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MELANOPHORE INDEX AS AN INDICATOR FOR JOINT HEAVY METAL TOXICITY IN FRESH WATER FISH *CHANNA PUNCTATUS*

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ABSTRACT

The study presents a correlation between melanophore index (MI) and oxidative stress in fresh water fish *Channa punctatus* treated with arsenic and mercury individual metal and also in the combination of both metals. *Channa punctatus* exposed to sub-lethal concentrations of arsenic, mercury and both metals together for 15 days time duration and the response via oxidative stress indicators and scale melanophores were assessed in accordance with standard protocols. Melanophore index decreased significantly ($p < 0.01$) in mercury and in combined metal fish. Oxidative stress increase was measured in the form of malondialdehyde (MDA) and reduced glutathione (GSH). Significant increase in reduced glutathione was observed in response to arsenic and mercury combined metal fish. Our findings demonstrated an inverse relationship between melanophore index and the oxidative stress indicators in a combined metal exposure.

KEY WORDS: **Melanophore Index, Oxidative stress, Arsenic, Mercury, Fresh water fish**

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INTRODUCTION

Both arsenic and mercury are released in aquatic ecosystem due to various natural and anthropogenic activities. Among all metals, these two metals are very toxic to aquatic organisms even when they are present even at very small amounts[1,2].These metals have ability to contaminate entire water streams and due to having nature of bioaccumulation and biomagnification through food chain they becomes a matter of global attention[3]. Two inorganic forms of arsenic i.e. trivalent arsenite (III) and pentavalent arsenate (V) are present in the environment and besides its carcinogenic activity it is also well known anticarcinogen[4,5]. In human being revelation to arsenic is linked with risk of developing tumors in various organs moreover, carcinogenic effect of arsenic, arsenicals are used for treatment of African trypanosomiasis and also can be receive as a medicine to treat acute promyelocytic leukaemia[4,6]. Mercury present in our surroundings basically in three different forms that is elemental mercury, inorganic mercury, and organic mercury however in terms of toxicity each one have their own toxicological consequences[7]. Main sources of mercury contamination are various industrial point sources, including present and former mining activities, dental amulgum, home exposures including fluorescent light bulbs, thermostats, batteries and skin lightening creams[8,9,10]. The symptoms of mercury toxicity in human are nervousness, insomnia, memory impairment, fatigue, other nervous system symptoms such as dizziness, peripheral neuropathy, tunnel vision tremors in coordination and depression[10,11]. Furthermore, excess amounts of free radicals are generated in the cell due to accumulation of these metals, the process involves the lipid peroxidation (LPO) and protein oxidation which cause changes in the oxidative state of cells[12]. Accumulation of these reactive oxygen species (ROS), cause significant changes in the fatty acids (FAs) profile due to lipid peroxidation which ultimately have a main reason for lower content in polyunsaturated FAs (PUFAs)[13].To counteract the impact of these generated reactive oxygen species fish have a defensive mechanisms comprise of antioxidants enzymes like reduced glutathione which detoxify generated reactive oxygen species converting itself into oxidized glutathione. It has been well established that skin is also one of the important route along with gills and gastrointestinal tract for metal absorption. Thus various pollutants such as heavy metals play a major role in the change of distribution of pigments and can be used as a potential indicator for primary recognition of pollutant in the aquatic system. Skin of fish performs so many vital functions and also it is very susceptible to infections due to change in water quality[14]. Thus scales embedded in the skin of fish and more particularly chromatophores in fish scale can serve as a useful biomarker for detecting the stress of pollution[14,15]. *Channa punctatus* is an air breathing fish belonging to the family Channidae and mostly found in south-east Asian countries. This species has important food source with high economic value for humans. Being to ease in transportation with easily maintained under laboratory conditions, make this species a worthy model for assessing the environmental toxicity[16]. In previous studies most of the researcher have focused on effect of single heavy metal in aquatic organisms while in reality at natural condition they are exposed to multiple heavy metals at a time, in present study our aim is to evaluate the correlation in melanophore index and oxidative stress in fish exposed to arsenic and mercury individually and in their combination.

MATERIALS AND METHODS

Fish collection and acclimatization

The freshwater fish *Channa punctatus* average wet weight and length 20±2.0 gm, 12±0.5 cm respectively, were procured from local outlets and to avoid any dermal infections they were given prophylactic treatment by bathing in 0.05% $KMnO_4$ solution for two minutes. Fish were acclimatized for 1 month. During acclimatization they were fed once a day with goat liver. Other parameters of water like temperature 22 ± 2°C, pH 7.1± .2 and dissolved oxygen concentration 6.8±.5 mg/l were measured during acclimatization and experiment.

Test chemicals

Present study involves all the reagents and chemicals of highest purity. Analytical-grade arsenic tri-oxide (As_2O_3)(99.5%) and Mercury Chloride ($HgCl_2$. H_2O) (99%), have been used. Thiobarbituric acid was purchased from Loba Chemie (Mumbai). 5-5-Dithiobis-2-nitrobenzoic acid, glutathione, bovine serum albumin and tricaine methane sulfonate were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Experimental procedure

Median lethal concentration of heavy metals for 96 h (96 h LC50 value) was calculated before starting the experiment by pursuing the 24 h renewal bioassay system according to Trimmed Spearman–Karber method. The acclimatized fish were divided into four groups. Group one was exposed with arsenic tri- oxide (1 mg/l, 10% of 96 h LC 50), a group second with mercury chloride (0.1 mg/l, 10% of 96 h LC 50), group third with arsenic tri oxide + mercury chloride (1mg/l +0.1 mg/l), and group fourth was maintained as control (without any heavy metal). Fish were exposed to the metals for 15 days and daily renewal of water as well as metals was done to maintain the concentration of metals in tanks. All groups contained five fish with 20 L of water in each aquarium. Experiments were performed in triplicates.

Sample preparation

After 15 days of metal exposure, fish of both experimental and the control groups were anesthetized by immersing in 1:4000 aqueous solution of tricane methane sulfonate. Liver tissue was carefully removed and washed with cold saline. Samples were immediately frozen into liquid nitrogen and preserved at -80°C for further analysis.

Estimation of Metals

100 mg of liver tissue was digested in 1 ml of concentrated nitric acid at 80°C for 1 h and metal concentration was analyzed by Atomic Absorption Spectrophotometer (Perkin Elmer AA800).

Assay of enzymes

Frozen liver samples were homogenized in chilled phosphate buffer [0.1 M, pH (7.0)] and centrifuged at 12,500 rpm for 15 min at 4ºC and supernatant were used for lipid peroxidation (LPO) and reduced glutathione (GSH) assay.

Analysis of oxidative stress parameters

Lipid Peroxidation assay (LPO)

The lipid peroxidation in the liver tissue had been assayed by determining malondialdehyde[17].The absorbance was taken at 532nm with spectrophotometer. Unit of LPO was expressed as nMMDA per mg protein.

Analysis of Antioxidant defense enzyme

Reduced Glutathione (GSH)

Reduced glutathione (GSH) in the liver tissue had been analyzed by using Ellmans' reagent[18]. Reaction of GSH and DTNB (5-5-dithiobis-2-nitrobenzoic acid) resulted in the formation of a yellow colored product 2-nitro-5-thiobenzoic acid. GSH concentration was determined by measuring the absorbance of yellow colored product at 412 nm. The unit of glutathione was expressed as nM GSH /mg protein.

Protein concentration

Protein was estimated in liver tissue by using bovine serum albumin as the standard[19].

Scales study

The scales were removed from the anterior dorsal side (nearer to head) of the experimental and control fish by using forceps and by scrapping with scalpel. The scales were stained with borax carmine and mount in DPX. Slides were observed under light microscope to calculate the melanophore index[20].

Statistical analysis

Statistical analysis was done by one way ANOVA followed by Tukey's post hoc test to compare the difference between mean of metal exposed group and control. Difference between mean at a 5% ($p<0.05$) level were considered as significant. All statistical analysis was run by SPSS 16.0 and results are expressed as mean±S.D.

RESULTS

Tissue concentration of arsenic and mercury

Mercury concentration in the liver was significantly ($P<0.05$) higher than in arsenic treated fish (Fig 1). Concentration of one metal was not affected by presence of other metal in combination group.

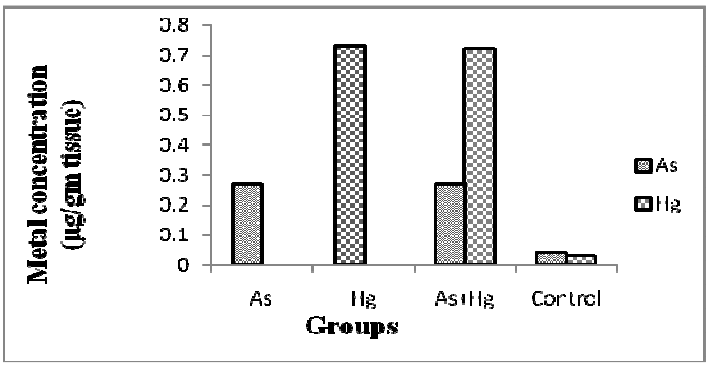

Figure 1

Metal concentration (µg/gm tissue) in liver of C. punctatus after exposure to arsenic and mercury individually as well as in combination for 15 days

Oxidative stress parameter

Peroxidation of lipids in metal treated group i.e. arsenic, mercury and arsenic+mercury is significantly ($p<0.05$) higher than control group (Fig 2A).

Antioxidants defense system

Reduced glutathione level was also significantly ($p<0.05$) higher in metal exposed group i.e. arsenic, mercury and arsenic+mercury than control (Fig 2B).

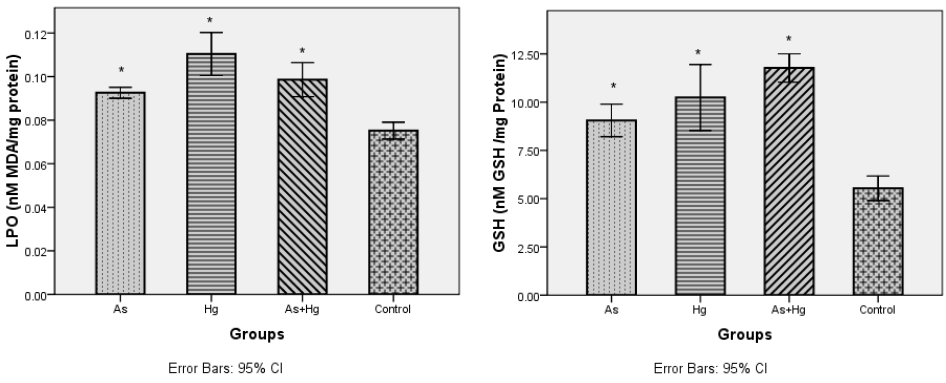

Figure 2A and 2B

Lipid Peroxidation and Reduced Glutathione level in liver of C. punctatus after 15-days exposure to metals. The significant difference in values of exposed fish over controls is indicated by Asterisk (*) (p <0.05). Data are reported as mean±SD (n=5)

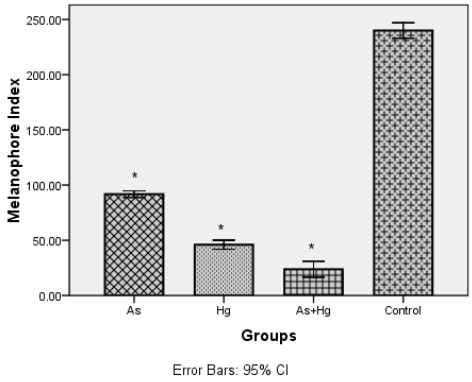

Figure 3

Melanophore Index of scales of C. punctatus after 15-days exposure to metals and control. The significant difference in values of exposed fish over controls is indicated by Asterisk (*) (p <0.05). Data are reported as mean±SD (n=5)

Chromatophores study

Chromatophores in control group were well arranged, densely packed with reticulated shape however, a number of chromatophores were decreased and some of the pigments were aggregated also (Fig-3 A). In mercury exposed fish, chromatophores were decreased in number and some chromatophores were stello-punctated type (Fig-3 B). Moreover, in combined metals group i.e. arsenic+mercury pigments of all the chromatophores were aggregated to form punctated type (Fig-3 C). The melanophore index (MI) significantly ($p < 0.05$) decreased in the arsenic and mercury exposed scales compared to the control (Fig. 3). Mixed metal group had least melanophore index.

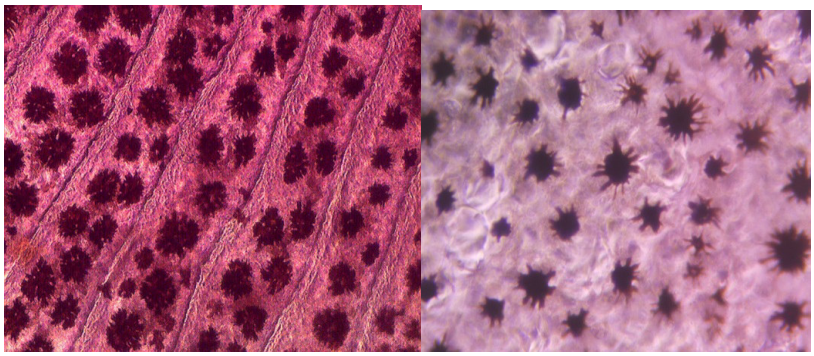

Figure 3A and 3B

Chromatophore pattern in scales of control (3A) and arsenic exposed group (3B)in C. punctatus.

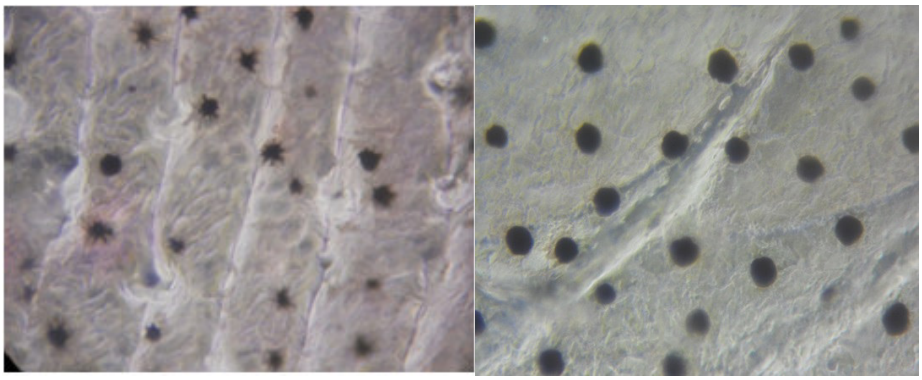

Figure 3C and 3D
Chromatophore pattern in scales of mercury (3C) and arsenic+ mercury (3D) exposed group in C. punctatus.

Pearson correlation analysis revealed a strong correlation between MI and MDA and GSH in fish exposed to arsenic. Correlation coefficient values of MI against MDA and GSH were found to be r = 0.467 and r = 0.224, respectively in arsenic treated fish. However for mercury exposure, the correlation coefficient values of MI against MDA and GSH were found to be r = -0.592 and r = .170 respectively. While for arsenic+mercury exposure, correlation coefficient values were observed to be r = -0.540 and r = -0.400 when MI values were correlated with MDA and GSH, respectively (Fig. 4 A, B).

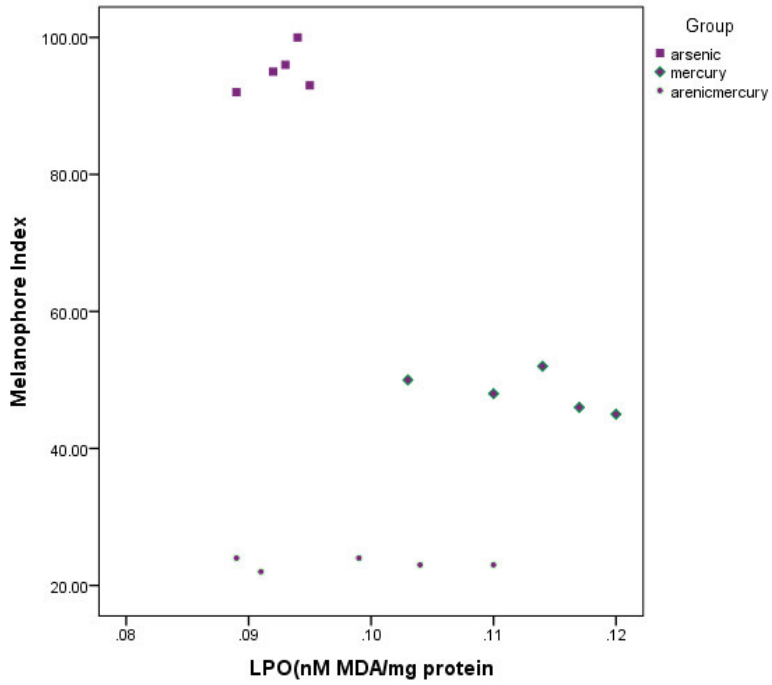

Figure 4A
Correlation of melanophore index with lipid peroxidation in liver of Channa punctatus treated with arsenic, mercury and arsenic + mercury together.

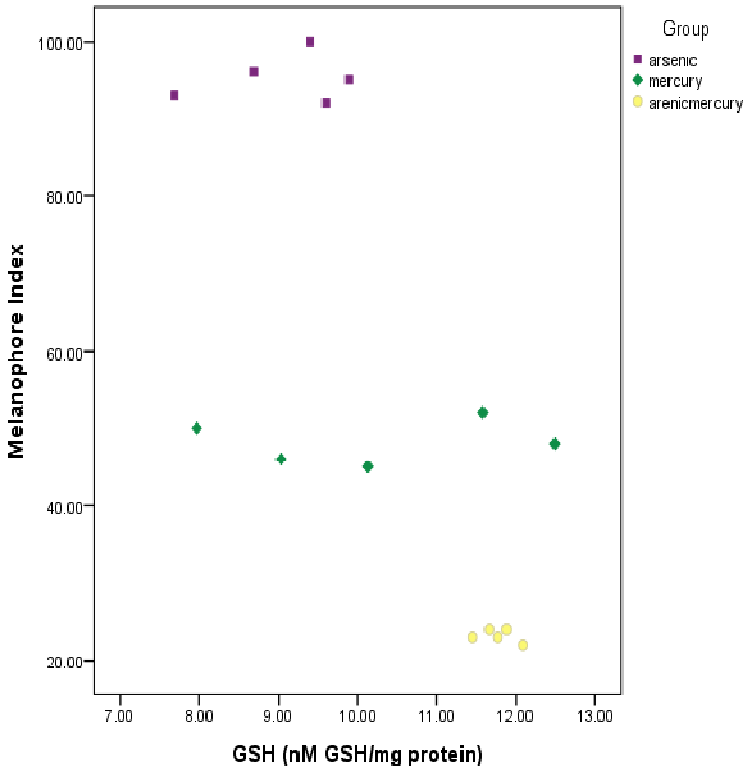

Figure 4 A
Correlation of melanophore index with reduced glutathione (GSH) in liver of Channa punctatus treated with arsenic, mercury and arsenic + mercury together

DISCUSSION

In aquatic ecosystem, heavy metals are typically present in combination with other inorganic contaminants[21]. Hence, single metal exposure based studies at laboratory condition does not meet to realistic empirical model. Liver is the major detoxification organ so major enzymatic reactions are altered in this organ therefore our study has given the emphasis on this organ only. Present study indicates that accumulation of mercury was significantly higher (0.73±.022 µg/ g tissue) as compared to arsenic (0.27±.022µg/ g tissue) in liver although metal accumulation was not affected by each other's presence. Heavy metals can induce the level of lipid peroxidation by interacting with cell membrane so they are responsible for alteration of major physiology of cell and our results have shown an elevation of malondialdehyde and altered reduced glutathione because of accumulation of metals in fish *Channa punctatus.* Both inorganic arsenic and monomethylmercury (MeHg) have been reported to have high rate of uptake and comparatively slow excretion, also both metals have tendency to bind and disrupt the normal function of sulfhydryl- containing hepatic proteins[22,1]. Reactive oxygen species such as superoxide anion radical ($O^{\cdot-}_2$), hydrogen peroxide (H_2O_2), lipid peroxide (LOOH) are produced due to accumulation of metals, also have an impact on vital physiological and biochemical functions[23]. Metal exposure to arsenic, mercury and mixture of both metals i.e (As+Hg) at sublethal concentration (10% of 96 h LC 50) showed loss of equilibrium, abnormal swimming, lightening of skin with aggressiveness. This changed response could have an adverse impact on survival and fitness which result in negative effect at a population level [24]. Lipid peroxidation result in formation of malonyldialdehyde which is a complex secondary end-product of lipid degradation. Metals induced lipid peroxidation, perpetuates in the disruption of cell integrity hence, its presence showed a reliable biomarker of oxidative stress. Therefore we conducted the evaluation of MDA content in the liver of metal treated and control fish. Since lipid peroxidation is an excellent biomarker of oxidative damage, present study clearly revealed that an exposure of arsenic and mercury individually as well as in combination are responsible for increased lipid peroxidation in the liver as compared to the control group after 15 days. Similar results were observed by[25,] they reported significant increase in malondialdehyde of Japanese flounder embryos and larvae after mercury exposure for10 days. Similarly iduction in lipid peroxidation in freshwater fish matrinxa˜, Bryconamazonicus after exposure to sub-lethal concentration of mercury chloride for 96 h in a static system was observed by [26]. Disparity in results was observed by[2], they found that dietary arsenic exposure did not have a significant effect on plasma lipid peroxide (LPO) concentrations in *Coregonus clupeaformis.* GSH a tripeptide (rich in thiol group), is one of the most

important antioxidant preventing damage to important cellular components caused by ROS generated by heavy metal toxicity. GSH neutralizes metals by forming GS- metal complexes through its thiolate sulphar atom[27]. In the present study GSH was significantly ($p<0.05$) increased in liver of fish exposed to arsenic, mercury and combination of both (arsenic + mercury). As 15 days exposure of metal to fish is not a long duration, and in this short duration, increase in GSH can be considered as part of first line of antioxidant defense against metal induced toxicity. Similar results were observed by[28], they found that 200µM arsenite resulted in an increase of liver content of GSH in *C. auratus* after 7 days exposure. Distribution of melanin is responsible for physiological color changes rather than changes in the amount of pigments in dermal chromatophores. Various stimuli are involved in either aggregation or dispersion of chromatophores[29]. Fish chromatophore study is a suitable indicator for the estimation of environmental stress due to its sensitivity to various environmental contaminants even at sub-lethal doses[29,30]. Present study is an attempt to correlate the non-invasive, external bio-indicator; fish scales (melanophore) with oxidative stress indicators. In present study, there is more aggregation of pigment granules in combined metals exposed group as compared to arsenic and mercury treated individually. Significant decrease in the melanophore index was observed after arsenic and mercury exposure to the fish (Fig. 3). Mercury treated group have more aggregation of pigments than arsenic group after 15 days exposure. Similar results were also reported by[31] they observed, during early exposure to arsenic i.e. on 7th day and 15th day pigments become dispersed but on later periods melanophore granules get aggregated in fresh water fish *Channa punctatus.* In another study[32], it was reported that mercury treatment causes an increase in the number and size of the pigment cells at early exposure and subsequently degenerated and lysed to release pigment granules and successively regenerate in a cyclic manner. Therefore, dispersion or aggregations of granular pigments, suggest the toxicity and sensitivity of metals towards chromatophores. Melanophore aggregation or dispersion in the fish scale is highly channelized process[33], while melanophore aggregation is mediated by inward movement of Ca^{2+} into the cell through voltage-dependent calcium channels, melanophore dispersion is facilitated by the melanocyte-stimulating hormone(MSH)/(G protein) Gsα mediated increase in intracellular cAMP[34]. This suggests that the decrease in the melanophore index (MI) observed in our study could be due to alterations in the melanophore aggregation/dispersion pathways induced by arsenic and mercury toxicity. Although current study here in is unable to establish this pathway, but we suggest further investigations to understand the specific mechanism involved in the decrease of melanophore index(MI). In our study, significant increase in the level of MDA and GSH and decrease in melanophore index (MI) was observed in combined metal as well as arsenic and mercury individually treated fish (Figs.2A and 2B). Exposure to heavy metals like cadmium result in decrease in melanophore index in fish scale, this had already been reported by[35] our study have also shown an inverse correlation in level of MDA and MI as well as GSH and MI. Based on the above findings we hypothesized that the elevated levels of MDA, GSH and decrease in melanophore index in combined metal exposed fish could be due to alteration of inward movement of Ca^{2+}into the cell through voltage-dependent calcium channels. Similar decrease in melanophore index was observed upon exposure of *O.mossambicus*to lead nitrate and phenol[36]. However, this hypothesis needs extensive validation in exposure to different test compounds.

CONCLUSION

The findings from this study clearly showed significant inverse correlation of the primary stress indicator (melanophore) with oxidative stress indicators (MDA and GSH) in fish exposed to arsenic and mercury combined metals. MDA induction was found to be specifically elevated in arsenic and mercury mixed treated fish with inverse correlation to MI. Variations in the lipid peroxidation depend on the nature of tested chemicals which may depend on their specific mechanisms of actions. Thus, considering the wide range of contaminants in the aquatic ecosystem, stress indicators that may respond to these varieties of contaminants need to be investigated for proper understanding of the basic mechanisms of aquatic pollutants induced stress in organisms. In conclusion, variation in melanophore index (MI) in fish together with the oxidative stress indicators facilitates a better measurement of environmental stress caused by environmental contaminants. This will not only help in eliminating erroneous results but also aid in establishing contaminant specific physiological responses in the aquatic animal models.

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