Journal of Fish Health Vol. 3 (2) - Dec 2023
https://doi.org/10.29303/jfh.v3i2.3249

EVALUATION OF OXIDATIVE STRESS ON ZEBRAFISH UNDER DIFFERENT pH AND DISSOLVED OXYGEN LEVELS USING MDA AS BIOMARKER

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Received : 2023-08-26
Accepted : 2023-12-28

Keywords :
Hypoxia, zebrafish, malondialdehyde, acidic ambience

ABSTRACT

Oxidative stress on zebrafish (*Danio rerio*) has been evaluated for pH (four levels namely pH 4.5-5.5, 5.5-6.5, 7.5-8.5 and 8.5-9.5) and Dissolved Oxygen (DO 20-30%, 40-50% and 60-70%) in terms of oxygen saturation. Temporally, the exposure for pH levels were maintained from as 1h, 2h, 3h and 4h and for DO, it was (4h, 8h, 12h and 16h. The malondialdehyde (MDA) was used as biomarker of oxidative stress. It was observed that out of all tissues (skeletal muscle, liver, gill and brain) analyzed, zebrafish showed highest stress response in skeletal muscle against both environmental ambiances. In case of pH levels, significant increase in MDA level in skeletal muscle was recorded at pH 4.5-5.5, and the MDA level of skeletal muscle at 2h showed significant increase at this level of pH. Whereas, at DO 20-30% saturation level, skeletal muscle tissue significantly responded at this level of DO saturation and showed the maximum MDA level compared to the other tissues. On analysis for the duration of exposure, it was observed that skeletal muscle of zebrafish showed highest levels of MDA at DO 40-50% O_2 saturation for 12h. In conclusion, it has become evident that zebrafish may undergo oxidative stress at pH 4.5-5.5 when exposed for 2hours, similarly, for DO it is 20-30% saturation for 12 hour exposure.

INTRODUCTION

Acidification in water bodies, throughout the world for last forty years, largely in lakes has invited extensive and vigorous research approach regarding aquatic resource management (Mcdonald, 1983). In freshwater bodies, it has become a global problem involving Europe, USA and Asia (Schindler, 1988; Psenner, 1994). Aquatic animals including fish live in a narrow range of pH, which is near neutral, therefore, any change in pH range adversely affects their normal biological functioning (Mcdonald, 1983; Wood, 1989). Of these, the metabolic mechanisms are found to be the most susceptible biological functions (Bhaskar and Govindappa, 1985). A high alkaline or acidic stress may affect the physiological system, especially ion transporting cells in fish (Kwong et al., 2014). Thus, a sudden change in pH may lead to acute stress and a chronic pH state may cause death. Besides, acidic environment induced physiological impairments may disturb the overall homeostatic condition during growth in fish. Likewise, aquatic environments undergo a seasonal and sometimes daily

variation in dissolved oxygen (DO) and this aquatic hypoxia triggers decreased metabolic rates in fish (Copatti et al., 2019). It is an early known fact that fish migrates to a more tolerable regions when exposed to low DO. They often seem to avoid lethal levels when better oxygenated water is available (Dowling and Wiley, 1986). Not only fish, but the survival, growth and reproduction of other aquatic species like Daphnia, Hyalella, Gammarus and other aquatic insects also gets affected when exposed to hypoxia (Nebeker, 1972; Nebeker et al., 1992). In fish species like *Megaleporinus obtusidens* and *Paralichthys olivaceus* low oxygen levels increase stress affecting their growth performance and other physiological parameters, and the tolerance level of hypoxia includes metabolic and oxidative adjustments (Guo et al., 2016; Copatti et al., 2019). Hypoxic stress also affects the blood parameters, antioxidant enzymes, differential expression of hypoxia inducing factor 1 (HIF1) and other physiological responses in fish species like *Clarias batrachus* and *Micropterus salmoides* (Yang et al., 2017). The zebrafish (*Danio rerio*), a small teleost has become the most widely used animal model for the study of various biological phenomena of vertebrates. Recently, this fish was subjected to study under different stress conditions for stress related diseases (Steenbergen et al., 2011). However, very limited studies on the stress tolerance, especially with pH and DO are performed on zebrafish. Few reported studies addressed toxicological effect on embryos of zebrafish (Dave, 1984; Dave, 1985; Andrade et al., 2017). Zahangir et al. (2015), reported secondary stress responses of 'pH stress' on zebrafish where the haematological parameters were primarily assessed. It was also analysed that the fish showed behavioural distress at lethal levels of pH (Zahangir et al., 2015). The physiology of zebrafish gets highly affected when exposed to lower than tolerable range of oxygen in both adults and embryos (Feng et al., 2015; Lin et al., 2020). Fishes like Tor putitora, bonefish and *Oreochromis niloticus* showed behavioural fluctuations when exposed to hypoxic conditions (Xu et al., 2006; Shultz et al., 2011; Kalkhundiya et al., 2020).

In the present study, responses during oxidative stress caused by acidic and alkaline pH and reduced DO were analysed through oxidative stress indicators and specified biochemical parameters, especially malondialdehyde (MDA) level in fish body. Being a by-product of lipid peroxidation, MDA has been widely used as a biochemical index of oxidative stress in mammals (Eze et al., 2008). The results of MDA, therefore, supported the change in behaviour of the zebrafish at the effective levels of DO and pH.

METHODOLOGY

Fish collection and maintenance

Zebrafish stock was collected from a local supplier in Howrah, West Bengal. After collection, the stock was brought to the laboratory and was kept in aquarium under laboratory environment (pH 6.5-7.5, temperature 25-28°C, DO 7-10 mg/l). The work has been approved by the IAEC having IAEC approval No. IAEC/III-16/2020. The water of the aquarium was cleaned on a regular interval with proper aeration. The stock of zebrafish was provided with fish food (Tetra bits complete) three times in a day for their normal growth and development. The fish were acclimatized in this laboratory conditions for about one week.

Stress exposure to zebrafish

After one week of acclimatization, zebrafish (weight: 0.7±0.5 g, total length: 3.8±0.2 cm,) in the aquarium (35cm x 15cm x 20cm) was used for further studies. The experiments were set under two different conditions viz. variation in DO and variation in pH. Zebrafish were

exposed to four different levels of tolerable DO namely, three hypoxic levels (20%-30% of O_2 saturation, 40%-50% of O_2 saturation, 60%-70% of O_2 saturation) and one normoxic level (80% and above of O_2 saturation) (Feng et al., 2015) under four different experimental setups (each setup, n=30). The DO levels were regulated using nitrogen gas (25ml gas per sec) by following the methods of Butlet et al. (1994). A portable digital DO meter (Lutron DO-5510) was used to record the DO levels of the aquaria. Zebrafish were exposed to four different sublethal levels of pH (4.5-5.5, 5.5-6.5, 7.5-8.5 and 8.5-9.5) (Zahangir et al., 2015) with pH 6.5-7.5 as control, under five different experimental setups (each setup, n=30). The pH levels were maintained by application of weak organic acid following a regression model with original data Y = 7.675 – 0.008X (Y = pH and X = Volume acetic acid, µl, R^2 = 0.997) and by the application of a strong base (NaOH) following a regression model with original data Y = 7.3667 – 0.005X (Y= pH and X= Volume NaOH, µl, R^2 = 0.9774). A portable digital pocket-sized pH meter (HI98107P) was used to record the pH of the aquaria. Ammonia levels were tested and no abnormalities were found.

Further, analyses were done on each tissue (Skeletal muscle, Liver, Gill, Brain) against each DO levels at four different time periods (4h, 8h, 12h and 16h). Similar analyses were also performed on each tissue (Skeletal muscle, Liver, Gill, Brain) against each pH levels at four different time periods (1h, 2h, 3h and 4h). The time periods taken were determined by the analysis of mortality rate of the fish for the lowest tolerable level of DO, and for both lowest and highest tolerable levels of pH. It was found that after 16h of exposure to the lowest tolerable level of DO, more than 50% mortality occurred. Similarly, after 4h of exposure to lowest and highest tolerable levels of pH, more than 50% mortality of zebrafish occurred.

Tissue collection and processing

The pooled tissues (skeletal muscle, liver, gill and brain) were collected at different time periods of different levels of DO and pH and were kept in lysis buffer (phosphate buffer) and then homogenized using micro tissue homogenizer. The tissues homogenized were then centrifuged in 10000 g for 15 min. The supernatant was collected and used for further biochemical assessment.

TBARS assay

The biochemical assessment was performed for malondialdehyde (MDA). MDA assay was performed according to the method of Aust, (1985). MDA is a product of lipid peroxidation and reacts with TBA (thiobarbituric acid) to give a red species named TBARS (thiobarbituric acid reactive substance).

Statistical analysis

Homogeneity of variances of data sets was tested using Levene's statistics. The Kruskal-Wallis test was computed where Levene's statistics did not comply to $p>0.05$. Otherwise, Student's t test and One way ANOVA were performed. In case of all analyses, α level was fixed at 0.05. SPSS 16.0 and Minitab software were used for all statistical analyses.

RESULTS

MDA analysis at each level of pH

MDA was analysed for each level of pH in four different time intervals. The boxplots for all four tissues under control environment are shown in Figure 1 (A and C). In

both pH and DO treatments under control environment, maximum response in terms of MDA levels were obtained from muscle tissue (Figure 1B and 1D).

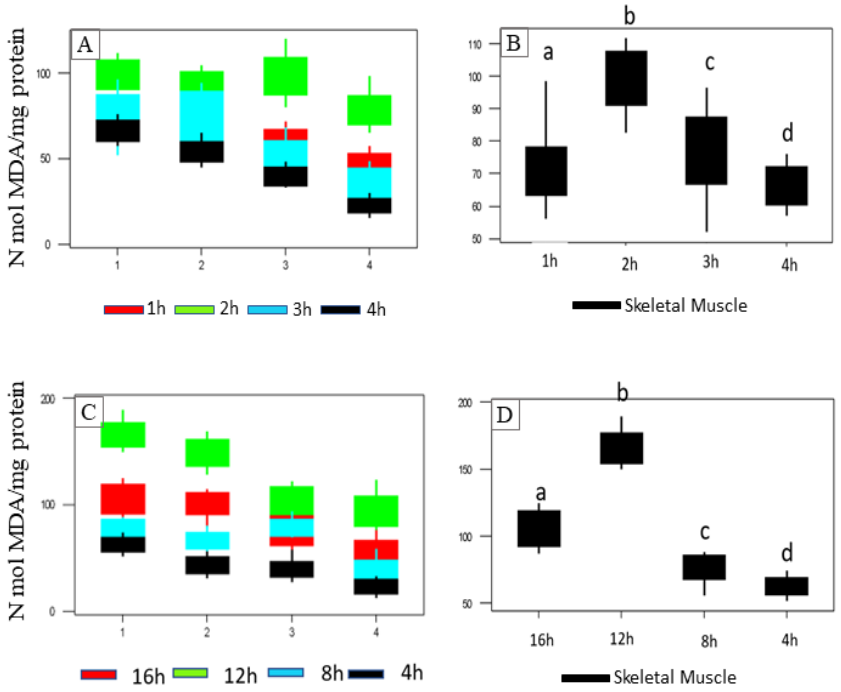

Figure 1. Boxplots showing MDA analysis for each tissue [skeletal muscle (1), gill (2), liver (3) and brain (4)]. The MDA levels at (A) pH 6.5-7.5 in all four tissues (Control) for 1h, 2h, 3h and 4h. (B) muscle MDA at pH 6.5-7.5 for 1h, 2h, 3h and 4h. The MDA levels at (C) DO at 80% and above (Control) for 16h, 12h, 8h and 4h, (D) muscle DO 80% and above and for 1h, 2h, 3h and 4h. The corresponding time intervals were selected within the time when survivability reaches less than 50%. Means (± SE) were compared using Kruskal Wallis test at $p<0.05$. Different lowercase alphabets indicate statistically significant difference at $p< 0.05$, n=10.

In all other sublethal pH levels, the MDA levels were found to be high compared to control (Figure 2A, 2C, 2E, 2G). At pH 4.5-5.5, it was observed that skeletal muscle tissue significantly responded at this level of pH and showed the maximum MDA level compared to the other tissues (Gill, Liver and Brain) (Figure 4B). Kruskal Wallis test confirmed the significant increase in MDA level in skeletal muscle compared to other tissues (Gill, Liver and Brain) induced by pH 4.5-5.5 at 2h (Kruskal Wallis test, $\chi^2=34.520$, df=3, $p<0.05$). Amongst all the other hours (1h, 2h, 3h and 4h), the MDA level of skeletal muscle at 2h showed a significant increase at pH 4.5-5.4 Kruskal Wallis test, $\chi^2=34.074$, df=3, $p<0.05$). At pH 5.5-6.5, it was observed that once again skeletal muscle tissue was significantly affected at this level of pH and showed the maximum MDA level compared to the other tissues (Gill,Liver and Brain) (Figure 4D). Kruskal Wallis test confirmed the significant increase in MDA levels in skeletal muscle compared to other tissues (Gill, Liver and Brain) induced by pH 5.5-6.5 at 2h (Kruskal Wallis test, $\chi^2=33.256$, df=3, $p<0.05$). Likewise, amongst all the other hours (1h, 2h, 3h and 4h), the MDA level of skeletal muscle at 2h showed a significant increase at pH 5.5-6.5 (One way ANOVA, Levene's test; p=0.099, F=251.770, df=3, $p<0.05$). At alkaline pH 7.5-8.5, it was observed that skeletal muscle tissue was again significantly affected at this level of pH and showed the maximum MDA level compared to the other tissues (Gill, Liver and Brain) (Figure 4F). Kruskal Wallis test confirmed the significant increase in MDA level in skeletal muscle compared to other tissues (Gill, Liver and Brain) induced by pH 7.5-8.5 at 2h

(Kruskal Wallis test, χ^2=36.585, df=3, p<0.05). Likewise, amongst all the other hours (1h, 2h, 3h and 4h), the MDA level of skeletal muscle at 2h showed a significant increase at pH 7.5-8.5 (One way ANOVA, Levene's test; p=0.189, F=256.189, df=3, p<0.05). Similarly, at a highly alkaline pH 8.5- 9.5, it was observed that skeletal muscle tissue was significantly affected at this level of pH and showed the maximum MDA level compared to the other tissues (Gill, Liver and Brain) (Figure 4H). Kruskal Wallis test confirmed the significant increase in MDA levelin skeletal muscle compared to other tissues (Gill, Liver and Brain) induced by pH 8.5-9.5 at 2h (Kruskal Wallis test, χ^2=36.585, df=3, p<0.05). Also, amongst all the other hours (1h, 2h, 3h and 4h), the MDA level of skeletal muscle at 2h showed a significant increase at pH 8.5-9.5 (One way ANOVA, Levene's test; p=0.180, F=160.276, df=3, p<0.05). Therefore, it could be said that, at allthe levels of pH (from pH 4.5-5.5 to 8.5-9.5) of the skeletal muscle showed maximumMDA levels when exposed for 2h.

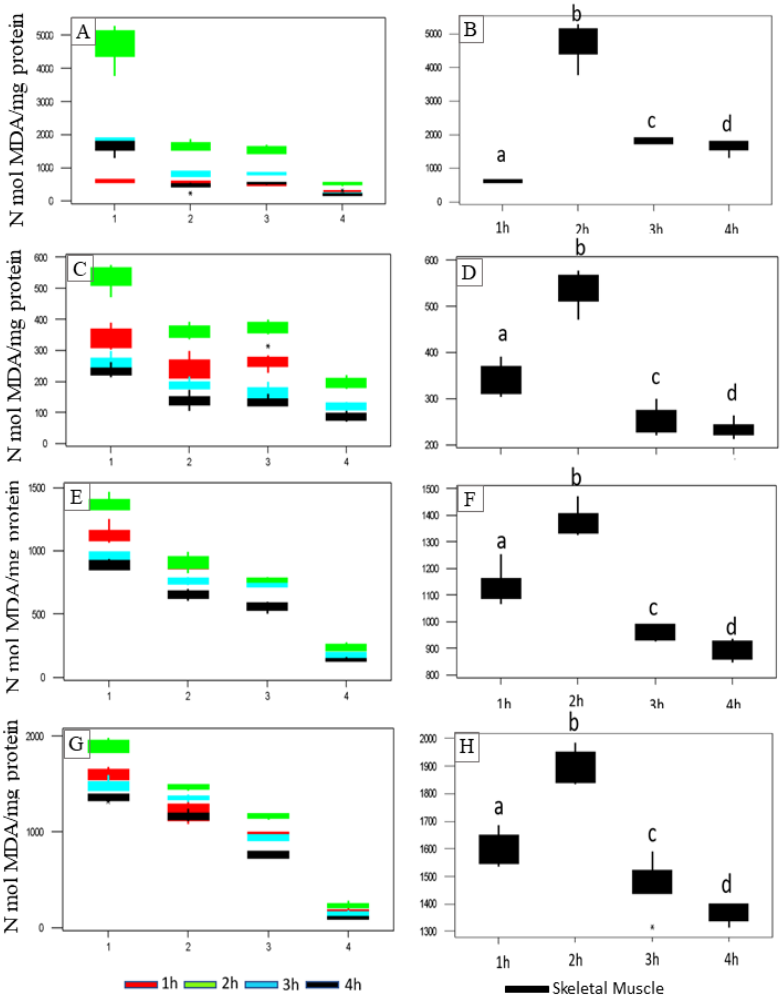

Figure 2. Boxplots showing MDA analysis for each tissue [skeletal muscle (1), gill (2), liver (3) and brain (4)] for different time intervals (pH: 1h, 2h, 3h and 4h; DO: 16h, 12h, 8h and 4h). The MDA levels at (A) pH 4.5-5.5 in all four tissues (B) pH 4.5-5.5 in skeletal muscle (C) pH 5.5-6.5 in all four tissues (D) pH 5.5-6.5 in skeletal muscle. The MDA levels at (E) pH 7.5-8.5 in all four tissues, (F) pH 7.5-8.5 in skeletal muscle (G) pH 8.5-9.5 in all four tissues, (H) pH 8.5-9.5 in skeletal muscle. The corresponding time intervals were selected within the time when survivability reaches less than 50%. Means (± SE) were compared using Kruskal Wallis test at p<0.05. Different lowercase alphabets indicate statistically significant difference at p< 0.05, n=10.

MDA analysis at each level of DO

Similar to pH levels, the MDA was analysed for each level of DO. The boxplots for all four tissues and also for muscle at DO 80% and above (Control) are shown in Figure 3C and 3D respectively.

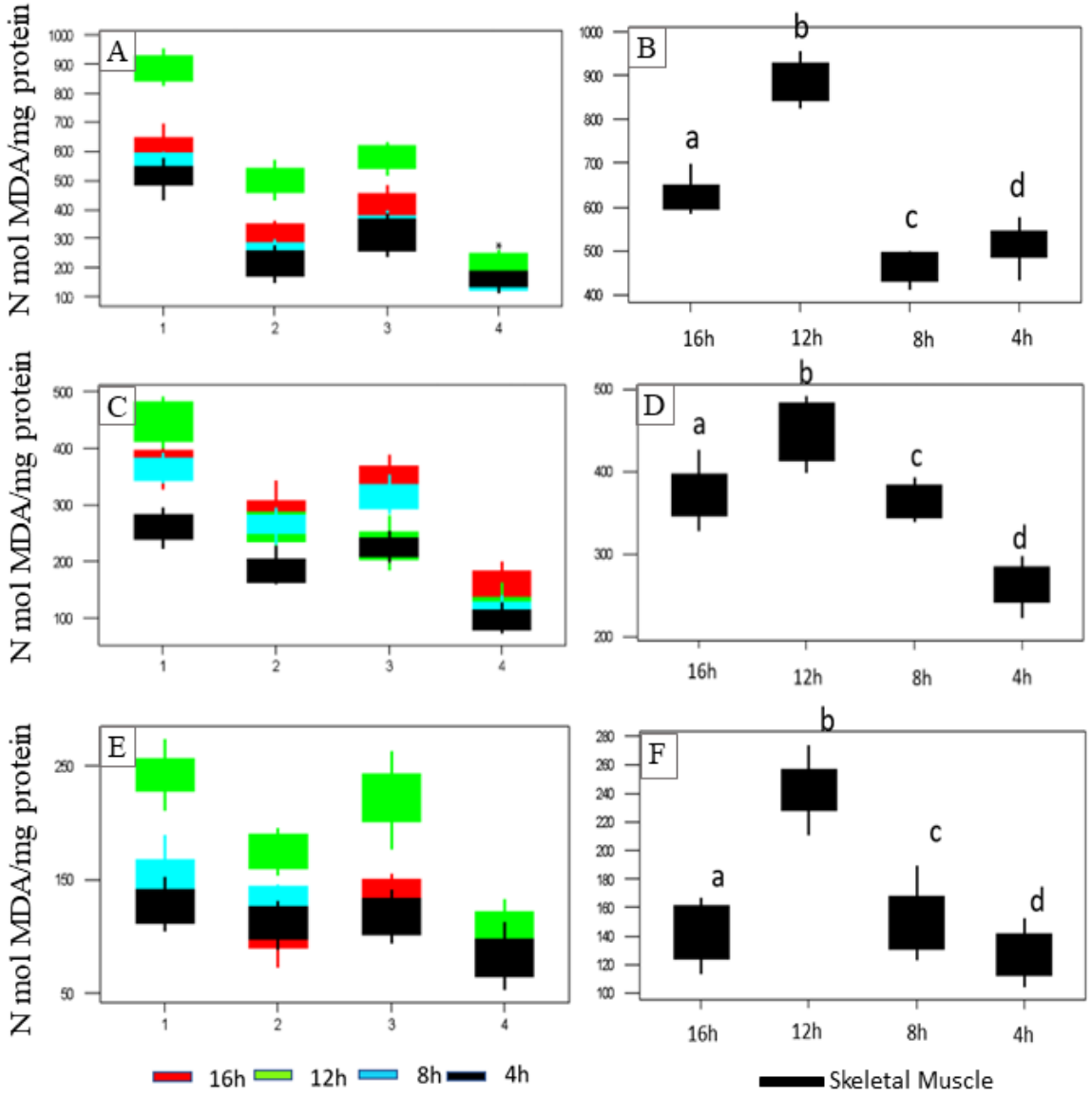

Figure 3. Boxplots showing MDA analysis for each tissue [skeletal muscle (1), gill (2), liver (3) and brain (4)] for different time intervals (16h, 12h, 8h and 4h). The MDA levels at (A) DO 20-30% in all four tissues (B) DO 20-30% in skeletal muscle (C) DO 40-50% in all four tissues (D) DO 40-50% in skeletal muscle. The MDA levels at (E) DO 60-70% in all four tissues (F) DO 60-70% in skeletal muscle. The corresponding time intervals were selected within the time when survivability reaches less than 50%. Means (± SE) were compared using Kruskal Wallis test at $p<0.05$. Different lowercase alphabets indicate statistically significant difference at $p< 0.05$, $n=10$.

At DO 20-30% saturation level, it was observed that skeletal muscle tissue significantly responded at this level of DO saturation and showed the maximum MDA level compared to the other tissues (Gill, Liver and Brain) (Figure 2A, 2C, 2E. 2F). One way ANOVA confirmed the significant increase in MDA level in skeletal muscle compared to other tissues (Gill, Liver and

Brain) induced by DO 20-30% O_2 saturation at 12h (Levene's test; p=0.958, F=360.145, df=3, p<0.05) (Figure 2B). Amongst all the other hours (16h, 12h, 8h and 4h), the MDA level of skeletal muscle at 12h showed a significant increase at DO 20-30% O_2 saturation (One way ANOVA, Levene's test; p=0.691, F=233.240, df=3, p<0.05). Similarly, at DO 40-50% saturation level, it was observed that skeletal muscle tissue significantly responded at this level of DO saturation and showed the maximum MDA levelcompared to the other tissues (Gill, Liver and Brain) (Figure 2D). One way ANOVA confirmed the significant increase in MDA level in skeletal muscle compared to other tissues (Gill, Liver and Brain) induced by DO 40-50% O_2 saturation at 12h (One way ANOVA, Levene's test; p=0.442, F=224.022, df=3, p<0.05). Amongst all the other hours (16h, 12h, 8h and 4h), the MDA level of skeletal muscle at 12h showed a significant increase at DO 40-50% O_2 saturation (One way ANOVA,Levene's test; p=0.118, F=73.558, df=3, p<0.05). At DO 60-70% saturation level, it was observed that skeletal muscle tissue significantly responded at this level of DO saturation and showed the maximum MDA level compared to the other tissues (Gill, Liver and Brain) (Figure 2F). One way ANOVA confirmed the significant increase in MDA level in skeletal muscle compared to other tissues (Gill, Liver and Brain) induced by DO 60-70% O_2 saturation at 12h (One way ANOVA, Levene's test; p=0.428, F=86.266, df=3, p<0.05). Amongst all the other hours (16h, 12h, 8h and 4h), the MDA level of skeletal muscle at 12h showed a significant increase at DO 60-70% O_2 saturation (One way ANOVA, Levene's test; p=0.792, F=72.312, df=3, p<0.05). Therefore, it could be said that, at all the levels of DO (from DO 20-30% saturation to 60-70% saturation), the skeletal muscle showed maximum MDA levels at 12h of exposure.

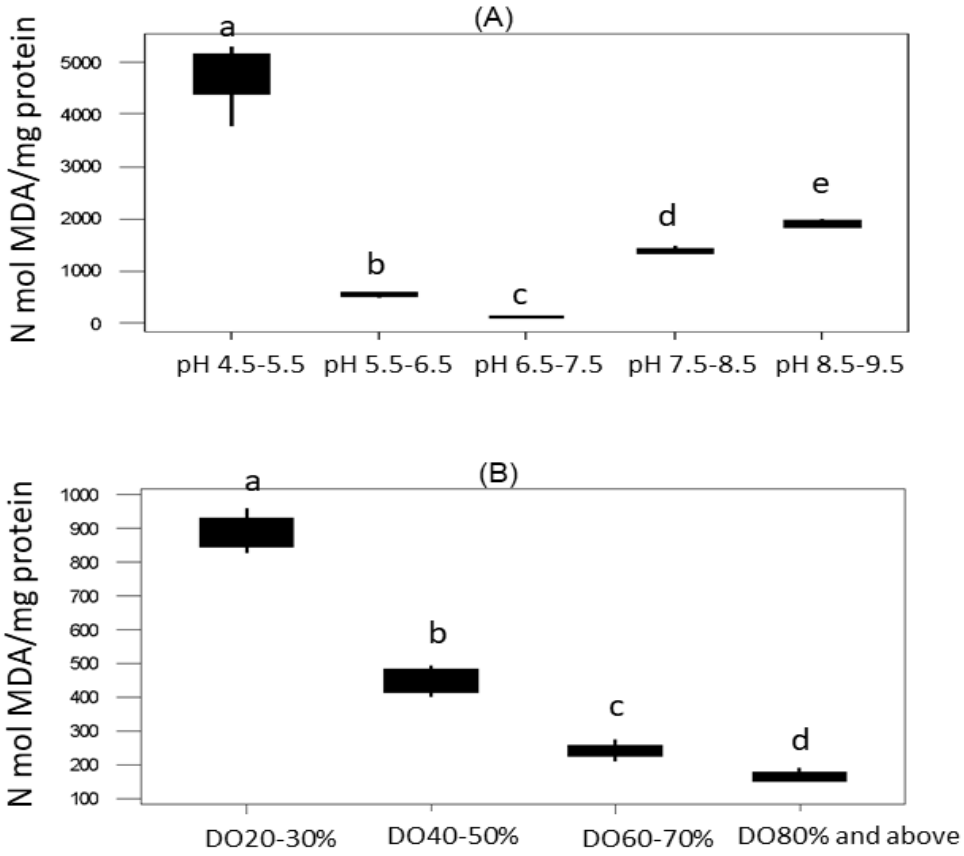

Figure 4. Boxplots showing MDA analysis of skeletal muscle at each level of (A) pH at 2h and (B) DO at 12h. Means (± SE) were compared using Kruskal Wallis test at p<0.05. Different lowercase alphabets indicate statistically significant difference at p< 0.05, n=10.

As it is clear from all the results here that skeletal muscle was strongly affected at 2h when exposed to pH and 12h when exposed to hypoxic ambience out of all the tissues tested, it was further analysed to see which level of pH and DO are comparatively more effective on inducing oxidative stress in skeletal muscle. Figure 4(A) showed clearly that at pH 4.5-5.5, the level of MDA is highest amongall the other levels of pH at 2h, showing its highest impact on creating oxidative stressin skeletal muscle at 2h (Kruskal Wallis test, χ^2=47.059, df=3, p<0.05). Similarly, Figure 4 (B) showed clearly that at DO 20-30% saturation the level of MDA is highest among all the other levels of DOat 12h, showing its highest impact on creating oxidative stress in skeletal muscle at 12h (Kruskal Wallis test, χ^2=36.585, df=3, p<0.05).

DISCUSSION

Studies have shown that lipid peroxidation (resulting MDA) can be considered as biomarker to understand oxidative stress (Abdelkhalek et al., 2014; Srivastava and Reddy, 2017). The present study aims at understanding whether tissues in zebrafish (Skeletal muscle, Gill, Liver and Brain) undergo oxidative stress (with MDA as biomarker) when 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) ambiances, and also to different levels of oxygen saturation (DO 20-30%, DO 40-50% and DO 60-70%) at different time intervals (1h, 2h, 3h and 4h for pH and 16h, 12h, 8h and 4h for DO saturations). Initially, two scenarios seem to be evident from the results- (i) At the maximum tolerable levels of highest (alkaline) and lowest (acidic) pH, when the mortality of the fish was greater than 50% (4h) for each level of pH, the MDA levels significantly increased in all the tissues (skeletal muscle, gill, liver and brain) and (ii) At lowest maximum tolerable level of DO (16h), the MDA levels significantly increased in all the tissues (skeletal muscle, gill, liver and brain). It clearly indicates that the fish undergoes hypoxia when pH levels were manipulated upto a tolerable limit. Similar explanation may be forwarded for DO when it is reduced to a tolerable limit. Beyond these limits survival of the fish is reduced below 50%. Thus the MDA levels were high for each tissue at different time intervals and also for the time when the mortality of fish was greater than 50% for each level of pH and DO. Ogueji et al. (2017), showed that Diazepam toxicity in *Clarias gariepinus* can cause oxidative damage indicated by elevated levels of MDA in tissues like gills and liver when exposed for 7, 14, 21 and 28 days. Even, environmental changes like change in temperature may cause oxidative damage in fish (Vinagre et al., 2012). Not only in fish, invertebrates like *Perna perna* and other bivalves when exposed to different environmental stressors in marine environment, they undergo severe oxidative damage which is indicated by the increased levels of MDA (Almeida et al., 2007). The MDA levels of juvenile seabass muscle tissue increases when exposed to a higher or lower temperatures with comparison to the optimum temperature for a period of 30 days (Vinagre et al., 2012). Not only temperature but hypoxia and acidic pH stress induce reactive oxygen species formation resulting in oxidative stress in skeletal muscles (Feng et al., 2015; Zahangir et al., 2015). Even in estuarine fish differential temperature causes oxidative stress which was evident from MDA levels of the skeletal muscle of the fish (Madeira et al., 2013). In the present study, too, skeletal muscle showed maximum effect compared to the other tissues (Gill, Liver and Brain), which was evident from the MDA levels at each level of pH and DO.

When compared among all the tissues evaluated for MDA with different levels of pH and DO, the skeletal muscle showed maximum MDA levels at 2h at pH 4.5-5.5 and for DO, it was maximum at 12h when the saturation level is 20-30%. It directly indicates an increase in time-dependent oxidative stress in the muscle tissue. Such time dependent study on MDA levels

was conducted in zebrafish exposed to dimethyl phthalate of different concentrations (Cong et al., 2020). Earlier studies in zebrafish have shown the lethal levels or the maximum tolerable levels of pH (acidic or alkaline) as well as DO (Feng et al., 2015; Zahangir et al., 2015), but the present study has shown a time dependent study of MDA where the maximum levels of DO and pH was determined exhibiting extreme oxidative stress.

Present study, thus, confirms that among all four tissues analysed, the skeletal muscle responded with maximum oxidative stress. Further, among all the studied hours of skeletal muscle it was evident from the MDA levels that 2h in case of pH and 12h in case of DO showed maximum oxidative stress response in all levels of pH and DO. Hence from the above study we got the most effective tissue (i.e. skeletal muscle tissue), most effective hour (2h for pH, 12h for DO) and the most effective level of pH (pH 4.5-5.5) and DO (DO 20-30%).

Further, the behaviour of zebrafish at these particular effective levels of pH and DO at the most effective hour were also studied. From the ethograms of the behavioural analysis, it was evident that the zebrafish remained maximum of the time towards the surface of the water when exposed to acidic stress of pH 4.5-5.5 and DO stress of 20-30% O_2 saturation. Similarly, from the study of (Yu and Li, 2011) it was observed that zebrafish under hypoxic stress shows an upright swimming movement towards the surface of the water. This may be due to hypoxic environment for which the fish goes under stress. Studies revealed that hypoxia induces aquatic surface respiration of zebrafish along with decreased swimming behaviour (i.e. distance, velocity and acceleration) (Braga et al., 2013; Abdallah et al., 2015). Exposure to other chemical stressors like deltamethrin induces increase in swimming speed, movement towards the surface and restlessness in zebrafish (Huang et al., 2014). Not only during hypoxia but studies have revealed that zebrafish undergoes movement towards the surface when exposed to sublethal levels of acidic pH (Magalhaes et al., 2012; Zahangir et al., 2015). This is similar to the present study where the zebrafish exposed to the effective pH level during the effective time showed a movement towards the surface.

CONCLUSION

The results of the research conducted can be concluded it has become evident that zebrafish may undergo oxidative stress at pH 4.5-5.5 when exposed for 2hours, similarly, for DO it is 20-30% saturation for 12 hour exposure.

ACKNOWLEDGMENT

Authors acknowledge National Agriculture Science Fund (NASF) [Poject Ref. NASF/ABA/6018/2016-17], The Indian Council of Agricultural Research (ICAR) for financial assistance and DIST FIST- II and CAS-II of Department of Zoology, Visva-Bharati for instrumental assistance. Authors are also thankful to DST-PURSE of Siksha Bhavana, Visva-Bharati for technical assistance.

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