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Original Article

Oxidative stress mediated antioxidant enzyme responses in tilapia (O*reochromis mossambicus*) and silver carp (*Hypophthalmichthys molitrix*) fingerlings during hypoxic transportation and reoxygenation

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ABSTRACT: Fish fingerling mortality due to transportation stress is one of the major problems in fisheries sector of Bangladesh. The present study aimed to understand the stress responses of fish fingerlings transported in a traditional way in Bangladesh. As indicators of oxidative stress, we monitored the production of hydrogen peroxide (H_2O_2) and activity of two antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPx) during hypoxic transportation and after reoxygenation events. Tilapia (*Oreochromis mossambicus*) and silver carp (*Hypophthalmichthys molitrix*) fingerlings were transported in hypoxic condition for 6 hours in aluminum vessels and subsequently released into normoxic (DO>5.0 mgL^{-1}) condition and reared for 16 days to observe delayed mortality. We found that Silver carp fingerlings were most susceptible to mortality during transportation and delayed mortality (51.93±8.06%) was found even higher than the Tilapia fingerlings. During hypoxic transportation H_2O_2 production was significantly ($p<0.05$) higher but SOD and GPx activities were found significantly ($p<0.05$) lower. However, at normoxic condition after initial increase up to 12 hours theH_2O_2 production gradually decreased while the GPx and the SOD activity increased gradually in the transported fingerlings. Our findings suggest that fish fingerlings transported in the traditional system suffer from oxidative stress, playing role in their early and delayed mortality even after release to normoxic condition.

KEYWORDS: Hypoxic; Reoxygenation; Fingerling transport, Hydrogen peroxide, Glutathione peroxidase, *Oreochromis mossambicus.*

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INTRODUCTION

Fisheries sector of Bangladesh is expanding as a potential economic subsector contributing 3.30% of Gross Domestic Product (GDP) of Bangladesh[1]. Fish seed production is increasing generously over the years; however, fish fry and fingerling transportation remains as a bottleneck for proper development of this sector in Bangladesh. Historically, transportation of live fish is a

common practice particularly in the rural areas of Bangladesh and often represents the only means of supplying fish fry/fingerlings for culture[2]. The traditional method which accounts for 95% of live fish fry and fingerling transportation in Bangladesh is simply the use of water filled small aluminum or plastic containers with continuous hand agitation for a duration of 1 to 6 hours[3].During transportation fish seeds face chronic and acute stress due to low dissolved oxygen (DO) concentration, high ammonia or nitrite levels, improper temperature or a high or low pH of the water; consequently fish have impaired growth[4,5] and unpredictable mortality[6].What is surprisingly observed is that the continuation of such mortality even after the termination of the stress causing delayed mortality [7], which often reached up to 90-100% in some freshwater fish like largemouth bass[8–10].Bangladesh loss a total of 6200 mt fish fingerlings annually due to transport related mortality of which 4-12% was reported as immediate mortality whereas delayed mortality was 27-49% [11].

The dissolved oxygen (DO) concentration is one of the most limiting factor in fish transportation systems. Most fish species can tolerate a drop in the DO below their minimum requirements for a short period. Hypoxia (dissolved oxygen concentration <2.0 mg/L) is a severe environmental stress that causes fish death [12,13].A mismatch between oxygen supply and its demand at the cellular level may result in a hypoxic condition. All aerobic living organisms including fish produces reactive oxygen species (ROS) namely superoxide (O_2•−), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$), lipid peroxides (ROOH) and hydroxyl radical (OH•) as a byproduct of aerobic respiration [14]. Under hypoxic conditions, the cellular ROS level is reported to be enhanced[15].The overproduction and/or mismanagement of ROS lead to the general phenomenon of oxidative stress that is implicated in aging and death [16,17]. However, aerobic organisms have developed a comprehensive antioxidant defense system to prevent excess oxidation and damage[18].The antioxidant enzymes such as superoxide dismutase (SOD) can act as the first line of defense against ROS by converting superoxide radical (O_2•−) to peroxide (H_2O_2); catalase which reduces H_2O_2 to water, and the scavengers like glutathione peroxidase (GPx) has role in detoxifying H_2O_2 and organic hydroperoxides[18].H_2O_2 serves as a key regulator for a number of oxidative stress-related states. The cellular half-life of hydrogen peroxide is reported about 1 millisecond which can diffuse away from its source and may damage other biomolecules of the organisms[14].H_2O_2 has comparatively longer half-life than other reactive oxygen species; so it could be trapped *in vivo* and as such can serve as a marker of oxidative stress. Antioxidant enzymes, such as SOD and GPx are considered to be potential markers for identification of stress caused by environmental factors [19].

Most studies on oxidative stress in fish so far have focused on the toxicological aspects, such as the effects of xenobiotics and heavy metals on the activities of antioxidant enzymes and the intensity of lipid peroxidation [20–22]. There are some comparative studies on the activity of antioxidant enzymes among various fish species [23,24]. The molecular aspects of stress especially on the fry and fingerlings during their transportation are underrepresented in literature. We hypothesize that monitoring the ROS production and the presence of antioxidant enzymes in cells during and after transportation could provide valuable information for better understanding the reasons behind the substantial mortality of fish fry and fingerlings. We will use silver carp (*Hypophthalmichthys molitrix*) and tilapia (*Oreochromis mossambicus*) fingerlings as model, the former representing one of the major culture species of Bangladesh whereas the tilapia represents a hardy culture species in Bangladesh. This study will focus on the effects of hypoxia and subsequent reoxygenation (hypoxia to normoxia) on H_2O_2 production and consequent antioxidant enzymes SOD and Gpx responses during transportation of Silver carp (*Hypophthalmichthys molitrix*) and tilapia (*Oreochromis mossambicus*) fingerlings in Bangladesh.

MATERIALS AND METHODS

Sampling and experimental fish

Tilapia (*Oreochromis mossambicus*) (6.7 ± 0.16cm and 3.97 ± 0.22g) and Silver carp (*Hypophthalmichthys molitrix*) fingerlings (9.36 ± 0.09cm; 5.71 ± 0.11g) were collected from the rearing pond of Government fish seed production farm of Debidwar, Comilla, Bangladesh. Fingerlings were conditioned in an artificially made hapa (net enclosure) in the rearing pond for 6 hours (h) before transportation.

Transportation

The preconditioned fingerlings were loaded in 30L aluminum vessel. Fingerlings were transported at 250 gL^{-1} loading density to simulate the traditional transportation method used in Bangladesh.Fingerlings were brought to the laboratory after 6 h of transportation. Hand agitation was performed during transportation to facilitate aeration same as the fish traders do during fish fry transport. To avoid reoxygenation, we did not exchange water during transportation. DO and temperature were measured at interval during transportation.

Reoxygenation

After 6h of transportation, fingerlings (n= 100 in each group) were released in three tanks (1000L) filled up with pond water to observe delayed mortality and reared for 16 days. The water was exchanged at every 24 h interval and continuous aeration was provided using aerators. Dissolved oxygen (DO) and water temperature were

measured every day (Table 1). Fish were reared with artificial feed at 2% of body weight in every 24 h interval.

Sampling

Fingerlings samples were collected at 0h, 1h, 3h and 6h of transportation and 0h, 12h, 24h, 48h, 96h, 192h and 384h after releasing (reoxygenation) in tanks. All samples were immediately frozen in liquid nitrogen and then stored at -80º C until analyzed.

Table 1. Average dissolved oxygen concentration (mg L^{-1}) and temperature (˚C) of water during transportation and reoxygenation (mean ± SEM).

Conditions	Dissolved oxygen (mg-L^{-1})	Temperature (˚C)
Transportation	1.02 ± 0.03	26.51 ± 0.33
Reoxygenation	5.31 ± 0.15	26.91 ± 0.25

Treatment of samples

Fish muscle tissue (0.5g) was soaked in 5 ml phosphate buffer saline (PBS, pH 7.4) for 3 minutes to wash blood and other undesirable substances. Then the tissue was homogenized in 3ml of chilled HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (20 mM HEPES, 1mM EGTA (ethylene glycol tetra acetic acid), 210 mM mannitol and 70mM sucrose, pH 7.2) for 3 minutes and centrifuged at 1500xg for 5 minutes for SOD measurement. For GPx, 0.5g of tissue was homogenized in 3ml of chilled Tris buffer (50mM Tris-HCl, 5mM EDTA, 1mM DTT, pH 7.5) for 3 minutes and centrifuged at 10,000xg for 15 minutes. In case of hydrogen peroxide (H_2O_2) measurement, 0.5g of tissue was homogenized in 3 ml of PBS for 3 minutes and centrifuged at 10,000xg for 5 minutes. After centrifugation, supernatants were collected and preserved at -80º C.

ANALYTICAL METHODS

SOD and GPx analysis were performed in Epoch micro-plate scanning spectrophotometer (BioTek instruments, USA) in triplicate in 96 well microplate. Hydrogen peroxide (H_2O_2) was measured in Nanodrop 2000 UV-Vis spectrophotometer (Thermo-Scientific, Wilmington, USA) in triplicates.

Measurement of Hydrogen peroxide (H_2O_2)

HYP01 assay kit (NWLSS™, USA) was used for the quantitative determination of hydrogen peroxide, which is based on the oxidation of ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) by peroxides. 1 part of Fe reagents were diluted with 100 parts of Xylenol Orange (XO) to prepare working XO/Fe reagent. 5µL catalase enzyme was added to each blank wells. 5µL dH_2O was added to each sample test wells and calibrator wells. Then 20µL of diluted sample or standard solutions of H_2O_2 (100mM) were added to each well as appropriate. The plate was agitated and incubated for 5 minutes at room temperature, then 200µL of XO: Fe reagent was added to each well. The plate was incubated for 45 minutes at room temperature. Finally, absorbance was taken at 570nm by using Nano drop spectrophotometer.

Measurement of Glutathione peroxidase

The Glutathione peroxidase assay kit (NWLSS™, USA) was used for detecting GPx in fish muscles. The NWLSS™ Glutathione peroxidase assay is an adaptation of the method of Paglia &Valentine [25]. A 150µL reaction mixture in each well was prepared by adding 50µL of diluted sample, 50µL of working NADPH, and 50µL of working H_2O_2. Absorbance at 340 nm was monitored in a plate reader with recording interval every 1 minute at 25º C. GPx activity was calculated from the net rate of reaction and was expressed in $mUmL^{-1}$.

Measurement of Superoxide Dismutase (SOD)

Cayman's superoxide dismutase assay kit (USA) was used for measuring SOD in fish muscle.This SOD assay measures all three types of SOD (Cu/Zn, Mn and Fe SOD). A standard curve was produced by diluting the supplied standard of SOD in assay buffer (50mM Tris-HCl, pH 8.0 containing 0.1mM di-ethylenetriaminepentaacetic acid (DTPA) and 0.1mM hypoxanthine). 10 µL of standard or sample were added to each well to reach reaction volume of 230µl. Reactions were initiated by adding 20µL of diluted xanthine oxidase to each well and incubated at 25º C on a shaker for 20 minutes and then absorbance was measured at 450 nm. SOD activity expressed in unit per milliliter (UmL^{-1}) of tissue homogenate. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

Statistical analysis

The results were statistically analyzed by using SPSS software version 11.5 with the level of significance at $p < 0.05$. Treatments were compared by one way ANOVA followed by Tukey's HSD post hoc for multiple comparisons.

RESULTS AND DISCUSSION

Mean DO level during fry transportation was in the range of 1.02 ± 0.03 mgL^{-1} in both groups of fish fingerlings, while it was 5.31 ± 0.15 mgL^{-1} after reoxygenation (Table1).The mean temperature of water was within 26ºC during transportation and after reoxygenation (Table 1). With the duration of transport, the physicochemical properties of the medium are changed and the quality deteriorates. Availability of dissolve oxygen and buildup of carbon dioxide and ammonia in transportation media are the limiting factors. Oxygen acts as a single critical factor in fish transportation, for instance, oxygen increases the LT_{50} (lethal time for 50% of the population) from 40h to 13h for turbot, *Scophthalmus maximus* (L.) transportation[28]. In the present experiment, the dissolved oxygen level reached as minimum to 1.02 ± 0.03 mgL^{-1} during transportation, which is in fact the hypoxic condition for the fish. The consequence of hypoxic

condition in cells is the accumulation of free electrons which are responsible for generation of high level of Reactive Oxygen Species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion and hydroxyl radicals[29].Hypoxia was shown to induce increased ROS production in pulmonary artery smooth muscle cells, cardiomyocytes, and several other cell types [30].

Mortality rate was higher for Silver carp than Tilapia fingerlings transportation

A great economic loss is associated with the fish seed mortality during transportation[3,26] and in the context of Bangladesh fisheries sector this is a crucial issue[11]. Delayed mortality of fish seeds, a relatively overlooked issue, also causes significant loss of fish seeds but poorly described in literature especially in the context of Bangladesh. In the present study, both the immediate mortality and the delayed mortality varied in Tilapia and Silver carp fingerlings.

At 1h and 3h of transportation, we observed no mortality of tilapia or silver carp fingerlings. However, after 6 h of transportation both species of fingerlings showed high rate of mortality. Mortality rate was higher for silver carp than tilapia fingerlings measuring 41.36±1.69 % and 18.40 ± 1.16% respectively. Silver carp fingerlings also showed high delayed mortality (51.93±1.5%) whereas few delayed mortality (8.06±0.61%) was found for Tilapia fingerlings (Figure 1). After 24h of reoxygenation, no additional delayed mortality was observed for tilapia fingerlings, but for silver carp fingerlings, delayed mortality was observed even after 8 days of reoxygenation.

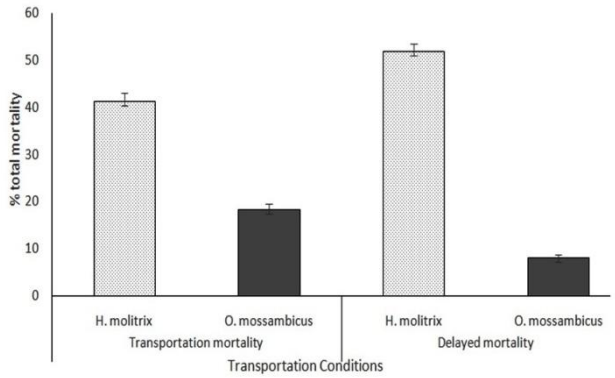

Figure 1. Cumulative transport mortality and delayed mortality of Tilapia (*O. mossambicus*) and Silver carp (*H. molitrix*) fingerlings during hypoxic transportation of 6 hours and subsequent reoxygenation.

Silver carp fingerlings were most susceptible to mortality due to transportation, notably the cumulative delayed mortality of this species was the highest (51.93±1.5%) between the two fish species studied. Several studies showed that fish seed mortality vary during transportation according to the species concern. *Labeo rohita* fingerlings, a fresh water fish for instance, in a transport simulation experiment was shown to have low level of immediate mortality (4 to 12%) but the delayed mortality was high (27 to 49%)[11], whereas 14% of total mortality was reported for silver carp fingerlings during transportation [27].Freshwater drums(*Aplodinotus grunniens*) transported for 6 hours showed 4% immediate mortality and 94% delayed mortality (over 1 to 2 weeks) [8].A total of 24% Tambaqui (*Colossoma macropomum*) juvenile immediately died while transporting in sealed bag with oxygen supply and the cumulative mortality of this fish was as high as 65% in a 10 hours transport simulation experiment at 312 kgm^{-3} loading density[26].

H_2O_2 production gradually increased whereas the GPx and SOD activity decreased during transportation

In our study live fish during hypoxic transportation showed significantly higher ($p < 0.05$) H_2O_2 production and it increases with the increasing duration of anoxic conditions.

However, the GPx activity and SOD activity decreased during transportation (Table 2). H_2O_2 production was significantly ($p<0.05$) higher at 6 h of transportation than 1h and 3 h of transportation while there was no significant ($p<0.05$) difference in H_2O_2 production at 1 h and 3 h of transportation. The highest content of H_2O_2 (8.36 ±0.45 µM) was recorded at 6 h of transportation in Tilapia fingerlings. GPx activity in Tilapia and SOD activity in Silver carp at 0h were recorded the highest measuring 11.38 ± 0.16 mU/mL^{-1} and 0.18± 0.01 UmL^{-1} respectively and then both activity decreased during transportation reaching to 7.93 ±0.88 $mUmL^{-1}$ and 0.12±0.02 UmL^{-1} respectively. SOD activity at 3h and 6 h of transportation was significantly ($p<0.05$) lower than 1h of transportation.

Hypoxia decrease GPx activity in muscle by 30% in fresh water carp fish, *Cyprinus carpio*[33]. A diminution in the expressions of GPx and SOD in response to hypoxia in Atlantic cod, *Gadus morhua*, a hypoxic-sensitive species was also reported [34]. The present study also revealed that during hypoxic transportation GPx activity was low, but after reoxygenation, GPx activity increased gradually. Down regulation of superoxide dismutase 2 (*sod2*), two glutathione peroxidases (*gpx1a* and *gpx4b*), and several other selenium-binding proteins with known or putative antioxidant functions were also reported in case of liver cell of stressed (starvation) zebra fish[39]. Hypoxia was shown to reduce the superoxide dismutase and glutathione peroxidase activities in isolated cardiac myocytes and it has been reported that low level of antioxidant reserve during hypoxia may contribute to the oxidative injury on reoxygenation[35,36].

Table 2. Production of H_2O_2 and activity of GPx/ SOD Tilapia (*O. mossambicus*) and Silver carp (*H. molitrix*) fingerlings during 1 h, 3 h and 6 h of hypoxic transportation (mean ± SEM). Values with different superscripts in same column are significantly ($p < 0.05$) different.

Transportation durations (hour)	*O. mossambicus*		*H. molitrix*		
	H_2O_2 (µM)	GPx (mUmL^{-1})	H_2O_2 (µM)	SOD (UmL^{-1})	GPx (mUmL^{-1})
0	6.83 ± 0.16^{c}	11.38 ± 0.16^{c}	6.30 ± 0.16^{a}	0.18± 0.01^{c}	9.35 ±0.13^{d}
1	7.67 ±0.34^{a}	9.07 ± 0.43^{a}	6.84 ±0.15^{a}	0.15 ± 0.01^{a}	8.61 ±0.29^{a}
3	7.93 ± 0.51$^{a, b}$	8.81 ±0.99^{a}	6.94±0.37^{a}	0.12±0.01^{b}	8.00 ±0.31^{b}
6	8.36 ±0.45^{b}	7.93 ±0.88^{b}	7.72±0.37^{b}	0.12±0.02^{b}	6.96 ± 0.59^{c}

H_2O_2 production gradually decreased whereas the GPx and SOD activity increased during reoxygenation

Immediate after reoxygenation, H_2O_2 content increased up to 12 h but then decreased gradually, however the GPx and SOD activity increased with the time of reoxygenation (Table 3). GPx activity increased significantly ($p < 0.05$) in both fish fingerlings. Between the two types of fingerlings, the tilapia fingerlings showed highest GPx activity (12.99 ± 0.46 µM). SOD activity was measured only for silver carp fingerlings, an increasing trends of SOD activity like that of GPx activity was recorded reaching the highest level measuring 0.210 ± 0.01 mUmL^{-1}after 384 hours of reoxygenation.

Table 3. H_2O_2 concentration and activity of GPx/SOD in Tilapia (*O. mossambicus*) and Silver carp (*H. molitrix*) fingerlings at 0, 12, 24, 48, 96, 192 and 384 h of reoxygenation followed by hypoxic transportation (mean ± SEM). Values with different superscripts in same column are significantly ($p < 0.05$) different.

Duration after reoxygenation (hour)	Tilapia (*O. mossambicus*)		Silver Carp (*H. molitrix*)		
	H_2O_2 (µM)	GPx (mUmL^{-1})	H_2O_2 (µM)	SOD (UmL^{-1})	GPx (mUmL^{-1})
0	8.36 ± 0.45^{a}	7.93 ± 0.88^{a}	7.72 ± 0.37^{d}	0.120 ± 0.02^{a}	6.96 ± 0.59^{a}
12	8.99 ± 0.52^{a}	8.61 ± 0.71^{a}	8.31 ± 0.47^{a}	0.147 ± 0.01^{a}	6.35 ± 0.61^{a}
24	7.99 ± 0.43^{b}	9.93 ± 0.82^{b}	8.13 ± 0.68^{a}	0.156 ± 0.01^{a}	6.67 ± 0.68^{a}
48	7.04 ± 0.24^{c}	10.66 ± 0.85^{c}	8.25 ± 0.67^{a}	0.190 ± 0.01^{b}	8.10 ± 0.55^{b}
96	7.14 ± 0.24^{c}	10.63 ± 0.54^{c}	7.84 ± 0.42^{a,b}	0.190 ± 0.01^{b}	9.05 ± 0.46^{c}
192	6.72 ± 0.15^{c}	12.41 ± 0.29^{d}	7.13 ± 0.28^{b,c}	0.193 ± 0.01^{b}	9.44 ± 0.25^{c,d}
384	6.51 ± 0.16^{c}	12.99 ± 0.46^{d}	6.96 ± 0.12^{c}	0.210 ± 0.01^{b}	9.71 ± 0.14^{d}

The sudden increase in oxygen availability after 6h hypoxic transportation induces higher metabolic rate, which might contribute to the higher production of H_2O_2 after reoxygenation. An increase of oxygen consumption in transgenic zebra fish is reported to be accompanied by an increase in ROS generation [31]. In response to sudden input of oxygen (O_2) after environmental hypoxia, the pacific white shrimp also showed an increased reactive oxygen species production [32]. Hypoxia - reoxygenation stress can initiate cell death events in terms of necrosis and apoptosis, and appearance of both dependent on increased reactive species production [37,38]. Higher H_2O_2 and, lower GPx and SOD activities in fish reveals that increased ROS induce cellular apoptosis process during hypoxic transport and subsequent reoxygenation might triggers mortality, specifically delayed mortality of fish fingerlings.

Changes in ROS level and antioxidant enzymes status in response to hypoxia–reoxygenation stress are crucial factors for oxidative damage and associated mortality. The oxidative stress mediated increase of H_2O_2 and reduced SOD and GPx activity might involve in fish fry mortality.

The findings of this study will contribute to better understanding the transport stress responses and the potential causes of mortality during and after fish fry and fingerlings transportation. Our findings suggest that fish fry and fingerlings should not be transported only with hand splashing or without oxygen supply. Furthermore, gradual adaptation to normoxic condition might reduce delayed mortality of fish fingerlings after hypoxic transportation.

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