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Nitrate Toxicity on Visceral Organs of Medaka Fish, *Oryzias latipes* : Aiming to Raise Fish from Egg to Egg in Space

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Abstract Histological survey was made to determine nitrate toxicity on the Medaka fish, *Oryzias latipes*. In order to investigate the effects of short-term exposure to nitrate, one-month-old Medaka fish was exposed to $NaNO_3$ at concentrations of 100 and 125 mg NO_3-N l^{-1} for 96 hours. At the end of the exposure period, survival rate was found to be 30% and 10%, for the 100 and 125 mg NO_3-N l^{-1} exposure concentrations, respectively. Histological examination of the organs showed that disruption of cell alignment was a common feature in the gills, intestinal ampulla, liver and kidney. A long-term exposure experiment was also carried out, whereby Medaka fish was exposed to $NaNO_3$ (100 and 125 mg NO_3-N l^{-1}) for three months from its egg stage. Eggs treated with $NaNO_3$ hatched within 10 days after fertilization. At the end of the exposure period, survival rate in the 100 and 125 mg NO_3-N l^{-1} treatments were 40% and 30%, respectively. Fibrosis of the hepatic cells and curved spinal column were observed in the juveniles subjected to long-term nitrate exposure. The results of our experiments suggest that the high mortality resulting from short-term acute exposure to nitrate is caused by general dysfunction throughout the whole body. The chronic toxic effects attributed to nitrate, following long-term exposure, were likely to have resulted from nutrient deficiency caused by hepatic dysfunction.

Keywords; Nitrate toxicity, Histological observation, Medaka fish, *Oryzias latipes*, Visceral organs

Introduction

Based on the space experiment conducted at the Second International Microgravity Laboratory (IML-2) in 1994, aboard the space shuttle (mission STS-65), Medaka fish (*Oryzias latipes*) was confirmed to be capable of normal reproductive processes, such as mating, fertilization, embryonic development and hatching at the absence of gravity (Ijiri, 1995). It suggests that Medaka fish can potentially complete its life cycle under microgravity. However, for this aim to be achieved, it is necessary to develop a biological filter system that can operate efficiently in space to maintain water quality at an acceptable level for the long-term breeding of Medaka fish. Conventional biological filter systems, being used in the past space missions (Nakamura *et al*., 1998; Nagaoka, *et al*., 1999; Shimura *et al*., 1999; Uchida, *et al*., 1999), relied only on the action of nitrifying bacteria for the removal of inorganic nitrogen compounds. Since these missions were short in duration (not exceeding two weeks), simple methods such as the conventional biological filter system could be employed to control water quality. Biological filter systems that rely on the nitrifying bacteria are prone to the buildup of nitrate produced by the nitrification of ammonia. For the long-term maintenance of aquatic animals such as Medaka fish, it is therefore necessary to include a denitrifying step, in order to convert nitrate into non-toxic nitrogen gas. Nitrate is considered to be the least toxic for fish among inorganic nitrogen compounds (Westin, 1974; Colt & Tchobanoglous, 1976; Tomasso & Carmichael, 1986). However, after exposure to nitrate at different life stages of Medaka fish, it was found that, even at relatively low concentrations, nitrate can affect embryonic development, growth, and egg-laying capacity. For Medaka fish, the median lethal concentrations for nitrate on three different stages, just hatching, one and two months after hatching, determined from a 96-h exposure experiment were found to be 156, 116 and 166 mg NO_3-N l^{-1}, respectively and the concentration considered to be safe for long-term exposure was found to be less than 25 mg NO_3-N l^{-1} (Shimura *et al*., 2000; Shimura *et al*., 2002). To assess the toxicity of nitrate to Medaka fish in greater detail, we studied histology of the gills, intestinal ampulla, liver and kidney, after acute and chronic exposure to nitrate. Toxicology of nitrate in other aquatic animals are also discussed in relation to the Medaka case.

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Materials and Methods

Acute and chronic toxicity experiments

Experiments were carried out using eggs and one-month-old fries of the Medaka fish, *Oryzias latipes*. For the acute toxicity evaluation, 10 fries (ranging in length from 9.2 to 10.2 mm) were maintained in 2 l vessels and exposed to sodium nitrate at two concentrations, (100 and 125 mg NO_3-N l^{-1}) for a 96-h period. For the chronic toxicity evaluation, 10 eggs were placed in 5 l vessels and exposed to sodium nitrate at the same two concentrations over a three-month period. The control groups, for both the acute and chronic experiments, were subjected to nitrate concentrations below 8 mg NO_3-N l^{-1}. In any experiment, the Medaka fish were maintained at 25 ± 1˚C, pH 7.4-7.8, and a 14:10 h light:dark cycle. The fish were fed daily with 8-10% of their weight in food. Fies were fed with Super Gold (Oriental Yeast) up to one month after hatching. Thereafter, TetraMin® (Tetra Werke) was provided as the food source for juveniles. The crude protein contents of Super Gold and TetraMin® were 50% and 45%, respectively. In the acute toxicity experiment, survival rate and behavioral characteristics were monitored by regular observation of the Medaka fish. In the chronic toxicity experiment, hatching time of eggs was also recorded, as was total body length and body weight of the fish at the end of the exposure period. A nitrifying biological filter with an activity of processing 5 mg NH_4-N day^{-1} (Shimura *et al*., 1999) was employed to prevent the accumulation of ammonia and nitrite during both experiments. Using this filter system, concentrations of ammonia and nitrite remained below 0.08 mg NH_4-N l^{-1} and 0.02 mg NO_2-N l^{-1}, respectively. Nitrate concentrations were controlled by exchanging 10% of the water in the vessels each week.

Histological observation

The fish were anesthetized with 0.1% tricaine methanesulfonate MS222 (Sankyo), sacrificed, and fixed with Bouin's solution. Serial paraffin sections (6-8 μm) were prepared by standard techniques. Sections were stained with hematoxylin and eosin prior to microscopic examination.

Statistical analysis

Data are represented as means ± S.E. Statistical significance was assessed by one-way analysis of variance follwed by Bonferroni's multiple comparison test. P values less than 0.05 were considered statistically significant.

Water quality analysis

Water samples were collected from the vessels daily before feeding the fishes. Ammonium, nitrite and nitrate concentrations were measured spectrophotometrically (Shimazu UV-3100) using the indophenol method, the Griess-Romijn method and the cadmium reduction method, respectively. Water samples were also analyzed for pH using a Horiba F-22 meter.

Results

Survival rate, behavior and growth

For the acute toxicity evaluation, the survival rate of fries exposed to nitrate concentrations of 100 mg NO_3-N l^{-1} (125 mg NO_3-N l^{-1}) over a 96-h period was 30% (10%). During the exposure to nitrate in the two cases, the fries exhibited appetite loss. For the chronic toxicity evaluation experiment, the eggs subjected to both the control and nitrate treatments hatched 9-10 days after fertilization. There was no significant difference in the hatching time between the experimental and the control group. Survival rate of the juveniles exposed to nitrate at concentrations of 100 mg NO_3-N l^{-1} (125 mg NO_3-N l^{-1}) over a three-month period was 40% (30%). The juveniles subjected to the nitrate exposure treatments displayed appetite loss and lethargic behavior at the higher rate compared to the control juveniles. These characteristics continued throughout the three-month exposure period. One of the juveniles exposed to nitrate at a concentration of 100 mg NO_3-N l^{-1} developed curvature in its spinal column (lordoscoliosis) 42 days after

Table 1 Histopathlogical effects of nitrate exposure on visceral organs of the Medaka fish

Organ	Short-term exposure [a]	Long-term exposure [b]
Gills	Disruption of cell alignment Hyperplasia Cell necrosis	normal
Intestinal amupulla	Necrosis and Disruption of cell alignment	normal
Liver	Decrease in cell number and Disruption of cell alignment	Decrease in cell number and Disruption of cell alignment
	Infiltration of lymphocytes	Infiltration of lymphocytes
	Dilation of interlobular veins	Fibrosis of tissue

a The fries aged 1 month were exposed to nitrate at concentrations of 100 and 125 mg NO_3-N l^{-1} over a 96-h period.
b Eggs to juveniles were continuously exposed to nitrate at concentrations of 100 and 125 mg NO_3-N l^{-1} over a three-month period.

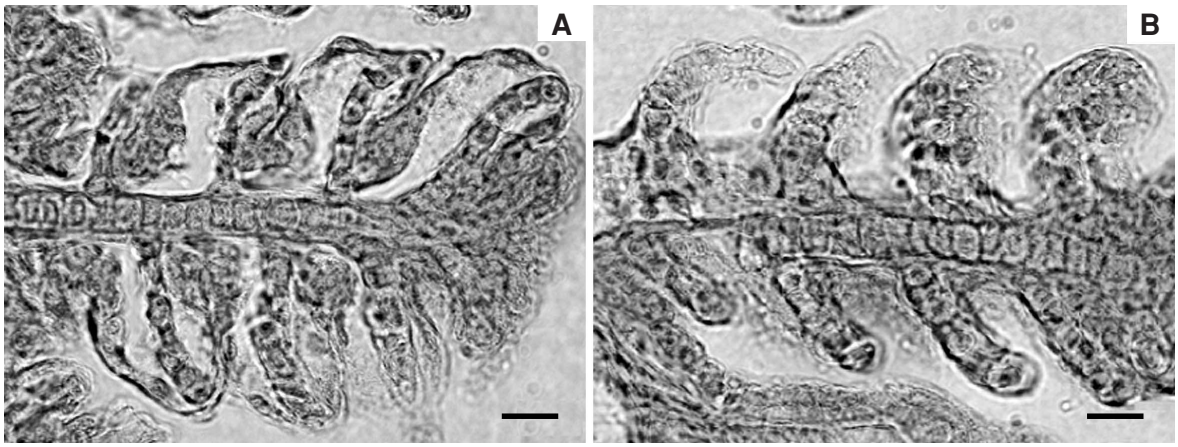

Fig. 1. Histopathological observations of the gills of Medaka fish exposed to nitrate over a 96-hr period. A: a control fry 1 month after hatching, B: a fry exposed to nitrate at a concentration of 100 mg NO_3-N l^{-1} over a 96-h period. Hemorrhage of the primary lamellae, disruption and rupture of the secondary lamellae, fusion of the adjacent secondary lamellae, hypertrophy and hyperplasia of the epithelial cells were observed. Bar: 10 μm

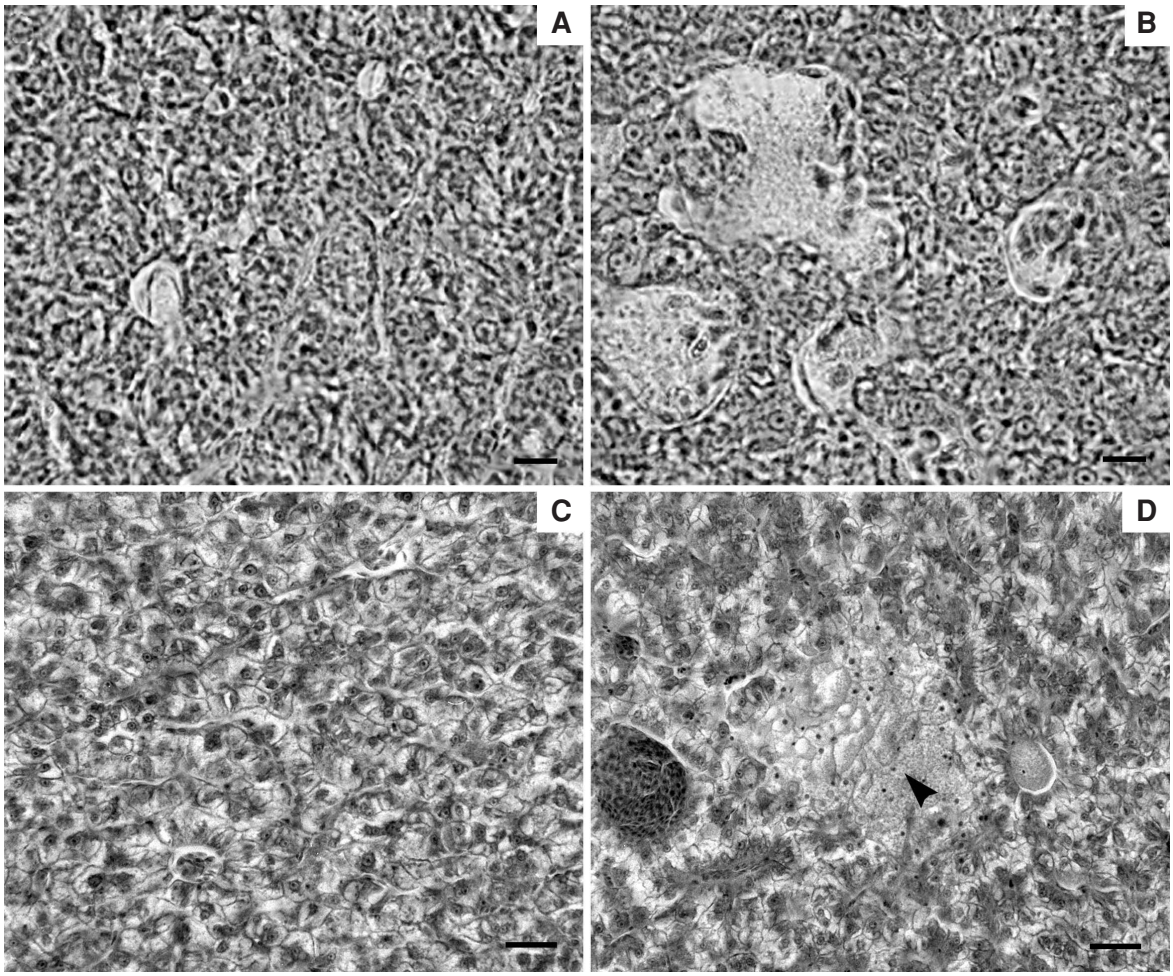

Fig. 2. Histopathological observations of the liver of Medaka fish exposed to nitrate for short-(96-hr) and long-(three-month) term periods. A: a control fry 1 month after hatching, B: a fry exposed to nitrate at a concentration of 125 mg NO_3-N l^{-1} over a 96-h period. The number of hepatic cells was reduced and cell alignment was disrupted. C: a control juvenile three months after hatching. D: chronic toxicity of nitrate at a concentration of 125 mg NO_3-N l^{-1} over a three-month period. Fibrosis of tissue is visible (arrow). The liver also showed symptoms of blood congestion in the sinusoids and hydropic swelling of the hepatocytes as well as vacuolation and dark granule accumulation. Bar: 10 μm

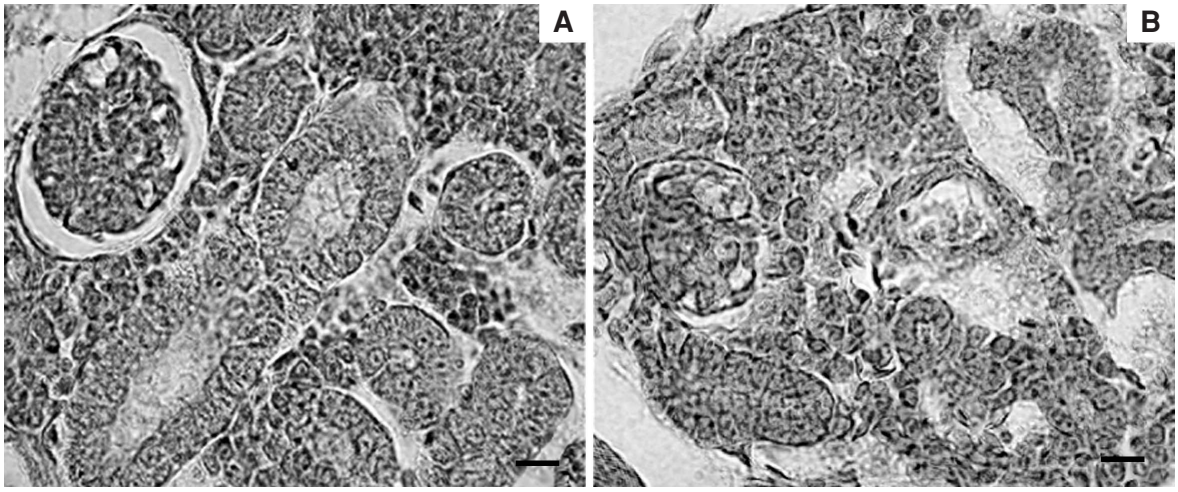

Fig. 3. Histopathological observations of the kidney of Medaka fish exposed to nitrate for 96-h. A: a control fry aged 1 month after hatching. B: a fry exposed to nitrate at a concentration of 100 mg NO_3-N l^{-1} over a 96-h period. Disruption of cell alignment was observed in the kidney. Bar: 10 μm

hatching, and died soon after. The juveniles from the control group displayed normal behavior during the three-month exposure period. No mortality occurred in the control treatment. Body weights of control, 100 and 125 mg NO_3-N l^{-1} nitrate treatments were 71 ± 2 (n = 10), 58 ± 3 (n = 4) and 57 ± 5 mg (n = 3) and total body length were 19.8 ± 0.3, 18.3 ± 0.8, and 18.5 ± 1.1 mm, respectively. There were significant differences in the body weight between control juveniles and NO_3-N nitrate treated juveniles ($p<0.01$). Total body length of juveniles exposed to nitrate was insignificantly smaller than that of control juveniles.

Histological observation of visceral organs

Table 1 shows the effect of nitrate exposure on the gills, intestinal ampulla, liver and kidney of Medaka fish. For the acute toxicity experiment, clear histological differences were observed in the gills, intestinal ampulla, liver and kidney of fries exposed for 96-h to nitrate. In addition, pathological changes were also observed in the gills of the individuals exposed to nitrate. In the gills, the most common symptoms of toxic exposure were necrosis and desquamation of the secondary lamellar epithelium, lifting of the epithelium, fusion of the adjacent secondary lamellae, haemorrhage at the primary lamellae, disorganization and rupture in the secondary lamellae and hyperplasia of the epithelial cells. In the gills of the fries exposed to nitrate, empty mucous cells and a reduction in mucous cell number were also observed (Fig. 1). In the intestinal ampulla, necrosis and disrupted alignment of the epithelial cells were observed. In the liver, vacuolation of hepatocytes and nuclear pyknosis were observed. A decrease in cell number, disruption of lobular cell alignment, infiltration of the lymphocytes, and dilation of interlobular veins were also observed (Fig. 2). In the kidney, disruption to cell alignment was observed in the proximal and distal tubules (Fig. 3). The livers of juveniles subjected to acute and chronic nitrate exposure showed similar symptoms (Fig. 2). Histological examination of the kidneys of juveniles exposed to nitrate over a three-month period showed symptoms of cell debris in the lumen of the renal tubules. No histological abnormalities were observed in the gills or intestinal ampulla of the individuals exposed to nitrate over a three-month period.

Discussion

In this study the histological affects of nitrate toxicity on the visceral organs of the Medaka fish were investigated. Acute toxic effects were observed in the gills, intestinal ampulla, liver and kidney. Regressive changes such the disruption of cell alignment, and the inflammation and necrosis of cells was also observed in many of the organs. The effects of chronic nitrate exposure were most evident in the liver, which displayed symptoms of fibrosis.

Short-term acute exposure to nitrate was found to invoke cellular disorders and metabolic disturbance. The pathological changes observed in many of the visceral organs were similar to what has been reported for chronic exposure to ammonia. Chronic exposure to un-ionized ammonia, which is known to be the most toxic form (U.S. EPA, 1999), has been reported to cause gill hyperplasia, and necrosis in the liver and intestine (Flis, 1968; WHO, 1986). It has also been suggested that the acute toxicity of nitrate can result in cytological damage that appears similar to the symptoms caused by some viruses that are known to cause lethal damage and systematic dysfunction in fish. Following three months of exposure to nitrate, however, no abnormalities were observed in the gills, intestinal ampulla, or kidney, although the liver showed some fibrosis of hepatic cells and infiltration of the lymphocytes.

In the chronic toxicity experiment, growth suppression in body weight, and curvature of the spinal column were observed in fish exposed to nitrate. These observations are in agreement with previous studies investigating the toxic effects of nitrate on different stages of the life cycle of the Medaka fish (Shimura *et al*., 2000; Shimura *et al*., 2002).

It has been reported that similar symptoms can result from the insufficient ingestion of essential compounds such as tryptophan (Harver, 1957; Harver & Shanks, 1960; Poston & Rumsey, 1983), linoleic acid and n-3 fatty acids (Phillips & Podoliak, 1957; Takashima, 1978; Takeuchi *et al*., 1991), and vitamins C and E (National Academy of Sciences, 1981; Sato *et al*., 1983). However, the food sources given to the fish in this study were rich in nutrients. Furthermore, no abnormalities were observed in the control group suggesting that the observed lesions resulted from nitrate-related toxicity. The available evidence also suggests the delayed growth and the curvature of the spinal column observed in juveniles subjected to chronic nitrate exposure may be related to liver dysfunction caused by nitrate exposure. It is well known that the liver plays many key roles in metabolism, and is responsible for the accumulation of excess nutrients, synthesis and conversion, and supply of various vitamins to other organs, as well as detoxification and synthesis of ovary components (Hamazaki *et al*., 1984). Based on the results of this study as well as previous reports, it is likely that chronic nitrate exposure can impair hepatic function and consequently lead to symptoms similar to those of nutritional deficiency.

On the International Space Station, efforts are made to maintain experimental animals under suitable conditions (Sakimura *et al*., 2003). In closed systems, it eccential to monitor water quality parameters during long-term experimental operations. Concentrations of inorganic nitrogen compounds derived from metabolic waste and excess food in water must be accurately regulated. For embryos of the African clawed toad (*Xenopus laevis*), nitrate concentrations of 56.7 mg NO_3-N l^{-1} have been reported to cause a reduction in growth rate during a 5-d exposure experiment (Schuytema & Nbeker, 1999a). Embryos of the red-legged frog (*Rana aurora*) are known to be even more sensitive. Decreased body length and weight of red-legged frog embryos has been shown to occur at nitrate concentrations of 29.1 mg NO_3-N l^{-1} and 235 mg NO_3-N l^{-1}, respectively, during a 16-d exposure experiment (Schuytema & Nbeker, 1999b). The present study showed that the acute and chronic exposure to nitrate, concentrations of 100 and 125 mg NO_3-N l^{-1} caused toxic effects on growth and some visceral organs of the Medaka fish. Clearly, nitrate toxicity is an important issue for the breeding of aquatic animals on the International Space Station. The present study also suggested that ammonia and nitrate might act synergistically to cause toxic effects on the Medaka fish.

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