

Effect of chronic exposure to sublethal of ammonia concentrations on NADP⁺-dependent dehydrogenases of Nile tilapia liver

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ABSTRACT

The effect of chronic exposure to sublethal ammonia (NH₃) on liver NADPH producing enzymes of Nile tilapia juveniles (*Oreochromis niloticus*) was studied. Fish with an initial weight of 15.0±1.4 g were reared in a static system and exposed to the total ammonia nitrogen (TAN) concentrations 5 or 10 mg L⁻¹ for consecutive 70 days at 26±0.5°C.

NADPH is mainly produced by glucose 6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), and the cytosolic form of NADP-dependent isocitrate dehydrogenase (IDH). These enzymes have been recently considered as an important factor in cellular defense against oxidative damage as they share in maintaining reduced glutathione (GSH) level. The activity of the enzymes G6PDH, 6PGDH, and cytosolic IDH in liver tissues of fish exposed to different concentrations of NH₃ showed significant increase. The degree of increase in activity was positively related to ammonia concentration. The increased activity of these enzymes resulted in higher NADPH availability. This may be interpreted as a defense to maintain a high level of GSH against the expected oxidative stress in ammonia-exposed fish.

Keywords: NADPH, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, cytosolic form of NADP-dependent isocitrate dehydrogenase.

INTRODUCTION

Ammonia is the main nitrogenous products excreted by teleosts, and mainly excreted as the unionized form NH₃ (Smith, 1929). The term ammonia refers to two chemical species which are in equilibrium in water (NH₃, unionized form and NH₄⁺, ionized form). Tests for ammonia usually measure total ammonia nitrogen (NH₃ plus NH₄⁺). The toxicity to ammonia is primarily attributable to NH₃, as opposed to NH₄⁺ and their equilibrium in water depending on pH and temperature (US EPA, 1999). The toxicity of ammonia to fish and other aquatic organisms is primarily attributed to NH₃ and most biological membranes are permeable to NH₃, but relatively impermeable to NH₄⁺ (Hargreaves and Kucuk, 2001; Randall and Tsui, 2002). The averages of the mean NH₃ acute toxicity values for 32 freshwater species were reviewed (Hargreaves and Kucuk, 2001; Randall and Tsui, 2002). The average value was 2.79 mg NH₃ L⁻¹ compared with 1.86 mg NH₃ L⁻¹ for 17 seawater species. The toxicity of ammonia to fishes varies in the different species; rainbow trout is more sensitive than carp, catfish, and silver perch.

Tilapia species are broadly cultivated in the tropical and subtropical regions of the world (FAO, 2007). Elevation in ammonia concentration is one of the limiting factors in fish farming (Randall and Tsui, 2002). Chronic ammonia exposures affect Nile tilapia in several ways, e.g. reduce growth rates (El-Shafai *et al.*, 2004, Hegazi and Hasanein, 2010), gill hyperplasia (Benli *et al.*, 2008), increased brain glutamine (Hegazi *et al.*, 2010b), and decreased brain monoamines and ATPase levels (Hegazi and Hasanein, 2010).

There are many studies that analyzed the contribution of different sources to the formation of reactive oxygen species (ROS) following acute ammonia intoxication in the brain of mammalian models (for reviews see Kosenko *et al.*, 2003 and Norenberg *et al.*, 2007). Recently, it was reported that ammonia exposure induced oxidative stress in brain and gills of the high ammonia tolerant fish mudskipper, *Boleophthalmus boddarti* (Ching *et al.*, 2009) and liver and white muscle of Nile tilapia (Hegazi *et al.*, 2010a).

The antioxidant system either enzymatic or non-enzymatic scavengers protect cells against oxidative damage (Winzer *et al.*, 2002). Most of these pathways use directly or indirectly NADPH to regenerate their reduced forms, especially GSH, to maintain their antioxidant capabilities (Spolarics, 1998). NADPH is one of the principal end products of several metabolic pathways and is also a crucial substrate of reductive biosynthetic reactions (Tomlinson *et al.*, 1988). NADPH produced at different rates in different tissues may indicate a possibility of differential protection against oxidative damage (Kehrer *et al.*, 1993; Lund *et al.*, 1994).

Putatively more important function of the enzymes glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), and cytosolic isocitrate dehydrogenase (IDH), are the main enzymes that generate a reducing equivalent as NADPH. These enzymes are important and acting in the protection of cells against oxidative damage (Winzer *et al.*, 2002), synthesis of most lipids for membranogenesis (Walzem *et al.*, 1991; Koh *et al.*, 2004) and are essential for liver lipogenesis in vertebrates (Koh *et al.*, 2004). The protection of cells against oxidative damage is mainly occurred through regeneration of GSH by glutathione reductase (GR) as it is the master antioxidant in body's organisms (Chae *et al.*, 1994; Dumaswala *et al.* 2001). GSH is a well-known antioxidant and free radical scavenger, which is usually present as the most abundant low-molecular-mass thiol in most organisms. It has various functions in the defense against oxidative stress and xenobiotic toxicity (Meister and Anderson, 1983). NADPH is an important cofactor in many biosynthesis pathways and in the regeneration of GSH. One of its major functions is the detoxification of toxic oxygen metabolites generated in cells (Meister 1981). GSH is involved in many cellular functions including antioxidant defense via direct interaction with ROS or via activities of detoxication enzymes like GSH peroxidases and GSH-S-transferases (Dickinson and Forman, 2002). The oxidized glutathione (GSSG) is reduced by GR in a NADPH-dependent reaction

and the normal implementation of this closed system (redox cycle) maintains a high intracellular GSH:GSSG ratio and protects cells against oxidative damage (Anderson, 1998). Livers of vertebrates are the main organs of biotransformation, and, therefore, they are the organs of choice for monitoring the effects of chemical pollution. The liver is the major source of GSH levels in blood and the chief detoxifier of externally derived (exogenous) toxins, and also is the main storage organ for glutathione prior to exportation to the other organs (Winzer *et al.*, 2002).

As such NADPH is critically important in maintaining the cellular defense against oxidative damage. NADPH is mainly produced by G6PDH, 6PGDH, the cytosolic IDH and its intracellular concentration modulates the oxidative damage to the cells (Lee *et al.*, 2002, Pandey *et al.*, 2003). Recently, it was demonstrated that the control of oxidative damage is one of the primary functions of NADPH. These enzymes provide the reducing equivalents required for GR conversion of glutathione sulfide back to reduced glutathione levels (Pankiewicz *et al.*, 2003, Erdoğan *et al.*, 2005, Kalpakcioglu and Şenel, 2008). In addition, NADPH participates in cell membrane protection and cell detoxification from xenobiotics through the glutathione reductase–peroxidase system for the former function and the mixed-function oxidases for the latter one. For these reasons, the effect of chronic exposure to sublethal concentrations of ammonia on Nile tilapia on the activity of these NADPH-producing enzymes in the liver of *O. niloticus* was studied.

MATERIALS AND METHODS

Fish husbandry

Fingerlings of Nile tilapia were obtained from the fish-hatching pond in Fowa city (Kafr El-Sheikh Governorate, Egypt). The fish were transported in oxygenated cellophane bags on December. Fish were graded, and those within the weight range of 15.0 ± 1.4 g were distributed in 24 glass aquaria of 33 L capacity (3 fish in each). Fish were fed on commercial fish pellets diet (25.2% protein, lipids, carbohydrate, and fibres) of Al- Gharbiyah Rice Mills Company (registered in the Egyptian Ministry of Agriculture, No 5872 at 4-5-2005), at the daily ration rate 3% of fish weight in the morning (10.00 AM). Aquaria were supplied with dechlorinated tap water, equipped with continuous aeration (in order to maintain oxygen level at 7 ± 0.2 mg L⁻¹), sponge biological filters (to provide self-cleaning of the tank) and controlled thermostatic heaters.

Experimental Design

The fish were acclimated to the rearing conditions of the laboratory for 7 days prior to the beginning of the experiment. After that the temperature was raised from the ambient temperature of winter ($15 \pm 2^\circ\text{C}$) by 2°C every two days till they reached to $26 \pm 0.5^\circ\text{C}$ which is the optimal temperature degree for *O. niloticus* maximal growth (Mishrigi and Kobo, 1978). Fish were then left for four weeks at this temperature degree to attain full thermal acclimation. After complete thermal acclimation, the aquaria were classified to three equal groups

and brought to contain one of the three different total ammonia nitrogen (TAN) concentrations 0 (controls), 5 (LL), and 10 (HH) mg L⁻¹ ammonium chloride (NH₄Cl, 99.5% purity) The experiment then was conducted for 70 consecutive days. The experimental ammonia toxicity conditions were conducted in the present experiment as described by Hegazi *et al.* (2010). The NH₃ concentrations in the different sublethal concentrations (Table 1) were calculated according to the equations of Albert (1973) and Emerson *et al.* (1975).

Table 1: Water quality in fish aquaria during the experimental period of chronic ammonia toxicity at 26 °C.

Parameter	Controls	5 mg TAN L ⁻¹	10 mg TAN L ⁻¹
pH	7.6 ± 0.05	7.8 ± 0.06	8.0 ± 0.08
DO saturation mg L ⁻¹	7±0.2	7±0.2	7±0.2
NH ₃ mg N L ⁻¹	0.053	0.185	0.575
Nitrite mg N L ⁻¹	0.042 ± 0.007	0.044 ± 0.005	0.46 ± 0.005
Nitrate mg N L ⁻¹	0.065 ± 0.008	0.079 ± 0.006	0.088 ± 0.007
Total hardness mg CaCO ₃ L ⁻¹	120 ± 1	120 ± 1	120 ± 1
Total alkalinity	127 ± 3	128 ± 4	129 ± 6

Cytosolic enzyme preparation

The procedure described by Moon and Ouellet (1979) and modified by Hegazi *et al.* (2010) was used for preparing the cytosolic enzymes. At the end of the experiment fish were killed by a rapid blow on the head. The right lobe of liver was excised for enzyme estimations to avoid the contamination by the left lobe of liver which contains the scattered pancreas. Liver tissue was homogenized (1:10 W/V) in cold buffer (4 °C) using all-glass manual homogenizer immersed in ice. The homogenizing buffer contained 20 N-2-hydroxyethyl-piperazine-N-2-ethane sulphonate (HEPES) pH 7.6 (adjusted by NaOH), 1mM EDTA, 30 mM β-mercaptoethanol 225 mM mannitol, 50 mM sucrose. The homogenate was centrifuged at 4 °C using a cooling centrifuge at 12000 x g. The resulting cytosolic supernatant was carefully collected avoiding the contamination with the upper lipid layer at the top of the tubes and immediately used for enzyme assays.

Enzymes assay

The enzymes were assayed by monitoring the increase in NADP absorbance ($\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ at 340 nm) using an UV/vis Spectrophotometer (JENWAY 6505 UK) at 25°C. The reaction was started by the addition of the

extracted cytosolic supernatant to the reaction mixture. The reaction was allowed to proceed for at least 4 minutes. All reactions were adjusted to be linear with respect to the reaction time, enzyme substrate to give V_{max} , and enzyme concentration during a detectable period for calculation of enzyme activity.

Assay of glucose-6-phosphate dehydrogenase (G6PDH) in tissue extract is interfered with by 6-phosphogluconate dehydrogenase (6PGDH) because 6-phosphogluconate formed by G6PDH is reoxidized by 6PGDH and forms another NADPH (WHO, 1967). G6PDH activity is determined by subtracting 6PGDH activity from the total activity of G6PDH and 6PGDH measured by spectrophotometric assay (WHO, 1967). The enzymes G6PDH and 6PGDH (Kagawa *et al.*, 1964) and NADP linked isocitrate dehydrogenase (decarboxylating) (Ochoa, 1955) were assayed.

- A. Total activity of glucose 6-phosphate dehydrogenase (G6PDH, E.C. 1.1.1.49) and 6-Phosphogluconate dehydrogenase (6PGDH, EC 1.1.1.44) was assayed in 68 mM imidazol-HCl (pH 7.4) buffer containing 1 mM EDTA, 2.5 mM MgCl₂, and 30 mM β-mercaptoethanol, 0.6 mM NADP⁺ and 5 mM G6P, and 5 mM 6-phosphogluconate.
- B. 6-Phosphogluconate dehydrogenase (6PGDH, EC 1.1.1.44) was assayed in 68 mM imidazol-HCl (pH 7.4) buffer containing 1 mM EDTA, 2.5 mM MgCl₂, 0.6 mM NADP⁺, and 5 mM 6-phosphogluconate.
- C. Isocitrate dehydrogenase (IDH, E.C. 1.1.1.42): was assayed in 68 mM imidazol-HCl (pH 7.4) buffer containing 1 mM EDTA, 2.5 mM MgCl₂, and 30 mM β-mercaptoethanol, 0.6 mM isocitrate and 0.4 mM NADP⁺.

Statistical Analysis

Data of enzyme activities at the end of the experimental period were analyzed by one-way, analysis of variance (ANOVA) to check the effect of ammonia concentrations on enzyme activity. The effect of NH₃ concentrations in comparison with controls were evaluated by Dunnett's test (compare all vs. controls) using a computer program (GraphPad InStat Software, Inc).

RESULTS

In the present experiment there were slight differences in pH values in fish-aquaria in the TAN concentrations 5 (LL) and 10 (HH) mg N L⁻¹. The calculated concentrations of NH₃ in the aquaria at the corresponding TAN concentration were 0.053 (controls), 0.185 (LL), and 0.575 (HH) mg L⁻¹ at pH 7.6±0.05, 7.8±0.06, and 8.0±0.08, respectively, at 26 °C.

The effects of chronic sublethal LL or HH TAN concentration on the activity of the enzymes G6PDH, 6PGDH, and cytosolic IDH in liver of Nile tilapia, when compared with the controls are presented in Fig. 1, 2, and 3. One-way ANOVA test indicated that the effect of the two TAN concentrations on fish were significant ($P < 0.001$).

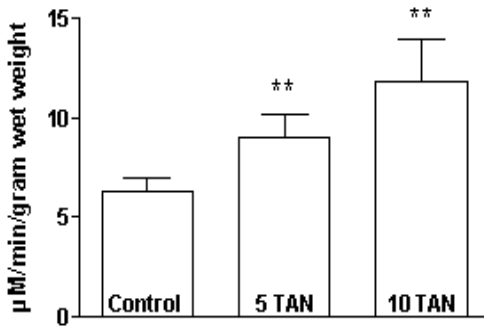


Fig. 1: Effect of NH_3 concentration on the activity of liver G6-PDH of *O. niloticus* juveniles** =significant at $P<0.01$ as compared with the corresponding control value.

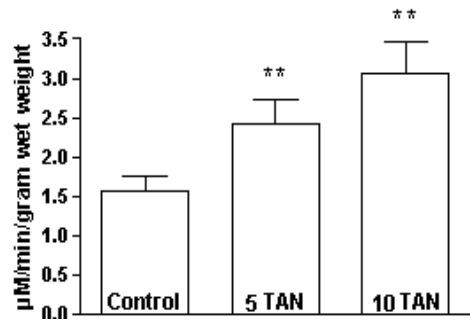


Fig. 2: Effect of NH_3 concentration on the activity of liver 6-PGDH of *O. niloticus* juveniles** =significant at $P<0.01$ as compared with the corresponding control value.

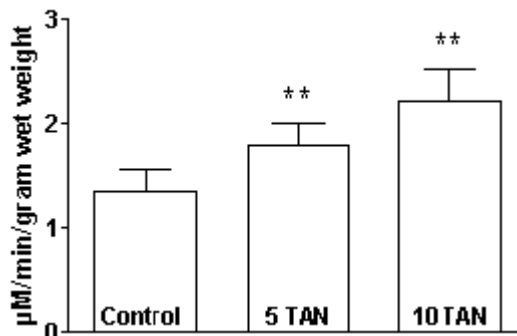


Fig. 3: Effect of NH_3 concentration on the activity of liver IDH of *O. niloticus* juveniles** =significant at $P<0.001$ as compared with the corresponding control value.

There was significant increase in the activity of these enzymes, with respect to LL or HH TAN exposures. The activity of G6PDH was significantly increased ($P<0.01$) by 43.6% and 89.3% in fish exposed to LL, and HH respectively (Fig. 1). The activity of 6PGDH was significantly increased ($P<0.01$) by 55.8% and 96.2% in fish exposed to LL, and HH respectively (Fig. 2). The activity of cytosolic IDH was significantly increased ($P<0.01$) by 33.3% and 64.4% ($P<0.01$) in fish exposed to LL, and HH respectively (Fig. 3).

DISCUSSION

The activities of G6PDH, and 6PGDH were significantly increased in the liver of fish exposed LL or HH, NH_3 concentrations in a manner positively dependent on NH_3 concentrations. To date, there was no available data concerning the effect of NH_3 exposure on these enzymes in fish, except the

effect of ammonia on red blood cell G6PDH of fish. Erdoğan *et al.* (2005) detected severe inhibition in the activity of purified G6PDH red blood cells of rainbow trout *Oncorhynchus mykiss*. Experiments with total ammonia nitrogen (2.2, 2.8 and 3.3 μM) showed the inhibitory effects on the enzyme, in vitro (determined in vitro by Lineweaver–Burk and regression graphs). However, in vivo studies in rainbow trout erythrocytes showed significant ($P < 0.01$) inhibition of G6PDH by total ammonia nitrogen 0.32 mM (5.44 mg L^{-1}) at 10 °C and pH 7.8 after 1, 2 and 3 h ammonia exposure. Free ammonia at these conditions was calculated as 0.063 mg L^{-1} which represents 12.3% of 96-h LC50 rainbow trout, *O. mykiss*. The 96-h LC50 of NH_3 was 0.512 mg L^{-1} for rainbow trout; *O. mykiss* (Person-Le Ruyet *et al.*, 1995, US EPA, 1999). The concentration of NH_3 calculated in the present experiment, represent 3.12% (LL), and 7.78% (HH), at 26°C. The 48-h LC₅₀ of NH_3 was 7.39 mg L^{-1} for Nile tilapia fingerlings (10.1±0.05 g) determined by Benli and Ksal (2005). The differences reported here between enzyme activity in rainbow trout and Nile tilapia may be related to dose level, exposure period, fish susceptibility to ammonia and/or tissue specificity. However, studies on fish species such as *Wallago attu* (Pandey *et al.*, 2003) and *O. niloticus* (Bainy *et al.*, 1996) collected from polluted sites showed that the activity of G6PDH was substantially higher in the fish liver collected from more polluted regions when compared with values collected from less polluted sites.

The first and rate-limiting reaction of the pentose phosphate pathway (PPP) is catalyzed by G6PDH and 6PGDH in addition to that it is considered as an important source for NADPH. Moreover, G6PDH activity is required to supply riboses for DNA and RNA synthesis, particularly in proliferating cells (Winzer *et al.*, 2002). For these reasons, at the cellular level, the continuous supply of ribose is essential to growth and proliferation processes. In addition, G6PDH is a regulatory enzyme in NADPH-dependent xenobiotic biotransformation and defenses against oxidative damage and is very sensitive to inactivation by chronic exposure to pollutants in highly contaminated marine habitats. Moreover, focal alterations in its activity provide a sensitive histochemical parameter to detect early stages of xenobiotic-induced hepatocellular carcinogenesis in humans and rodents before morphological changes appear (Stumpf and Bannasch, 1994). Therefore, G6PDH is not only a relevant experimental marker for carcinogenesis in mammals, but was also identified as a sensitive indicator of early steps of pollution-induced carcinogenesis in fish (Winzer *et al.*, 2002).

The activity of the cytosolic IDH was significantly increased in the liver of fish exposed LL or HH, of NH_3 concentrations positively increased with NH_3 concentrations. The cytosolic NADP^+ -IDH is shown to play an important role in cellular defense against oxidative damage as a source of NADPH (Jo *et al.*, 2001, Lee *et al.*, 2002, Kim and Park, 2003). It is known that vertebrate tissues contain three classes of IDH isozymes: mitochondrial NAD^+ - NADP^+ -dependent and cytosolic NADP^+ -dependent (Koshland *et al.*, 1985). The isocitrate

dehydrogenases catalyze oxidative decarboxylation of isocitrate to 2-oxoglutarate and require NAD^+ or NADP^+ , producing NADH and NADPH, respectively (Koshland *et al.*, 1985). NAD^+ -dependent IDH appears to play a major role in the tricarboxylic acid cycle, while the role of the NADP^+ -dependent enzymes, either cytosolic or mitochondrial, play an essential role in NADPH. Earlier study indicated cytosolic IDH in the rat liver was 16- and 18-fold more active in producing NADPH than G6PDH and malic enzymes, respectively (Veech *et al.*, 1969), suggesting an important role of this isozyme in the production of NADPH and eventually for the cellular defense against oxidative stress. Despite these earlier results, the biological role of the cytosolic IDH in the antioxidant defenses in vertebrate cells has not been established. The data of Lee *et al.* (2002) provide direct evidence for the protective role of the cytosolic IDH against cellular oxidative damage.

NADPH is also required for the formation of active catalase tetramers, where each catalase monomer contains one NADPH binding site necessary for its enzymatic activity (Kirkman and Gaetani, 1999). It was reported that mammalian G6PDH and cytosolic IDH are important in providing NADPH for β -oxidation of fatty acids for growth (Minard *et al.*, 1998, 1999).

The role of G6PDH, 6PGDH and cytosolic IDH in cellular defense against oxidative damage gains progressively escalating support. It is expected that the increase in the activity in these enzymes may strongly attain higher levels of NADPH in the liver tissue of *O. niloticus* juveniles. The protective role of these enzymes against oxidative damage may be attributed to their capability to provide the cellular demand of the reducing equivalent, NADPH, necessary for regeneration of glutathione in the cell and thereby play a key role in cellular defense against oxidative stress-induced damage. This may also indicate a higher lipogenic activity in liver tissues. It seems that the fish species under investigation is capable of producing high levels of NADPH provides higher protection against stress-induced damage for the fish reared at these ammonia concentrations. NADPH produced at different rates in different tissues may indicate a possibility of differential protection against oxidative damage. Increased α -ketoglutarate production augmented by increased IDH activity may share in ammonia trapping to glutamate and glutamate in the cell.

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