Effects of Organic and Inorganic Mercury on the Apoptosis of the Neural Tube Cells of Zebrafish Embryos (*Danio rerio*)

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With 2 figures & 2 Histograms received Feb., accepted for publication March 2017

Abstract

In this study, we tested the hypothesis that mercury-induced developmental toxicity was mediated via ectopic occurrence of apoptosis during embryonic development. We employed microscopy to acquire images of whole-mount staining of apoptotic cells in zebrafish embryo exposed to 5, 10, 50, 80, 100, 150 ppb me hg and 50, 100, 200, 300, 400, 500 ppb hg cl from 5 hours post fertilization (hpf) to 48hpf.

In mercury-treated embryos with varying degrees of gross developmental malformations, significantly higher numbers of apoptotic cells were detected with this method. In the degenerating neural tube of mercury treated embryos apoptotic cells were detected, while in the healthy neural tube of the untreated controls no apoptotic cells were found. The percentages of apoptotic cells increased with the dose in both Me hg and Hg cl exposure. Exposure to mercury, therefore, induced ectopic apoptosis at 84 hpf.

Keywords: mercury, Apoptosis, Zebrafish embryos

Introduction

Mercury is a heavy metal that affects the activity of several enzymes, ion channels or receptors (Prabhu and Salama, 1990; Boraso and Williams,1994; Hulme et al., 1990; Chiamvimonvat et al., 1995) by combining with SH groups (Halbach, 1990) that are necessary for their normal function.

At 1 mM HgCl, an increase in 2 force occurs, probably as a result of a digi-

talis like effect inhibiting the activity of the plasma membrane Naq, Kq-ATPase (Halbach et al., 1981; Magour et al., 1987; Anner et al., 1992).

Mercury neurotoxicity causes a variety of neurological and behavioral effects in humans including central hearing loss, vestibular dysfunction, autism, mental deterioration, speech difficulty, impaired vision, weakness of the extremities and ataxia, and in some cases has proven to be fatal (Carpenter, 1994; Chang, 1977; Musiek, and Hanlon, 1999; and Takeuchi et al, 1968). Several investigations have reported hearing loss and auditory comprehension difficulties in humans exposed to mercury (Harada et al., 1977; Myers et al., 1995).

In the brain, methylmercury is converted to inorganic mercury most likely by in situ demethylation (Vahter, 1985). Inorganic mercury compound is shown to inhibit a variety of metabolic events in the brain by potentiating secondary neurotoxic events, (Albrecht, 1996).

Organic and inorganic mercury are reported to disrupt ion channel functions (which play a crucial role in cellular homeostatsis) and, in turn, affect processes such as synaptic transmission and growth cone elongation (Sirosis, 1996).

Materials and methods

Zebrafish Embryos

Adult fish were raised in the Department of Biology at Texas A&M University and kept under standard laboratory conditions at a room temperature of approximately 28.0°C (Westerfield, 2000). Male and female adult zebrafish were paired in the evening and fertilized embryos were obtained at 10 to 11AM the following morning. All embryos were held in an incubator held at 28.5°C Embryo medium, consisting of ultrapure water containing low concentrations of specific ions and adjusted to pH 7.2, was used to maintain the developing zebrafish embryos and was freshly prepared for each experiment according to Westerfield (2000). All zebrafish embryos were staged and fixed at specific hours post fertilization (hpf) as described by Kimmel et al. (1995). Both adult and embryonic zebrafish embryos were maintained according to protocols that were carried out in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No. 85-23, revised 1996).

Methylmercury preparation and exposure

Methylmercuric chloride (95% purity) was obtained from Alfa Aesar (Ward Hill, MA, USA). Methylmercuric chloride (methylmercury) was initially dissolved in sterile deionized water to a concentration of 0.1 mg/ml) and fur-

ther diluted with embryo medium for ZFE expsosure . All methylmercury stock solutions were stored at 4ºC until used.

ZFEs were exposed to different concentrations of methylmercury using 24-well flat bottom plates with low evaporation lids (BD Biosciences, San Jose, CA, USA). The total volume of embryo medium in each well was 2 ml. In each experiment some ZFEs were place in embryo medium without methylmercury to serve as negative controls. Additional ZFEs served as positive controls and were exposed to embryo medium with 2% ethanol (Mindel 2000). Two to three zebrafish embryos were added to each well and the 24-well plates were prepared in triplicate. A total of 12 ZFEs were tested. The 24-well plates were covered with low evaporation lids and incubated for up to 48 h at 28.5◦C (Thelco Laboratory Incubator; Cole-Palmer Instrument, Vernon Hills, IL, USA).

Mercuric chloride Preparation

Mercuric chloride Preparation was conducted in 24-well flat bottom plates with low evaporation lids (BD Biosciences, San Jose, CA, USA). The total volume of zebrafish medium in each well was 2 ml. Four wells on each plate were used for negative controls and contained only zebrafish medium. Four wells on each plate were used as positive controls and contained zebrafish medium with 2% ethanol (Mindel, 2000). The remaining 16 wells were divided into four wells, each containing 2 ml of zebrafish embryo medium and one of four different doses of mercuric chloride (5, 10, 50, 80, 100, 200, 500,600, and 700 ppb hg cl), described in the previous section. Two - four zebrafish embryos 6 hpf were added to each well, the 24-well plates were prepared in duplicate and a total of 96 zebrafish embryos were tested. The 24-well plates were covered with low evaporation lids and incubated for 48 h at 28.5◦C (Thelco Laboratory Incubator; Cole-Palmer Instrument, Vernon Hills, IL, USA).

Whole mount immune histochemistry

Acridine orange staining Embryo cell apoptosis was identified using AO staining. AO is a nucleic acid-selective metachromatic stain useful for studying apoptosis patterns (Chan and Cheng, 2003). Ten larvae from each beaker (n = 3) were washed twice in 30% Danieau's solution (58m MofNaCl, 0.7m MofKCl, 0.4mMofMgSO4, 0.6mM of Ca (NO3)2, and 5mM of HEPES, pH 7.4), then transferred to 5ugml−1 of AO dissolved in 30% Danieau's solution for 20 min at room temperature. The larvae were then washed with 30% Danieau's solution three times

for 5 min each. Before examination, the embryos were anesthetized with 0.03% MS-222 for 3 min. Apoptotic cells were identified with a fluorescence

Statistical analysis

The presence of significant differences between the mean values was determined using analysis of variance (ANOVA). Each value is the mean ±SD from three to six experiments. The level accepted for statistical significance in all cases was $P<0.05$.

Results

In 48-hpf embryo, we saw the distribution of apoptotic cells throughout the living embryo. Briefly, apoptotic cells gradually increased with the increase with the dose of mercury (Me hg or Me cl). In case of Me hg we found that the significant deference between the control and treated embryos appears at the dose 50 ppb Me hg (Figs 1& 3). This means that the doses 5 and 10 ppb do not affect the cells significantly but have minor effect on the developing cells. The effect of the dose 50 and 80 ppb Me hg differs significantly from all doses. The effect of the doses 100 and 150 ppb Me hg differs significantly from all doses while there is no significant difference from each other. In case of Hg cl we found that the significant deference between the control and the treated embryos appears at the dose 300 ppb Hg cl (Figs 2&4). This means that the doses 50, 100, and 200 ppb do not affect the cells significantly but have minor effect on the developing cells. The effect of the doses 400 and 500 ppb Hg cl differs significantly from all doses and also differs from each other. The effect of the dose 300 ppb Hg cl differs significantly from 50 ppb Hg cl. There is no significant difference between the effect of the doses 50, 100, and 200 ppb Hg cl. Few apoptotic cells were seen in the mesodermal tissue and notochord. Apoptotic cells were also found at sites of somite formation. The total number of apoptotic cells in each embryo was counted. No apoptotic cells were detected in the untreated embryo. This dynamic pattern of apoptosis was consistent in all the embryos tested, although the actual numbers of apoptotic cells differed from embryo to embryo. Having established the pattern of distribution and the baseline number of apoptotic cells at 48 hpf, we investigated the distribution of apoptosis in mercury-treated embryos. In mercury-treated embryos with gross malformations, localized regions of apoptosis were found in tissues developing abnormalities. For example, in an embryo with improper regionalization of the brain and stunt tail bud (Figs 3&4), focal groups of apoptotic cells were found in the correspond-

ing regions (Figs 3&4). In an embryo that failed to develop a proper body axis, resulting in more or less undifferentiated anterior and posterior regions (Figs 3&4), high numbers of apoptotic cells were found throughout the body with the highest numbers being found in the anterior and posterior ends (Figs 3&4). In the two embryos with tail malformations (Figs 3&4), localized apoptosis was found in the tail regions (Figs 3&4). Some embryos show no obvious malformation of the neural tube while there is a high number of apoptotic cells were observed there (Figs 3&4). Ectopic patterns of apoptosis were also observed in mercury-treated embryos that did not show any gross signs of malformations (Figs 3&4).

Discussion

We used the occurrence of apoptosis as a mean to study the developmental toxicity of mercury exposure during zebrafish embryonic development. The spatial and temporal patterns of apoptosis in vertebrate embryonic development are tightly regulated events (reviewed in Jacobson et al., 1997). Disruption of the regulated occurrence of apoptosis as a result of genetic mutations or exposure to toxicants leads to developmental abnormalities (Mirkes 2002; Zakeri and Ahuja 1997). In this study, our focus was to obtain a quantifiable measurement of the occurrence of apoptosis in the whole zebrafish embryo. To achieve this goal, we employed microscopy and adapted the acridine orange staining methodology for detection of apoptosis in zebrafish embryos. By doing so, we obtained accurate patterns of the distribution of apoptotic cells during the course of embryonic development. Quantification of the number of apoptotic cells was then achieved by counting the dead cells. This methodology has given us a clear picture of the dynamics of apoptosis in zebrafish development. Any increase in the number of apoptotic cells could be measured. We have also investigated the effect of exposing the embryos to different doses of Me hg and Hg cl for 24 h. We further showed that apoptosis occurred in tissues with malformations and, hence, provided a mechanism link to mercury-induced developmental abnormalities. Our study showed that apoptosis occurred in malformed tissues. This observation supports previous studies, which showed that zebrafish mutant embryos with neurodegeneration resulting in malformed central nervous system have apoptosis in their neural tissues (Furutani-Seiki et al. 1997). Our study was one of the first investigating the dynamics of apoptosis induction by mercury in whole organisms. With the combination of methodologies described here, our work has not on-

ly corroborated other published work on the induction of apoptosis by mercury but has also provided additional information on mercury toxicity. We were able to show that mercury affected distribution of apoptotic cells in 48-hpf embryos.

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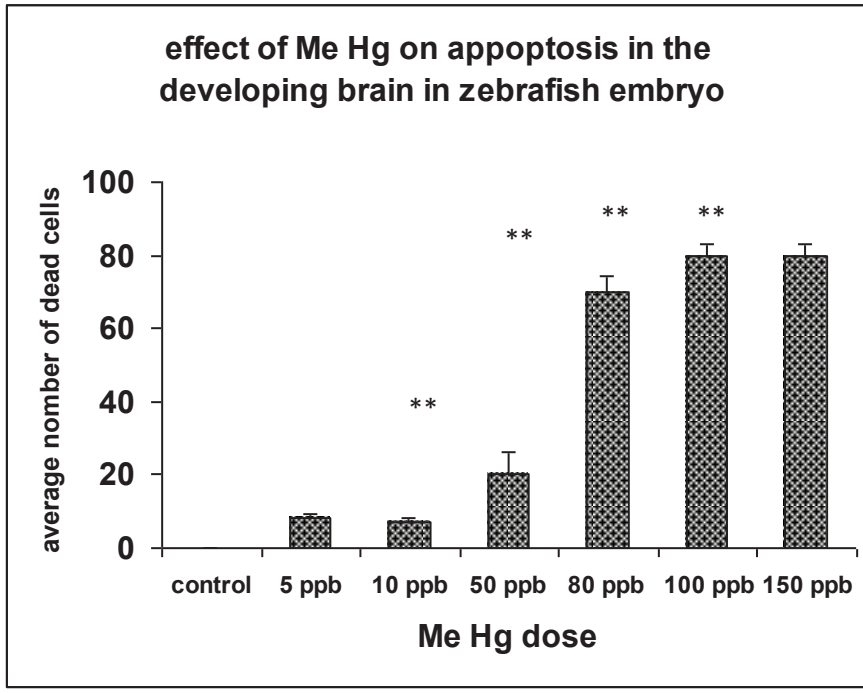

Fig (1): A histogram showing the high number of dead cells of the developing brain of the zebrafish which indicates the neurotoxic effect of the Me Hg on the developing neuron. ** highly significant.

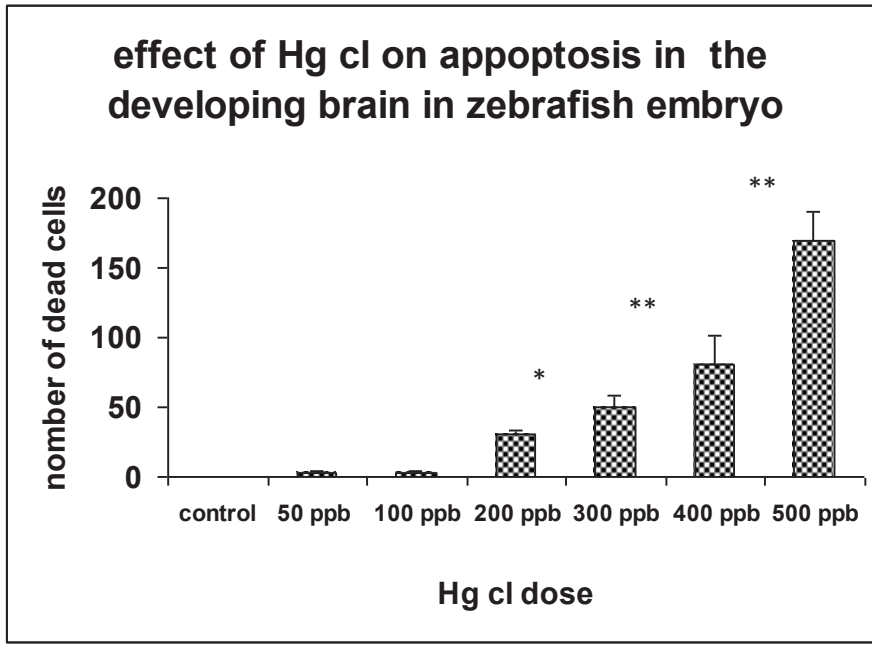

Fig (2): A histogram showing the high number of dead cells of the developing brain of the zebrafish which indicates the neurotoxic effect of the Hg cl on the developing neuron. * significant, ** highly signify
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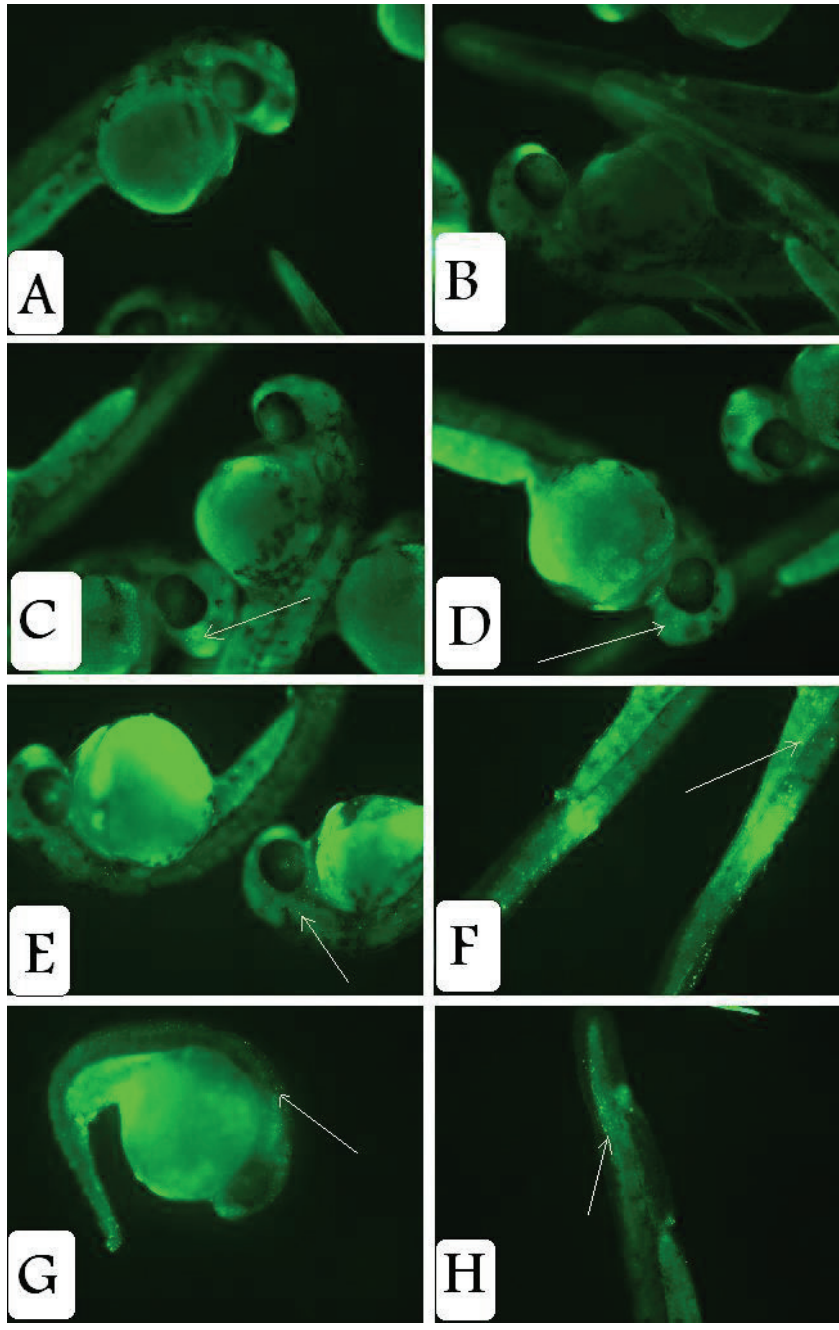


Fig (3): Flurecent micrographs of acridine orange stained zebrafish embryos: control (A), Mehg treated embryos 5 ppb (B), 10 ppb(C), 50 ppb(D), 80 ppb(E&F), 100 ppb(G&H). apoptic cell (arrow)

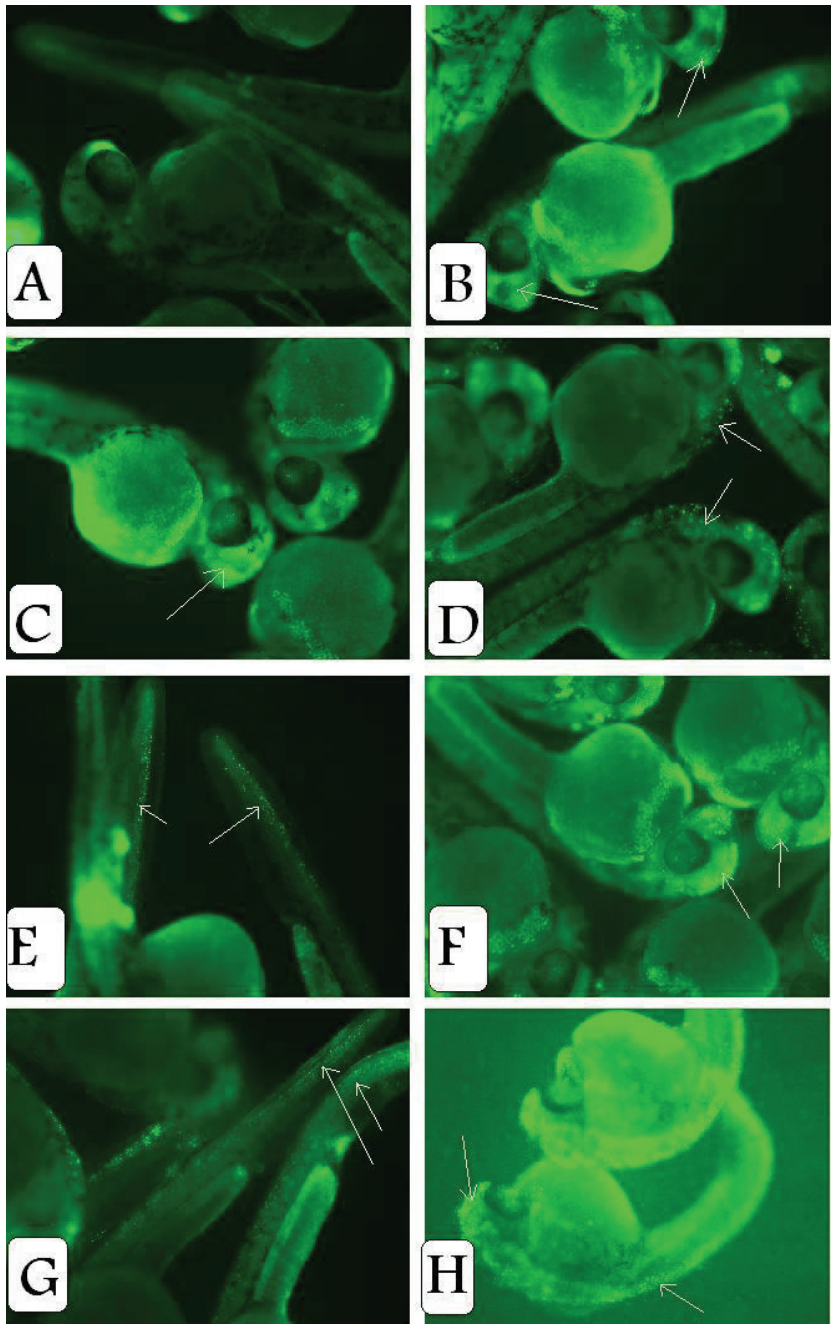
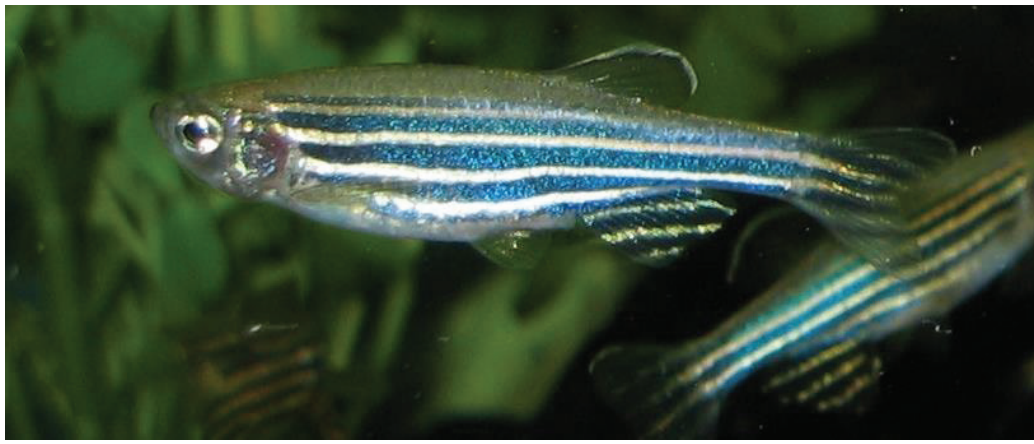


Fig (4): Flurecent micrographs of acridine orange stained zebrafish embryos: control (A), Hgcl treated embryos 50 ppb (B), 100 ppb(C),200 ppb(D&E), 300 ppb(F&G), 400 ppb(H). apoptic cell (arrow).

Animal species in this Issue

zebrafish (*Danio rerio*)



Kingdom: Animalia & Phylum: Chordata & Class: Actinopterygii & Order: Cypriniformes & Family: Cyprinidae & Genus: *Danio* & Species: *D. rerio* (F. Hamilton, 1822)

The **zebrafish** (*Danio rerio*) is a tropical freshwater fish belonging to the minnow family (Cyprinidae) of the order Cypriniformes. Native to the Himalayan region, it is a popular aquarium fish, frequently sold under the trade name **zebra danio**. The zebrafish is also an important and widely used vertebrate model organism in scientific research, and was among the first vertebrates to be cloned (frogs were cloned decades earlier). It is particularly notable for its regenerative abilities, and has been modified by researchers to produce many transgenic strains.

The zebrafish is named for the five uniform, pigmented, horizontal, blue stripes on the side of the body, which are reminiscent of a zebra's stripes, and which extend to the end of the caudal fin. Its shape is fusiform and laterally compressed, with its mouth directed upwards. The male is torpedo-shaped, with gold stripes between the blue stripes; the female has a larger, whitish belly and silver stripes instead of gold. Adult females exhibit a small genital papilla in front of the anal fin origin. The zebrafish can grow to 6.4 cm (2.5 in) in length, although it seldom grows larger than 4 cm (1.6 in) in captivity. Its lifespan in captivity is around two to three years, although in ideal conditions, this may be extended to over five years.

Source: Wikipedia, the free encyclopaedia