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Effect of Two Aquatic Ambient Factors (pH and Dissolved Oxygen) on Antioxidant Generation in Skeletal Muscle of Zebrafish (*Danio rerio*, Hamilton 1822)

Sabarna Chowdhury[1] and Surjya Kumar Saikia[1*]
[1]Departement Zoology, Visva Bharati University, Santiniketan, Bolpur, West Bengal 731235, India

*Correspondence :
surjyasurjya@gmail.com

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Abstract

Ambient factors in the aquatic body are often responsible for oxidative stress in fish. Zebrafish have been frequently targeted for study to understand such hypoxic effects. In this study, two ambient factors, viz. DO saturation (20-30%, 40-50%, 60-70%, and, 80% and above) and pH (4.5-5.5, 5.5-6.5, 6.5-7.5, 7.5-8.5 and 8.5-9.5) were experimented for generation of antioxidant (Catalase, SOD, and Glutathione) in the skeletal muscle of zebrafish. The oxidative stress was marked by the levels of MDA in the skeletal muscle. These conditions were tested against the treatment period (in hr) from 4hr to 16 hr for each DO saturation level and 1hr to 4 hr for each pH level. The analysis of data shows that, for DO saturation, the skeletal muscle of zebrafish suffered maximum at 12 hr of treatment period having the highest level of MDA against 20-30% of saturation level. Similarly, the period for pH was 2hr and the treatment level was 4.5-5.5. Although alkaline ambiance (pH 8.5-9.5) also exerted strong oxidative stress at 2 hr, it was significantly low in MDA generation. In all cases, the antioxidant levels spiked after post maximum generation period i.e. 16 hr for DO saturation and 2 hr for pH level. This indicates a time point where the fish undergoes oxidative stress and initiation of a counter mechanism during the post-oxidative stress period in these two ambient conditions. A two-way ANOVA has explained that the effect of the treatment period and the level of ambient factors significantly contributed to the antioxidant generation in zebrafish. The outcome of this work will directly help in the management of fish culture when the aquatic body turns hypoxic or acidic.

INTRODUCTION

Reactive Oxygen Species (ROS) are reactive molecules and can damage cellular structures resulting in an imbalance between the oxidants and the antioxidants to form a state called oxidative stress (Birben *et al*., 2012). Antioxidants, being either exogenous or endogenous compounds that eliminate oxidative stress and its consequences from the biological systems play a major role as bioindicators in cellular responses against oxidative stress (Kurutas, 2016; Caliskan and Caliskan, 2021). Similarly, in fish, the

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imbalance between the production of ROS and the antioxidant defense system can cause oxidative stress resulting in DNA hydroxylation, protein denaturation, lipid peroxidation, apoptosis, and ultimately cell damage (Hoseinifar *et al*., 2020). Previous studies have evaluated oxidative stress markers like lipid peroxidation and antioxidant enzymes to understand the oxidative stress mechanisms under various conditions (Karadag *et al*., 2014; Camkurt *et al*., 2017; Sahreen *et al*., 2021).

Oxygen and pH are effective ambiance factors that often push an organism towards physiological stress conditions. For example, when there is insufficient oxygen (hypoxic situation), in order to cope with hypoxic stress a transcriptional response is mediated by Hypoxia Inducible Factors (HIFs) in mammals (Majmundar *et al*., 2010). Aquatic species like fish often suffer from hypoxia due to exposure to low oxygen availability and sudden changes in pH in water. Studies have shown that in aquatic organisms like fish HIF1α (a subunit of HIF) gets upregulated in fish (Pelster and Egg, 2018) to form HIF1α-HIF1β heterodimer that undergoes post-translational modification and promotes downstream gene expression related to hypoxia (Hudson *et al*., 2002). Hypoxia can cause severe mortality, and reduce growth rates, fish reproduction and development, and the behavior of fish which may lead to reduced abundance, diversity, and harvest of fish in aquatic environments (Breitburg, 2002; Wu, 2009).

In a study on zebrafish, Chowdhury *et al*. (2020) highlighted that acidic ambiance impairs the growth of zebrafish under an experimental condition in a laboratory. Recently, Chowdhury and Saikia (2022), in an extensive review have suggested zebrafish as a potential animal model for studying oxidative stress.

With this background, in the present study, the response of oxidative stress was evaluated by determining the levels of antioxidant enzymes (Superoxide dismutase (SOD) and Catalase) and antioxidant (Glutathione), when exposed to reduced DO and variable pH (both acidic and alkaline) levels. Such knowledge is necessary, especially in zebrafish for its wider acceptability as an experimental model and for the possibility discussed by Chowdhury and Saikia (2022). A counter mechanism is a must if oxidative stress occurs in animals. Chowdhury *et al*. (2020) have reported that out of several other tissues (gill, brain, and liver), skeletal muscle is confirmed as highly and promptly affected tissue in zebrafish. Based on such report, the present study was performed on the skeletal muscle tissue of the zebrafish.

METHODOLOGY

Ethical Approval

The work presented here has been approved by the IAEC having IAEC approval No. IAEC/III-16/2020.

Place and Time

This research was conducted between 2020 to 2022 in the Department of Zoology, Visva-Bharati University, Santiniketan, West Bengal, India.

Research Materials

The portable digital pocket-sized pH meter (HI98107P) and portable digital DO meter (Lutron DO-5510) were used for instant recording of the water pH and DO in all aquaria. An N_2 gas cylinder was used to regulate dissolved oxygen saturation in the experimented aquaria for Oxygen stress. A Bead mill micro tissue homogenizer (Omni International) was used to homogenize pooled tissue from the fish samples. All biochemical assays were performed in a UV/VIS Spectrophotometer (Shimadzu).

Research Design

Four levels of percent DO saturation (viz. 20%-30%, 40%-50%, 60%-70%, and 80% and above) and five levels of pH (4.5-5.5, 5.5-6.5, 6.5-7.5, 7.5-8.5, and 8.5-9.5) were set for experiments on zebrafish. In each experiment, a total of 30 zebrafish were deployed. The experiment was computed in triplicate. Three aquarium sets were used to experiment with each level of ambient factor

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(DO and pH). For hypoxic stress (DO as an ambient factor) the duration of the experiment was up to 16 hours, at which the survivability of zebrafish was reduced to 50% (Chowdhury and Saikia, 2023). In the case of acidic stress (pH as an ambient factor), the duration was up to 4 hours at which the survivability was reduced to 50% (Chowdhury *et al*. 2020).

Work Procedure

Fish Collection and Maintenance

Zebrafish were collected locally from a commercial supplier in West Bengal, India. All fishes were stocked for one week in an aquarium in a laboratory environment (pH 6.5-7.5, temperature 25-28 ºC, DO 7-10 mg/l). Regular washing and cleaning of the aquarium was performed. Continuous aeration was provided to the stocked fish. Commercially available food (Tetra bits complete) was supplemented three times a day.

Stress Exposure to Zebrafish

After one week of acclimatization, adult zebrafish (weight: 0.7±0.5g, total length: 3.8±0.2 cm,) in the aquarium were used for oxidative stress experiment. Two different experimental conditions were set, viz. four levels of percent DO saturation (viz. 20%-30%, 40%-50%, 60%-70%, and 80% and above) and five levels of pH (4.5-5.5, 5.5-6.5, 6.5-7.5, 7.5-8.5, and 8.5-9.5). In each experiment, a total of 30 zebrafish were deployed. The experiment was computed in triplicate.

The oxygen saturation was regulated using N_2 gas (25ml N_2 gas per sec) (Butler *et al*., 1994). A portable digital dissolved oxygen meter (Lutron DO-5510) was used to monitor the DO levels in the aquaria. Similarly, the pH levels were maintained using weak organic acid as independent variable (X) in the regression model Y = 7.675 – 0.008X (Y = pH and X = weak organic acetic acid (μl) (R^2 = 0.997) and volume (μl) of strong base (NaOH) in the regression model Y = 7.3667 – 0.005X (Y= pH and X= Volume NaOH, μl, R^2 = 0.9774). A digital pH meter (HI98107P) was used to monitor the pH of the aquaria. A time-dependent observation on the survivability of zebrafish showed 16 hrs of treatment as the maximum tolerable level of hypoxic stress, beyond which more than 50% mortality of zebrafish occurred. Similarly, after 4 hours of pH treatment, more than 50% mortality was observed in the zebrafish population. It is, therefore, the maximum treatment time has been fixed at 16 hrs for DO saturation and 4 hrs for pH levels.

Tissue Collection and Processing

Tissue-specific pooled samples (skeletal muscle, n=10) were collected (Mumford *et al*., 2005) kept in a lysis buffer (phosphate buffer), and then homogenized using a microtissue homogenizer. The homogenized tissues were then centrifuged in 10000 g for 15 min and supernatant was collected for all biochemical analysis.

Biochemical Assays

For SOD, the antioxidant enzyme SOD assay was performed (Ewing and Janero, 1995), and catalase assay and reduced glutathione quantification were performed using microplate assay kits (G-Biosciences, ITAK1061 and ITAK1006).

Data Analysis

One-way ANOVA was performed to see any effect among the means of the treatments. A two-way ANOVA was performed to understand if there is any effect of treatment time (in hours) and ambient factors (DO or pH levels) or interactions of both on the generation of Catalase, Glutathione, and SOD in the skeletal muscle of zebrafish. In the case of all analyses, α level was fixed at 0.05. The SPSS 16.0 and Minitab software were used for all statistical analyses.

RESULTS AND DISCUSSIONS

MDA Analysis for pH and DO Level

The MDA results indicated that the skeletal tissue had undergone oxidative stress during the treatments. Out of all DO

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saturation levels, a significant increase in MDA at DO 20-30% oxygen saturation level was observed compared to the control (i.e. 80% oxygen saturation and above) (Figure 1A). Likewise, except for the first hour, all the treatments of the skeletal muscle showed a significant increase in MDA at pH 4.5-5.5 compared to the control (i.e. pH 6.5-7.5) (Figure 1B).

Effect of pH on Antioxidants

Catalase (CAT)

In the case of catalase, its levels in skeletal muscle tissue were reduced in all levels of treatments of pH compared to the control, with the lowest level at pH 4.5-5.5 in all the treatments hours (i.e. 1hr, 2hr, 3hr, and 4hr) (Figure 2A). Amongst the treatment hours, 2 hr of treatment at pH 4.5-5.4 caused reduced generation of catalase in skeletal muscle (Figure. 2A, superscribed letters). It was then followed by pH 8.5-9.5 and pH 5.5-6.5.

From these results, it could be said that at all the levels of pH, the skeletal muscle showed minimum catalase levels at 2hr of exposure and it reduced significantly at pH 4.5-5.5. Beyond 2 hr of treatment, generation of catalase showed a gradual increase.

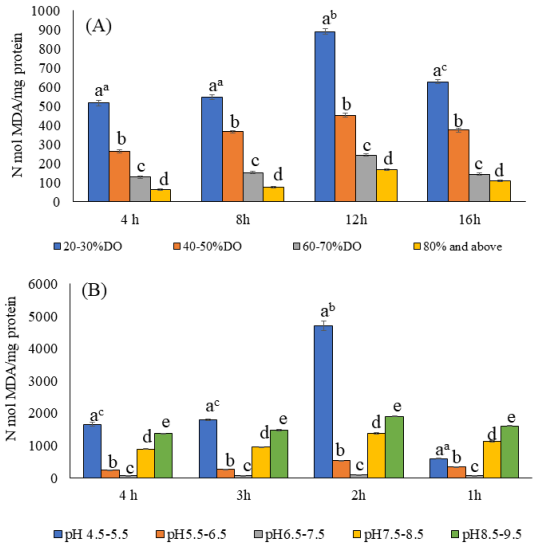

Figure 1. Bar graph showing levels of MDA in the skeletal muscle of zebrafish (n=10) treated with different levels of pH and DO.

Description: (A) different pH levels over different time durations (1hr, 2hr, 3hr, and 4hr); (B) different DO saturation levels over different time durations (4hr, 8hr, 12hr, and 16hr). Means (± SE) were compared using One way ANOVA at $p<0.05$. Different lowercase alphabets indicate statistically significant differences at $p<0.05$ within the means of groups. Means with different superscripted letters show statistically significant differences at $p<0.05$ between the means of different groups.

Glutathione

Analyzing the generation of glutathione at different levels, it was observed that the skeletal muscle tissue was significantly affected at all levels where the levels of glutathione were reduced compared to the control (Fig. 2B). Within each treatment group for a particular treatment period, it was highly reduced in pH 4.5-5.5 level followed by the alkaline pH 8.5-9.5. A comparison across the group (1hr, 2hr, 3hr, and 4hr) showed the highest reduction when treated for two hours at pH 4.5-5.5 (Fig. 2B, superscripted alphabets). Therefore, it could be said that at all the levels of pH (from pH 4.5-5.5 to 8.5-9.5) the skeletal muscle showed minimum glutathione levels at 2hr of exposure. Its generation beyond 2 hr of treatment showed a gradual increase.

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SOD

The generation of SOD was analyzed for each level of pH. It was observed that at pH 4.5-5.5, the skeletal muscle tissue responded significantly with a minimum SOD level compared to the control. One-way ANOVA within each group of treatments based on time (hour) showed a significant decrease in SOD level in skeletal muscle compared to control (Fig 2C). Amongst all the treated hours (1hr, 2hr, 3hr, and 4hr), the SOD level of skeletal muscle at 2hr showed a significant decrease when treated with pH 4.5-5.4 (Fig 2C, superscripted alphabets). Except for the first hour, the generation of SOD against alkaline levels with pH 7.5-8.5 in all the other treatments was significantly low, but not as high as the acidic level (pH 4.5-5.5).

The treatments with different levels of pH clarified that the fish underwent stress at an acidic ambiance (pH4.5-5.5) compared to the rest four pH levels tested. The maximum effect in terms of generation of SOD was observed at pH 4.5-5.5 (Fig 2C, superscribed letter) for 2 hr of treatment. Its generation beyond 2 hr of treatment showed a gradual increase.

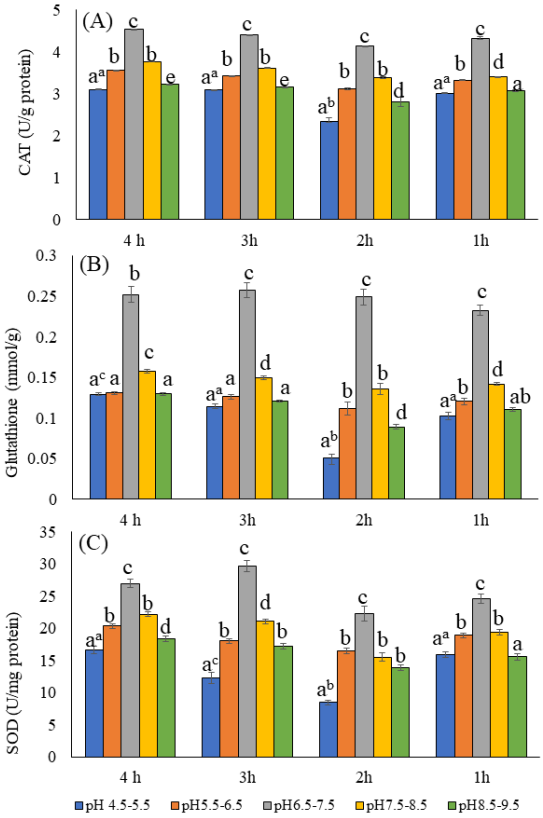

Figure 2. Bar graph showing levels of antioxidants in the skeletal muscle of zebrafish treated with different pH levels over different time durations (1hr, 2hr, 3hr, and 4hr). Different lowercase alphabets indicate statistically significant differences at $p<0.05$ within the means of a group. Means with different superscripted letters show statistically significant differences at $p<0.05$ between the means of different groups.

Description: A) Catalase, (B) Glutathione (C) SOD

Effect of DO Saturation on Antioxidants

Catalase (CAT)

On analysis for each level of DO, the generation of catalase reduced in skeletal muscle at all DO saturation levels compared to the control. Out of all the

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oxygen saturation levels, the 20-30% DO saturation was marked with a significant reduction of catalase (Fig 3A). A one-way ANOVA confirmed the significant decrease in catalase level in skeletal muscle at 12 hr treatments (DO saturation level 20-30%) compared to the control (Fig 3A, superscribed letters). This observation clarifies that at all the DO saturation levels (from 20-30% to 60-70%), the skeletal muscle showed minimum catalase levels at 12hr of exposure to the 20-30% DO saturation level. Its generation beyond 2 hr of treatment showed a gradual increase.

Glutathione

For DO, the glutathione levels were reduced at all the saturation levels of DO compared to the control (Fig 3B). The reduction at DO 20-30% saturation level in skeletal muscle tissue was low compared to the rest of the DO saturation levels. Amongst all the other hours (4hr, 8hr, 12hr, and 16hr), the glutathione level of skeletal muscle at 12hr showed a significant decrease at DO 20-30% saturation level. (Fig 3B, Superscribed letters). It can be, therefore, assumed that at all the levels of DO (from 20-30% to 60-70% saturation), the skeletal muscle showed minimum glutathione levels at 12 hours of exposure. Its generation beyond 12 hr of treatment showed a gradual increase.

SOD

In the case of hypoxic treatments, the generation of SOD was found to be significantly low in all treatment hours than the control (Fig. 3C). Out of all the DO saturation levels, 20-30% of saturation level showed a statistically significant effect on the tissue with minimum SOD level compared to control and all the other treatment hours. With the lowest SOD level, the 20-30% DO saturation was incrementally followed by 40-50% and 60-70% respectively.

Analysis among the treatment groups showed that the skeletal muscle was strongly affected when the fish was subjected to a hypoxic ambiance (20-30% saturation) at 12hr out of all the levels tested for different time durations (Fig. 3C, superscribed letters). Its generation beyond 12 hr of treatment showed a gradual increase.

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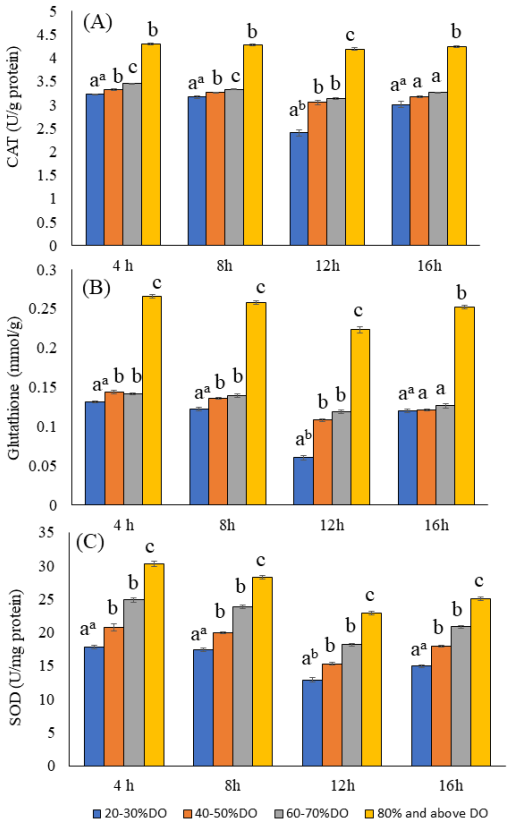

Figure 3. Bar graph showing levels of antioxidants in the skeletal muscle of zebrafish treated with different DO saturation levels over different time durations (4hr, 8hr, 12hr, and 16hr). Different lowercase alphabets indicate statistically significant differences at $p<0.05$ within the means of groups. Means with different superscripted letters show statistically significant differences at $p<0.05$ between the means of different groups.

Description: (A) Catalase (B) Glutathione and (C) SOD.

MDA Versus Antioxidant Levels

Fig 4 clearly shows that the generation of catalase (Fig. 4A), glutathione (Fig 4B), and SOD (Fig 4C) were at their lowest level at 12 hr when treated with 20-30% DO saturation levels. Similarly, for pH at acidic ambiance (pH 4.5-5.5), Fig 4 shows that the catalase (Fig. 5A), glutathione (Fig 5B), and SOD (Fig. 5C) were at the lowest when kept under acidic ambiance (pH4.5-5.5) for 2 hrs.

These lowest levels of antioxidants are marked with the highest formation of MDA in the tissue. The generation of antioxidants then started showing a gradual increment in case pH levels, and for DO saturation levels, all these antioxidants showed an increase in 16 hr of treatment.

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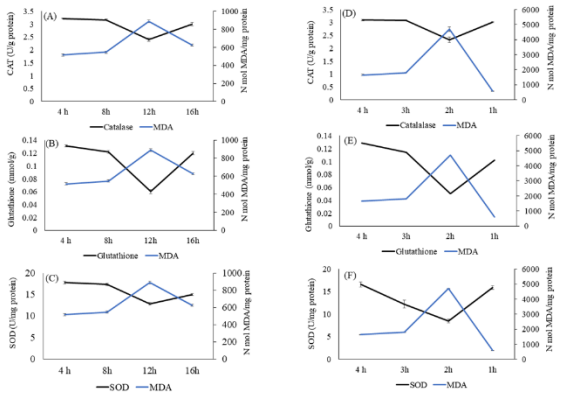

Figure 4. Line graph showing MDA versus anti-oxidant levels in the skeletal muscle of zebrafish treated with 20-30% DO saturation level and pH 4.5-5.5 over different time durations.

Description: (a) For DO 20-30%, the effects are shown as (A) Catalase, (B) Glutathione, (C) SOD; (b) For pH 4.5-5.5, the effects are shown as (D) Catalase, (E) Glutathione and (F) SOD.

Interaction of Time and Ambient Factors

Table 1 suggests a summary of the effect of Time (in hours) and different levels of ambient factors or both on the formation of antioxidants in the skeletal muscle of zebrafish. The η represents the effect size and it explains that the effects of time and ambient factors (pH and DO saturation levels) were individually and statistically significant on the production of all antioxidants in the skeletal tissue. The interactions of Time and ambient factors also have a significant effect on it, except that the effect sizes of the interaction are found to be smaller than the individual effects.

Table 1. Two-way ANOVA showing effects and interactions between ambient factors and treatment time on the generation of antioxidants. The critical value (α) considered is 0.05.

Type	S	TypeIII-SQ	df	MS	F	*p*	η
			Time (Hours) versus pH levels				
Cat	T	6.536	3	2.17	17.656	0.000	0.815
	pH-L	51.270	4	12.8	103.866	0.000	0.972
	TxpH-L	1.481	12	0.12	8.612	0.000	0.366
Glu	T	0.031	3	0.10	5.995	0.010	0.600
	pH-L	0.566	4	0.14	81.810	0.000	0.964
	TxpH-L	0.021	12	0.002	6.606	0.000	0.307
SOD	T	861.213	3	287.071	10.716	0.001	0.728
	pH-L	3432.856	4	858.214	32.036	0.000	0.914
	TxpH-L	321.537	12	26.795	8.190	0.000	0.354
			Time (Hours) versus Dissolved Oxygen				
Cat	T	3.375	3	1.125	127.554	0.000	0.727
	DO_L	38.823	3	12.941	0.0014	0.000	0.968
	TxDO-L	1.955	9	0.217	24.632	0.000	0.606
Glu	T	0.043	3	0.014	292.970	0.000	0.859
	DO-L	0.499	3	0.166	0.00038	0.000	0.956
	TxDO-L	0.010	9	0.001	22.952	0.000	0.589
SOD	T	910.571	3	303.524	405.436	0.000	0.894
	DO-L	2640.748	3	880.249	0.001176	0.000	0.894
	TxDO-L	27.883	9	3.098	4.138	0.000	0.205

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Cat, Catalase; Glu, Glutathione, SOD, Superoxide dismutase; T, Time; pH-L, pH levels; DO-L, DO levels; S, Source; SQ, Sum of Square; MS, Mean Square; η^2, partial eta square.

The present study intends to confirm the effect of ambient factors on the skeletal tissue of fish taking zebrafish as a model. Here, an attempt was made to understand the changes in the levels of antioxidant enzymes and antioxidants when the fish is exposed to different levels of acidic (pH 4.5-5.5, pH 5.5-6.5) and alkaline pH (pH 7.5-8.5, pH 8.5-9.5) levels, and to different DO saturation levels (viz. 20-30%, 40-50%, 60-70% and above 80%) at different time intervals (1hr, 2hr, 3hr and 4hr for pH and 16hr, 12hr, 8hr and 4hr for DO saturations). It was clear from the results that the generation of SOD, catalase, and glutathione were significantly decreased at different time intervals within the time when the mortality of fish was <50% for each level of pH and DO. This pattern of decrease in antioxidant level is opposite to the MDA level where it increases with time of exposure. Earlier studies in mammals and fish exposed to various stressors have shown a similar contrasting pattern of change in MDA levels with antioxidant levels (Mukherjee *et al*., 2019; Mukherjee *et al*., 2022).

The MDA levels as shown in Fig 4, evidently explained the status of oxidative stress of zebrafish with its increased formation in the skeletal muscle and subsequent reduction against the rising levels of antioxidants in the muscle with progress in the duration of the treatments. For DO saturation levels, such an effect was visible at 12 hours of treatments, and for pH levels, it was 2 hours of treatments. This is obvious that the fish suffered from oxidative stress and it can be understood biochemically at 12 hours in case of DO saturation level and 2 hours in case of pH levels. An increase in MDA indicated the onset of oxidative stress situation in both cases, and the subsequent release of antioxidants causing a decrease in MDA formation is indicative of a counter mechanism.

A recent study has revealed that oxidative stress plays a vital role in maintaining skeletal muscle myogenesis. In mammals, an alteration in the redox homeostasis in muscle can lead to various muscle disorders (Lian *et al*., 2022). As can be seen from the present study, oxidative stress in all DO saturation levels started from 12 hours of treatment and for all pH levels, it appeared to be 2 hours of treatment. As it became evident that the zebrafish muscle underwent oxidative stress at pH level 4.5-5.5, the lowest levels of antioxidants at this level also confirmed the effect. A similar explanation may be forwarded for the DO saturation level at 20-30%.

Earlier, in mammals, Arsova-Sarafinovska *et al*. (2009) revealed that under altered oxidative status, lipid peroxidation significantly increased with a lower level of catalase and glutathione. A similar observation was also reported by Kasapoglu and Ozben, (2001) where antioxidant levels decreased against increasing MDA with the increase in aging in mammals. A study in *Heteropneustes fossilis* exposed to sodium fluoride showed a reduced level of antioxidants compared to the control (Yadav *et al*., 2015). In another fish, *Ctenopharyngodon idellus* oxidative stress response in the liver, gills and kidneys exposed to chlorpyrifos has shown reduced levels of antioxidants compared to the control, which is opposite to the levels of lipid peroxidation (Kaur and Jindal, 2017). In the present study, too, lipid peroxidation increased whereas antioxidant levels decreased when fish were exposed to pH levels and DO levels on temporal gradients. Earlier, a similar observation was reported from the liver tissue of rats (Mak *et al.*, 1983). This can be, therefore, concluded that the skeletal muscle of zebrafish undergoes oxidative stress at the level of pH 4.5-5.5 when exposed for 2 hours and DO 20-30% when exposed for 12 hours. Beyond these hours,

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its level started decreasing. However, the situation may not be the same in the case of chronic exposure to such ambient situations.

This is also important to know which factor, level of pH/DO saturation or time of treatment, exerted a significant effect on change in anti-oxidant levels in skeletal muscle. Ludke *et al*. (2017) reported a time-dependent generation of ROS when cardiomyocytes were treated with doxorubicin in 24 hours. In the present study, a 2-way ANOVA depicted the effect of the length of treatment (in hours) when the fishes were kept under different pH or DO saturation levels. At the same time, it is also well known that antioxidants can attenuate the damaging effects of oxidative stress and delay cellular processes that contribute to cellular dystrophy. This is because anti-oxidants neutralize the effect of oxidative stress reacting with Reactive Oxygen Species in cells (Poljsak *et al*., 2013; He *et al*., 2017). The effect of stressors (pH levels and DO saturation levels) on the generation of antioxidants is statistically significant. However, although the interaction between the two factors (effect of time and Stressor levels) showed a statistically significant effect on anti-oxidant generation, the effect size in these cases is very low, projecting a weak interaction (considering $\beta \approx 0.08$). It may have a significant effect, but to validate such a hypothesis more stress levels need to be tested.

CONCLUSION

In conclusion, it can be accepted that both ambient factors exert oxidative stress on the skeletal tissue of fish in a time-dependent manner. In the case of pH, the highest oxidative stress was observed at 2 hrs, with subsequent neutralizing effect by antioxidants. Similar conclusions can be made in the case of DO saturation levels. In the case of DO saturation levels, the treatment at 12 hrs showed the highest oxidative stress following attenuation by antioxidants. This information has established that ambient-induced (pH and DO saturation) oxidative stress in zebrafish is primarily a time-dependent phenomenon.

CONFLICT OF INTEREST

Authors have no conflict of interest to declare.

AUTHOR CONTRIBUTION

SC conducted experiments, produced, compiled, and analyzed data, and prepared the first draft of the manuscript; SKS conceived the idea, supervised the work, monitored data compilation and analysis, suggested statistical analysis of data, and reviewed the draft.

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