levels were measured using Sigma diagnostic kits.

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 by Peter et al. (2000). Briefly, about 100 mg each of gill filaments scrapped from the gill arch, kidney and anterior portion of intestine were separately homogenized in 0.25 *M* SEI buffer (pH 7.1) and centrifuged at 700 x g. The supernatant was used to measure the specific activity of Na^+, K^+-ATPase. Saponin (0.2 $mg.mg^{-1}$ protein) was routinely added to optimize substrate accessibility. The samples were incubated at 37ºC in a medium containing 100 mMol. L^{-1} NaCl, 30 mMol L^{-1} imidazole, 0.1 mMol L^{-1} EDTA, 5 mMol L^{-1} $MgCl_2$ (pH 7.4) 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 placing in ice-cold 8.6% TCA solution and the liberated inorganic phosphate, P*i*, was quantified spectrophotometrically. 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 Pi^{-1} h^{-1} mg $protein^{-1}$.

Statistics

Data were collected from six animals in each group. Before statistical analyses, data were checked for normal distribution and variance homogeneity. All data were submitted to two-way analysis of variance (ANOVA) followed by Student-Newman-Keul's test when required, at $P<0.05$. Significance between groups was tested by ANOVA and SNK comparison test at 5% significance level (GraphPad Instat 3, San Digeo). Values are in mean ± standard error of six fish.

Results

Thyroidal response

The plasma T_3 showed a significant ($P<0.05$) decline after 62 µ*M* Fe(III) loading for 48 h (Fig. 2A). However, fish kept for 96 h recovery in freshwater after Fe(III) treatment had no effect on plasma T_3 (Fig. 2A). Loading of Fe(III) in water produced a decline in the plasma T_4 concentration in a dose-dependent manner (Fig. 2B). Catfish pre-treated with Fe(III) and kept for recovery reversed the downregulated thyroid activity as was evident from the increased plasma T_4 (Fig. 2B).

Fig. 1A

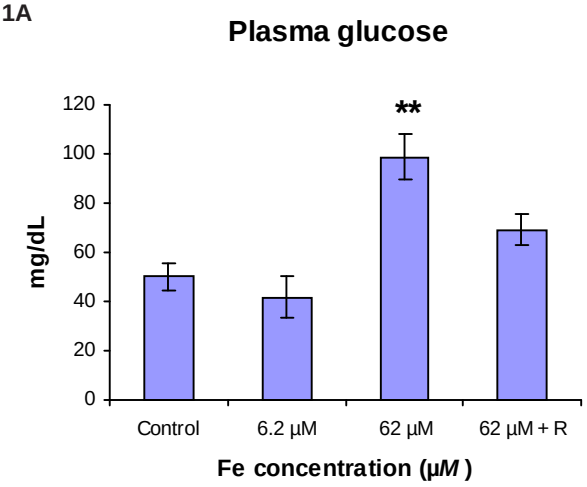

Fig. 1B

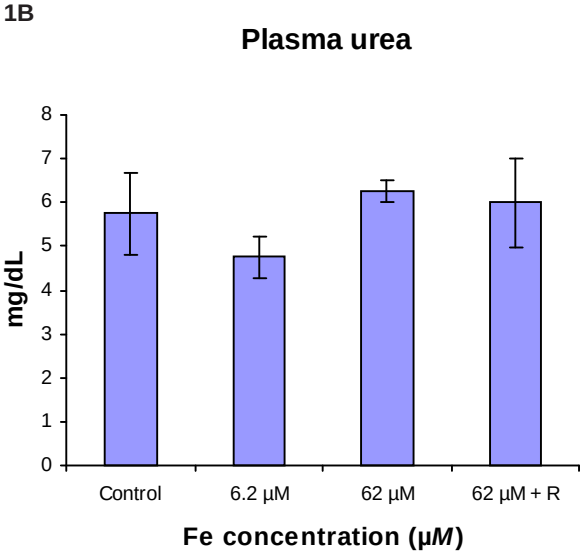

Fig. 1. Plasma glucose (A) and plasma urea (B) levels in Fe(III) (6.2 and 62 µ*M* $FeCl_3$) treated *Clarias gariepinus* for 48 h with or without 96 h recovery (R). Each column represents mean ± SEM for six fish.

** $P < 0.01$ compared to control fish

Fig. 2A

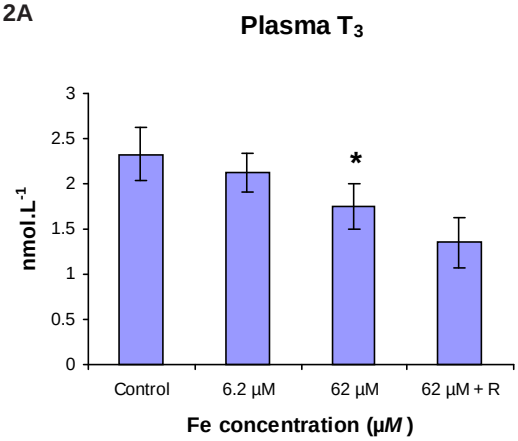

Fig. 2B

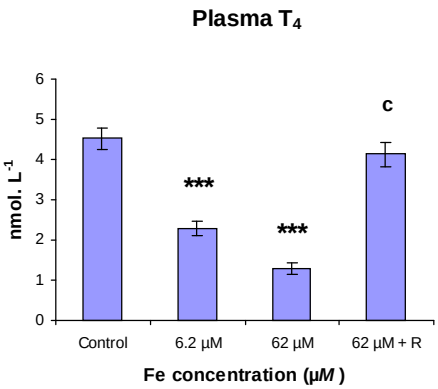

Fig. 2. Plasma T_3 (A) and T_4 (B) levels in Fe(III) (6.2 and 62 µ*M* $FeCl_3$) treated *Clarias gariepinus* for 48 h with or without 96 h recovery (R). Each column represents mean ± SEM for six fish.
* $P < 0.05$; *** $P < 0.001$ compared to control fish
c: $P < 0.001$ compared to 62 µ*M* Fe(III)-treated fish.

Fig. 3A

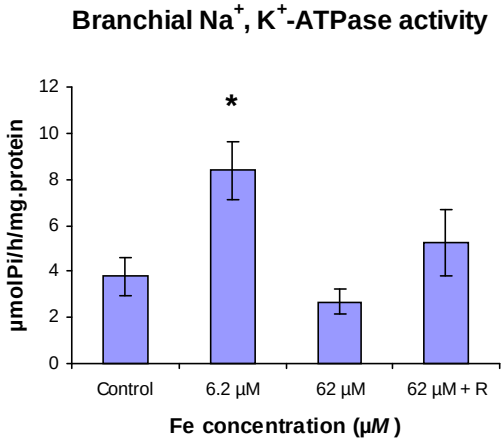

Fig. 3B

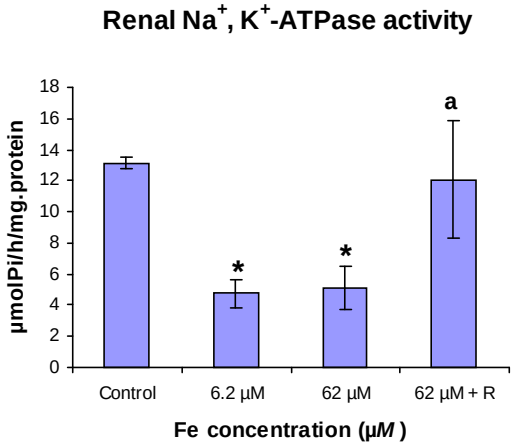

Fig. 3. Na^+, K^+-ATPase activity in the gill (A) and kidney (B) of Fe(III) (6.2 and 62 µ*M* $FeCl_3$) treated *Clarias gariepinus* for 48 h with or without 96 h recovery (R). Each column represents mean ± SEM for six fish.
* $P < 0.05$ compared to control fish
a: $P < 0.05$, compared to 62 µ*M* Fe(III)-treated fish.

Fig. 4A

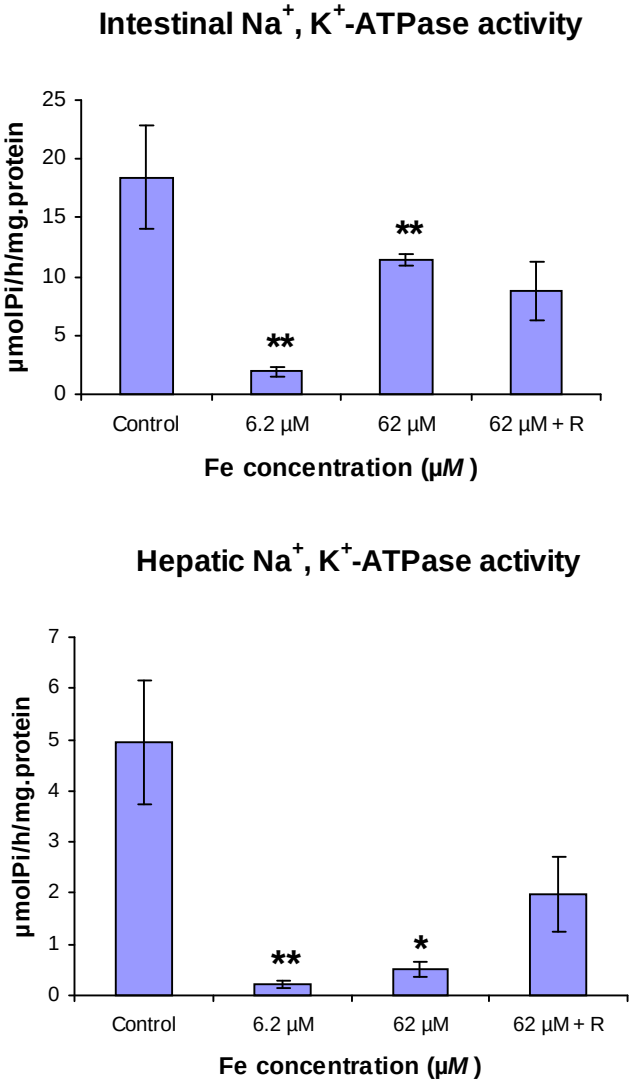

Fig. 4. Na^+, K^+-ATPase activity in the intestine (A) and liver (B) of Fe(III) (6.2 and 62 µ*M* $FeCl_3$) treated *Clarias gariepinus* for 48 h with or without 96 hr recovery (R). Each column represents mean ± SEM for six fish.

* $P< 0.05$; ** $P< 0.01$ compared to control fish

Metabolic response

A significant ($P<0.01$) hyperglycemia occurred in 62 µ*M* Fe(III)-treated catfish, whereas low dose (6.2 µ*M*) produced little effect (Fig. 1A). A decreased plasma glucose concentration was recorded in the fish kept for 96 h recovery after 62 µ*M* Fe(III) (Fig. 1A). The plasma urea concentration remained unaffected in both iron-loaded fish and the fish kept for recovery (Fig. 1B).

Hydromineral response

Low dose of water-borne Fe(III) produced a significant ($P<0.05$) increase in the branchial Na^+, K^+-ATPase activity (Fig. 3A). Neither a high dose of Fe(III) nor its recovery produced any change in the branchial sodium pump activity (Fig. 3A). Significant decrease in the Na^+, K^+-ATPase activity in the renal (Fig. 3B), intestinal (Fig. 4A) and liver tissues (Fig. 4B) was observed in Fe(III)-treated fish. A reversal of Na^+, K^+-ATPase activity in the liver (Fig. 4B) was observed in fish kept for recovery. However, intestinal Na^+, K^+-ATPase activity failed to reverse after recovery (Fig. 4A).

Water-borne Fe(III) at 6.2 µ*M* concentration reduced plasma Na^+, but not with 62 µ*M* Fe(III) treatment (Table 1). Conversely, plasma K^+ showed a significant increase at the lower dose, whereas the higher dose had little effect. Plasma Ca^{2+} increased, whereas plasma PO_4^{2-} decreased after iron loading (Table 1). The fish kept for recovery in clean water for 96 h, however, did not show any change in the plasma minerals (Table 1).

Discussion

This study is the first to demonstrate that thyroid is involved in iron handling in catfish. In addition, the results also reveal a disturbed metabolic and hydrominieral regulations in Fe(III)-treated fish.

Thyroid hormones (THs) are known for their osmoregulatory and metabolic effects (Leatherland, 1994; Peter et al., 2000; Oommen et al., 2006), and are involved in the regulation of stress tolerance in fish (Leji et al., 2007; Peter et al., 2007; Peter and Peter, 2007). Our results indicate that thyroid is involved in the handling of iron as evident in the fall of T_4 in iron-treated fish and its subsequent rise in the fish kept for recovery after iron treatment. It is likely that fish kept for recovery relied on T_4 to handle the iron-induced toxicity as enhanced antioxidant production by THs has been demonstrated in perch tissues (Oommen et al., 2006). Despite the extensive studies relating thyroid function and iron uptake in mammalian systems (Campbell et al., 1992; Shvets et al., 1997; Eftekhari et al., 2006a, b; Dabbaghmanesh et al., 2008), little is known about the thyroid activity in relation to iron handling in fish. Iron deficiency has been shown to disrupt thyroid metabolism in mammals. For example, iron deficiency anemia brings about decreased circulating levels of T_3 and T_4 by 20–40% (Hess et al., 2002). In the rat, iron deficient anemia leads to decrease in plasma T_3 and T_4, reduced activity of hepatic thyroxine deiodinase, impaired peripheral conversion of thyroxine to

Table 1. Plasma ions (mmol.L^{-1}) in *Clarias gariepinus* treated Fe(III) as $FeCl_3$ for 48 h with or without 96 h recovery (R). Values are mean ± SEM of six fish.

Exptl Status	Na^+	K^+	Ca^{2+}	PO_4^{2-}
Control	116.10 ± 1.10	5.33 ± 0.41	2.34 ± 0.18	1.87 ± 0.08
Fe(III) 6.2 µM	97.10 ± 2.50*	10.53 ± 1.40*	2.66 ± 0.08	1.07 ± 0.05*
Fe(III) 62 µM	109.50 ± 2.80	6.06 ± 0.81	3.29 ± 0.21*	1.49 ± 0.07
Fe(III) 62 µM + R	118.30 ± 4.40	7.40 ± 0.11	2.70 ± 0.10	1.58 ± 0.09

triiodothyronine, and blunting of thyrotropin response to TRH (Beard et al., 1998). It is likely that a compensatory endocrine mechanism exists in the iron-exposed catfish which may demand a lowered T_4.

The involvement of thyroid in iron handling is also substantianted by the remarkable changes in the osmotic and metabolic regulations in this fish. It is argued that catfish handles excess iron by changing T_4 availability and thus modifies its action on metabolic and osmotic regulations. Studies on the sensitivity of thyroid to many toxicants have gained much attention and a number of endocrine disrupting compounds have been identified for their thyroid disrupting ability (Schmutzler et al., 2007). Similarly, disturbed thyroid function has been demonstrated in a number of fishes 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 inhibition of thyroid activity clearly points to the sensitivity of thyroid axis to iron handling in fish.

Hyperglycaemia is often considered as a reliable index of stress response in fish (Wendelaar Bonga, 1997). An indication of stress and a high energy demand during the initial phase of stress response could thus be ascribed to the observed hyperglycaemia in Fe(III)-loaded catfish. This further implies that catfish experiences stress and may depend on hypothalamo-pituitary-interrenal (HPI) or brain-sympathetic-chromaffin (BSC) axes to release cortisol or adrenalin as hormonal support, since induction of hyperglycemia by these hormones has been demonstrated earlier (Balm et al., 1994; Wendelaar Bonga, 1997; Iwama et al., 2006). Increase in plasma glucose is partly due to catecholamine surges since it mobilizes energy resources to fuel stress response in fish (Reid et al., 1998). Although this metabolic adaptation enables the stressed fish to derive more energy, as has been suggested earlier (Peter et al., 2004), supplementing excess iron appears to be stressful to fish. On the other hand, absence of a change in the plasma urea indicates an undisturbed metabolic status in catfish even with a high dose Fe(III) loading, though increase in blood urea appears to be a part of stress response in fish (Barton and Schreck, 1987).

As an essential sodium ion transporter generating transmembrane Na/K gradient across cell membrane, Na^+, K^+-ATPase is involved in the transport of many ions and regulates many cellular functions (Evans, 1998). The upregulation of branchial sodium pump activity observed at 6.2 µ*M* water-borne Fe(III) associated with declined plasma Na^+ and elevated plasma K^+ levels, suggests a disturbed Na^+ homeostasis in this catfish. The chloride cells in the gill epithelia that harbor Na^+, K^+-ATPase become the target site for Fe(III) handling. It appears that an increased gill permeability resulting in the increased iron absorption at its low dose by the gill epithelia occurs in the Fe(III) treated fish, although high dose does not favor its absorption through the gill epithelia. Interestingly, at this iron concentration, sodium pump activity in the intestine, kidney and liver reflected down-regulation, indicating a decline in iron handling through these tissues. The down-regulated Na^+ pump activity in these tissues, except gill, further supports the notion that excess iron disturbs the hydromineral balance. It appears that freshwater catfish possesses compensatory physiological mechanisms to maintain iron homeostasis through regulating gill iron acquisition since gills have been shown to absorb iron (Cooper and Bury, 2007; Peter et al., 2007), though the exact mechanism of iron acquisition by this tissue remains unknown.

Overall, our results suggest that thyroid hormones are involved in iron handling in catfish by way of

suppressing the thyroid axis upon Fe(III) exposure and its activation during withdrawal. Disturbances in metabolic and hydromineral homeostasis are also the consequences of excess Fe(III) exposure .

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