

Effect of CO₂ exposure on the antioxidant enzymes activity and gill histology of adult zebrafish (*Danio rerio*)

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Abstract

Increase in carbon dioxide level is considered as a threat to all living organisms and in particular aquatic organisms are vulnerable. Although a number of studies were carried out to predict the effect of increased CO₂ with marine organisms, fresh water fish were the least concerned ones. Therefore, the aim of this study was to study the effect of antioxidant enzymes in zebrafish exposed to acute concentration of CO₂ (based on prediction for 2300). Three ranges of pH were selected below the range where no fatality was observed, 6.0 – 6.5, 5.5 – 6.0 and 5.0 – 5.5. Antioxidant enzyme analysis of superoxide dismutase (SOD), catalase (CAT), lipid peroxidation (LP), glutathione reductase (GR) and glutathione peroxidase (GPx) was performed for the tissues, liver, gills and muscles. Although a significant decrease in SOD activity was observed in all three tissues, only a slight variation in CAT activity was found in all the tissues. MDA content which is an indicator of lipid peroxidation showed an increasing pattern in all the tissues. A slight increase in GR activity was found in all the tissues; however, a significant decrease in GPx activity was noted. Further, the histology of gills exposed to CO₂ showed marked damage and injury including lamellar fusion and necrosis. Our study concludes that exposure to CO₂ has led to oxidative stress which has resulted in cellular injury and damage.

Keywords: carbon dioxide acidification, zebrafish, antioxidant, oxidative stress, cellular damage.

Introduction

An increase in carbon dioxide concentration in water body including marine and freshwater has devastating implications. Industrial revolution has resulted in contributing a high concentration of CO₂ into atmosphere resulting in acidification of the surrounding environment due to variations in pH. Over the years, a number of studies have been accumulated to study the effect of dissolved CO₂ on marine organisms (Ferrari et al. 2011). These studies show that ocean acidification could have an adverse effect on marine fish and other organisms which will be realized greatly at the end of this century as opposed to earlier studies which reported the fish's ability to compensate for hypercapnic acid-base disturbances. The predicted level of CO₂ values of 1,000 μatm by 2100 and μatm 1,900 by 2300 (Meehl et al., 2007) will result in detrimental effect to wide array of organisms including behavioural changes, impacts on neurosensory, mitochondrial function and metabolic rate. Now, it is realized that similar pattern of valuable information is lacking with freshwater system and its associated life forms (Ou et al. 2015; Jesus et al. 2018). This area of study is crucial as climate change and/or acidification are predicted to have adverse effects on the organisms in freshwater habitat (Jesus et al. 2018) as this system is more sensitive to change in pH.

Although many fish species cannot survive the acute or chronic exposure of acidic water, there are some species that can thrive such environment and thus can serve as a useful model to study the effect of ionic-base balance. One such fish species which is adopted widely by researchers worldwide is the zebrafish (*Danio rerio*) (Hwang et al. 2011). Zebrafish is a temperate animal that lives in tropical temperature ranging from 24 to 35 °C and pH from 6.6 to 8.2 (McClure et al. 2006). The adaptation of the zebrafish including tolerance of pH up to 4.0 (Kwong and Perry 2013), wide range of temperatures, P_{CO_2} and P_{O_2} enables the effective study related to interaction of different environmental factors.

Moreover, the small size of the zebrafish, low-cost maintenance, transparent physiology and availability of abundant genetic information makes the animal a suitable model for acidification studies. Previous studies of acidification on zebrafish have documented the paracellular losses of Na⁺ (Kwong and Perry 2013) and increased mRNA expression of bronchial H⁺-ATPase (Chang et al. 2009).

Antioxidant system is the most accepted and adopted approach to study the effect of stress in organism, reactive oxygen species being the best indicator of stress (Sevcikova et al. 2011). The generation of ROS triggers the antioxidant system where cascades of antioxidant enzymes are produced by the cells to counterbalance the ROS and to reform the oxidant balance. However, if there is an excess of ROS, the enzymes fail to perform its function effectively leading to cellular injury, damage and loss of integrity. With this background, the present study aims to study the effect of CO₂ acidification on the antioxidant enzymes of adult zebrafish.

Materials and Methods

Maintenance of zebrafish

Adult zebrafish of the short-fin wild-type zebrafish (*Danio rerio*) AB strain were purchased from a local aquarium farm (Sirago Aquafarm, Mettur, Tamil Nadu, India) and housed in mixed-sex groups in static 20-L glass tanks with airlift-driven filtration at 25°C on an ambient 14-hr/10-hr light–dark photoperiod. The diet for the adult zebrafish consisted of dry micropellets and brine shrimp (hatched from 25 g of eggs in 2 L salt water) daily along with rotifers every two days. The ratio of food per day was maintained at 4% of body weight of the adult fish.

Experimental set-up and CO₂ treatment

Three 10-L tanks were maintained with 100 fish in each tank. The three different experimental condition included, ambient air with pH 6.8 and two experimental treatments were established in accordance with the future atmospheric predictions of IPCC of 1500 μatm with pH range of 6–6.5 and 2200 μatm of P_{CO_2} levels with pH range of 5–5.5 for the years 2150 and 2300, respectively (Krinner et al. 2013). The atmospheric P_{CO_2} levels were achieved by bubbling the water in the respective tank using a Boyu ceramic CO₂ diffuser. With stable temperature at 22°C, the pH level was measured using a pH meter. The healthy zebrafish was exposed acutely in the acidified tanks, and the experiment was replicated thrice.

Sample preparation

Fishes were divided into four main groups with 80 individual in each group. Group 1 is the control which does not receive any CO₂. Groups 2, 3 and 4 are the fish with the acidified water due to infusion of CO₂ with pH of 6 – 6.5 and 5.5 – 6.0 and 6.0 – 6.5, respectively. The groups 2, 3 and 4 received CO₂ for a total period of two weeks on a basis of 30 minutes continuous CO₂ followed by 90 minutes of aeration during the day time. Triplicates were maintained for each group. Certain parameters such as swimming pattern, stress and toxicity were recorded during the experimental period. Fish were starved 24 h before dissection.

After the treatment period, the fish were collected both from the control and treated tanks and washed with distilled water and blotted dry with tissue paper. Liver, gills and muscle samples were dissected out carefully from the fish and individually homogenized in 1.0 ml of Tris-HCl buffer for each 100 mg of tissue sample, followed by centrifugation at 10,000 rpm for 15 min at 4 °C. The supernatants were transferred to sterilized vials and stored at -20 °C until further use.

Antioxidant assays

Catalase assay

Catalase assay was done following the colorimetric method of Sinha (1972). Hydrogen peroxide (0.2 M) was mixed with the each homogenate of liver, gills and muscles, followed by the addition of dichromate/acetic acid reagent (5% dichromate with glacial acetic acid at a ratio of 1:3). The reagent mixture was heated, and the remaining hydrogen peroxide at different time intervals was read at 583 nm.

Superoxide dismutase

The activity of superoxide dismutase was determined according to the method of Marklund and Marklund (1974) with few modifications. Homogenate of 50 µl of liver, gills and muscles was added to the reaction mixture (50 mM, Tris-HCl buffer [pH 8.4]; 1 mM EDTA; 2.6 mM pyrogallol) and incubated for 5 min. After the incubation period, the absorbance was measured at 460 nm and the unit of relative enzyme activity was expressed in U/mg protein.

Lipid peroxidation

Lipid peroxidation method relies on the measurement of thiobarbituric acid-reactive substances (TBARS) by following the method of Devasagayam and Tarachand (1987). For the assay, approximately to 100 µl from each tissue homogenate, 100 µl of 5% trichloroacetic acid (TCA) was added followed by incubation in ice for 5 min. Then, the samples were mixed with 100 µl of 0.67% thiobarbituric acid and centrifuged at 3500 rpm for 10 min at 4°C. Further, about 250 µl of supernatant was collected in a test tube and boiled in a water bath for 10 min, which was then allowed to cool at room temperature, and the absorbance was read at 535 nm using the Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

Glutathione peroxidase

Glutathione peroxidase assay was performed following the procedure designed by Paglia and Valentine (1967). Briefly, each 880 μ l of reaction mixture consisting of 150 μ M of NADPH, 1 mM GSH, 100 mM sodium azide in potassium phosphate buffer and pH 7.0 was added to 20 μ l each of liver, gills and muscle tissue homogenate. The mixture was transferred to 24-well titre plate and the absorbance was read at 340 nm for 1 min using ELISA microplate reader.

Glutathione reductase

Glutathione reductase activity of the tissue homogenate was determined following the method of (Staal et al. 1969). Reaction mixture consisting of 0.3 M sodium pyruvate (pH 6.0), 25 mM EDTA and 3 mM oxidized glutathione was added to each of the tissue homogenate and the change in absorbance was monitored at 340 nm for 3 min using ELISA microplate reader. The activity was expressed as micromoles of NADPH oxidized per minute per milligram protein.

Histopathology studies

After 14 days of experimental duration, the fish from each group was collected and dissected to collect the gills. Then, the tissue was fixed with 10% buffered formaldehyde (pH 7.4) at room temperature overnight. The samples were then dehydrated using a graduation of ethanol followed by clearing using xylene and embedded in blocks with paraffin wax. Sectioning of the blocks was done using microtome and stained with hematoxylin and eosin and examined under trinocular microscope attached with monitor.

Results and Discussion

Carbon dioxide is ever increasing in the atmosphere due to several undesirable anthropogenic activities that are relative to the elevating CO₂ level in aquatic environment making it more acidic. This continuous acidification on the aquatic organisms implies a devastating effect on economic activities such as fishing and tourism, which also indicates a negative impact on the food chain. Measuring and studying the impacts of increased CO₂ will assist the future researchers and other authorized personnel to plan and implement essential steps to mitigate these effects on the aquatic life forms. In this line, the present study was focussed to study the effect of predicted level of CO₂ on a model freshwater fish, zebrafish (*Danio rerio*) by measuring the antioxidant enzyme activity on exposure to acute level of CO₂. Studying the antioxidant enzyme activity is a widely adopted method to determine the status of oxidative stress in the organism (Kyrtopoulos 2006). In support of this statement, our study shows that the levels of antioxidant proteins are altered which are indicative of oxidative stress. Similar damages in marine organisms due to oxidative stress has been reported by a few researchers earlier (Pimentel et al. 2015; Wood et al. 2016).

In this study, we found the amount of carbon dioxide infusion which drops the water pH to 4.5 – 5.0 is lethal to the fish (Table 1). Hence the three pH range below the toxic level was selected for further studies. The fish in the group of pH 6.0 to 6.5 were found to be active and occupied the middle and lower half of the tank as well as about 103 embryos were obtained from the CO₂ exposed fish of group 2. However, the fish in the group 3 and 4 were found to be less active and occupied the lower half of the tank most of the time and produced significantly less number of embryos of 67 and 30, respectively.

SOD and CAT enzymes are involved in protecting the cells against the damage caused due to excessive free radicals generated as a result of stress inducted by carbon dioxide in this study. SOD is the first-line defence and play an important role in

detoxification of the generated free radicals and H₂O₂ (Arun and Subramanian 1998). As shown in Fig. 1, a decreasing trend of SOD was found with the reduction in pH from 6.5 to 5.0 consistently for all the three tissues, liver, gills and muscle. This indicates that enhanced amount of CO₂ depletes the SOD enzyme. Similar decrease in SOD activity was reported by Muazzam et al. (2019) when zebrafish was exposed at high concentration of endosulfan (1 µg/L) and imidacloprid (1 mg/L) where an inhibition of SOD enzyme was reported. However, for the CAT enzyme, only a slight decrease in activity was reported (Fig. 2) in liver and gills at all pH ranges; whereas in muscle tissues, a slight increase in the activity was recorded at pH 6 – 6.5 and was found to be normalized at further decrease in pH. It can be thus predicted that carbon dioxide had least effect on the activity of the zebrafish as they are capable of stress tolerant.

Lipid peroxidation was determined by measuring the MDA level and is considered as a biomarker of pollution. As shown in Fig. 3, there had been a significant increase in the MDA content in the liver and gills tissues at various pH ranges; however, in muscle tissue, a comparatively lower MDA content was observed than the control. An increased value in liver and gills indicates the induction of lipid peroxidation in the cell membranes. Our data correlate with the findings of Jin et al. (2010) and Muazzam et al. (2019) where increased MDA content was observed on exposure of zebrafish with highest concentration of atrazine and pesticides, respectively.

Our results show that the GR activity was significantly increased in all the three tissues: for liver and gills at pH range of 5.5–6.0 and for muscle at the pH range of 5–5.5 (Fig. 4). Glutathione reductase regenerates the reduced glutathione from its oxidized form and hence is an important oxidant. Increased activity denotes an overconsumption of glutathione for cellular reactions. Similar increase in GR activity was documented by

Plhalova et al. (2013) when zebrafish was exposed to acetylsalicylic acid in the concentrations of 0.004, 0.4, 40, 120 and 250 mg/L.

GPx is mainly accountable for mitigating the damage caused by lipid peroxidation as it reduces hydrogen and lipid peroxides (Winston and Di Giulio 1991). In our study, we found a notable increase in GPx at the pH range of 6.0 – 6.5 in both liver and gills followed by a decreasing trend (Fig. 5). However, a dose-dependent decrease in the enzyme activity was observed in muscle tissue. An increase in GPx activity was documented by other researchers working on ibuprofen (Bartoskova et al. 2013), while Bartoskova et al. (2014) reported no increase in the GPx activity when zebrafish was exposed with norfloxacin.

The morphology of the gills of zebrafish unexposed to CO₂ showed intact morphological structure with filaments and secondary lamellae. The gills of the fish exposed with CO₂ of pH range 6 -6.5 exhibited lamellar fusion and oedema, while those with the pH range 5.5 – 6.0 and 5.0 – 5.5 showed hypertrophy and proliferation of erythrocytes of cartilaginous core (Fig. 6). These changes are the result of defence mechanism exhibited by the organism against the environmental contaminant (Scown et al. 2010). Similar changes were recorded by Mansouri et al. (2016) where the combined effect of silver nanoparticles and mercury were studied on zebrafish gills. In addition, maduramicin, an antibiotic, also showed similar changes in the gills of zebrafish when exposed to different concentrations for 14 days (Ni et al. 2019).

Conclusion

Exposure of CO₂ to zebrafish has caused damage to antioxidant enzymes. A modulation in the activities of these enzymes reflects a significant cellular injury as a result of oxidative stress. The CO₂ has also shown to cause significant cellular damage in the gills of the fish which has resulted in necrosis of the cells. It is important to note that several other

important species are vulnerable to increased CO₂ in the environment and hence efforts should be taken to mitigate the anthropogenic activities that results in increased CO₂ in the atmosphere.

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Table. 1. Effect of different concentration of CO₂ on zebrafish

pH range	6.5 – 6.0	5.5 – 6.0	5.0 – 5.5	4.5 – 5.0	4.0 – 4.5
Parameters					
Mortality	0	2 ± 0.03	5 ± 0.09	No survival	No survival
Movement	Active	Less active	Less active	-	-
Positioning	Middle half	Upper half	Upper half	-	-
Number of embryos	103 ± 5.3	67 ± 3.7	30 ± 4.5	-	-

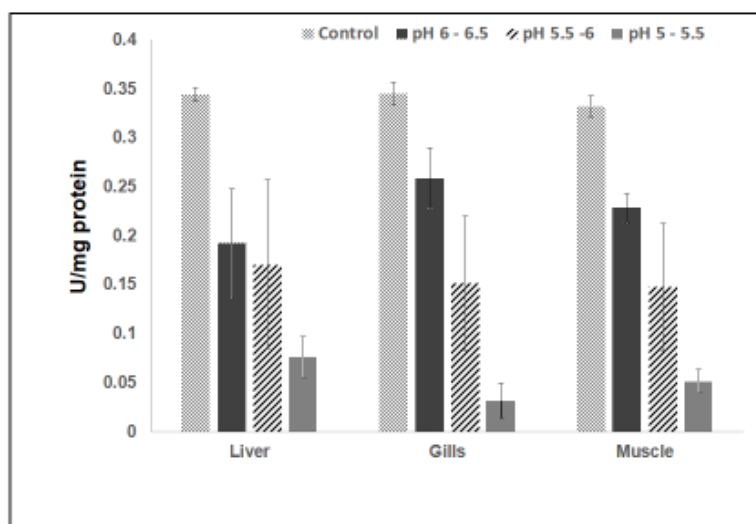


Figure 1. Level of superoxide dismutase (SOD) activities in liver, gills and muscle tissue of zebrafish exposed to CO₂. Values are the mean \pm SD of three independent experiments.

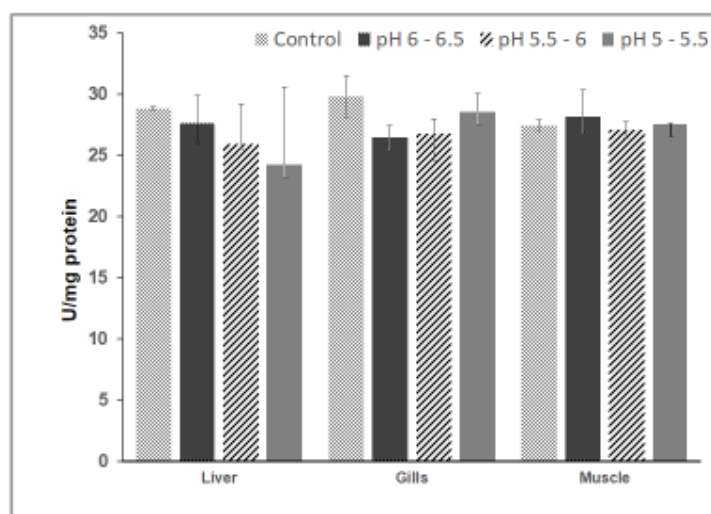


Figure 2. Level of catalase (CAT) activities in liver, gills and muscle tissue of zebrafish exposed to CO₂. Values are the mean \pm SD of three independent experiments.

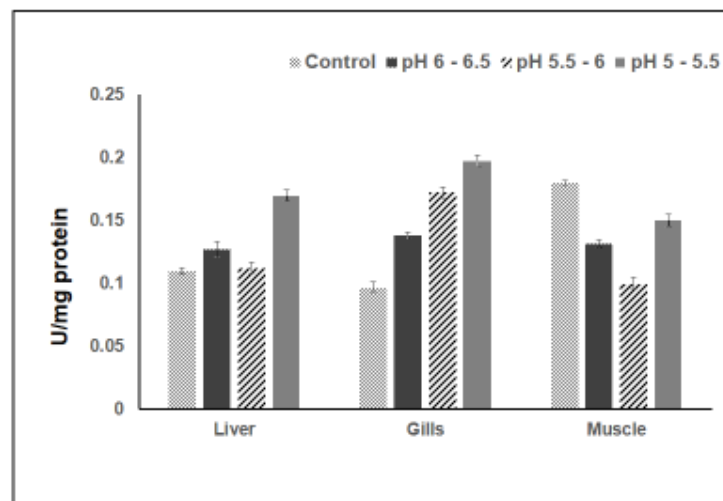


Figure 3. Level of MDA content in liver, gills and muscle tissue of zebrafish exposed to CO₂. Values are the mean \pm SD of three independent experiments.

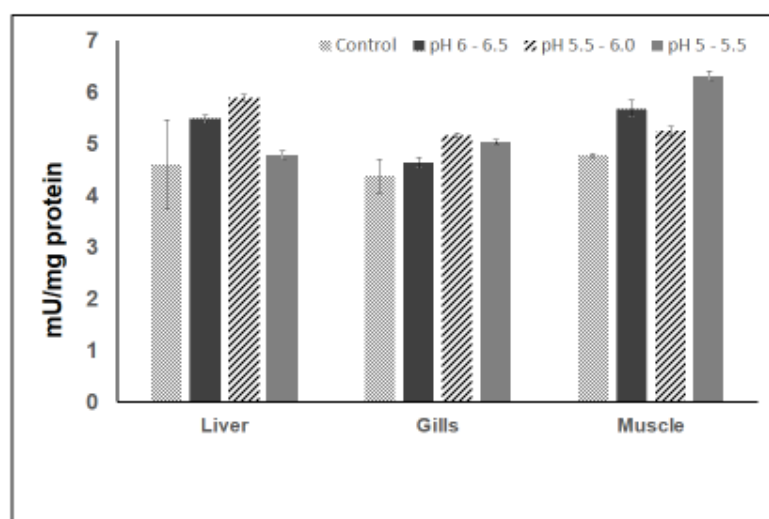


Figure 4. Glutathione reductase (GR) activity in liver, gills and muscle tissue of zebrafish exposed to CO₂. Values are the mean \pm SD of three independent experiments.

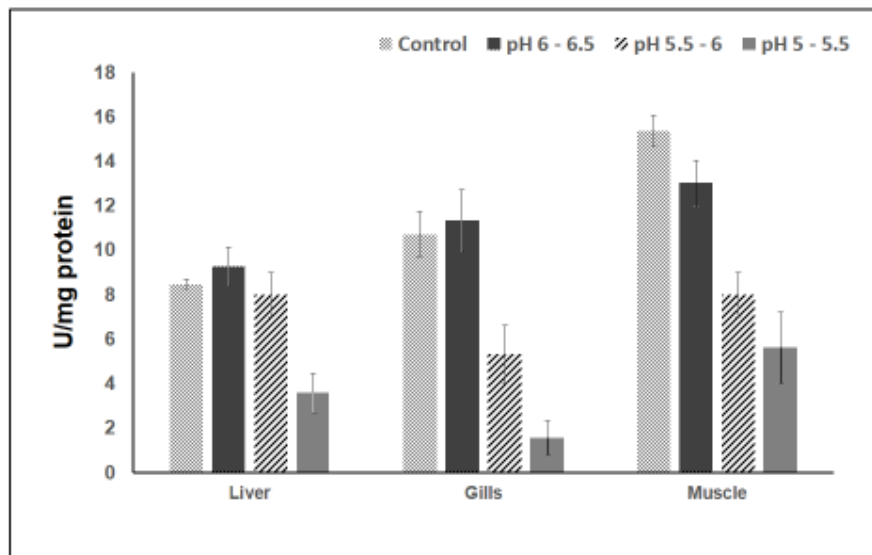


Figure 5. Glutathione peroxidase (GPx) activities in liver, gills and muscle tissue of zebrafish exposed to CO₂. Values are the mean \pm SD of three independent experiments.

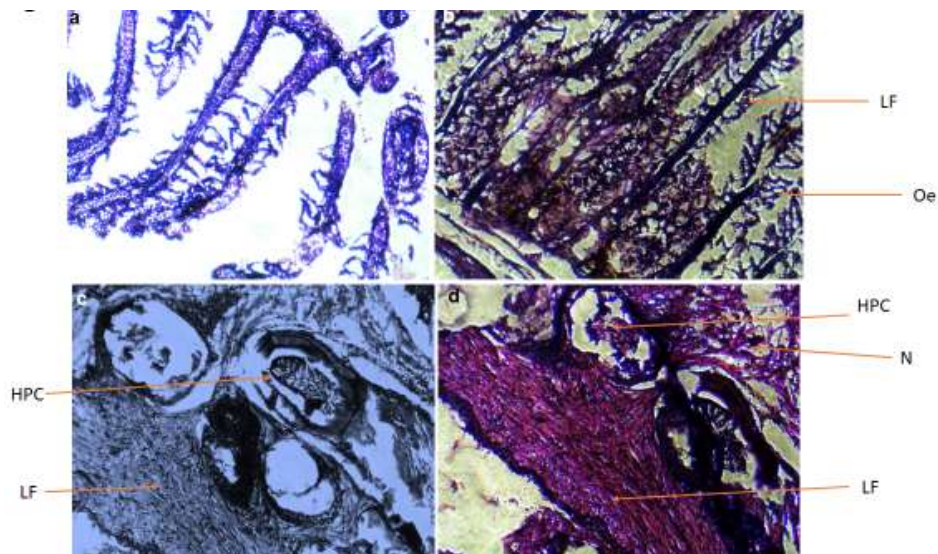


Figure 6. Gill morphology in the zebrafish after exposure with CO₂ with pH range (a) normal (pH 7.0), (b) 6.0 – 6.5, (c) 5.5 – 6.0 and (d) 5.0 – 5.5. LF, Lamellar fusion; N, Necrosis; Oe, odema; HPC: Hypertrophy and proliferation of erythrocytes of cartilaginous core.