

Molecular characterization and expression profiling of arsenic mediated stress responsive genes in *Dawkinsia tambraparniei* (Silas, 1954)

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ABSTRACT

An endemic fish *Dawkinsia tambraparniei* in River Tamiraparani happen to extinct for reason of anthropogenic activity and natural events that occurs. Among them, heavy metal arsenic stands as major toxic contained by aquatic systems until date. Therefore, the present study intended to explore the impacts of (As) heavy metal on *D. tambraparniei* through molecular characterization and expression profiling of stress responsive genes. Following, lethal concentration (LC₅₀) of arsenic trioxide (As₂O₃) and sodium arsenite (NaAsO₂) was determined by probit analysis. Then, fishes were exposed for 42 days with As₂O₃ (22.60 ppm) and NaAsO₂ (31.65 ppm) with control (0.0 ppm). Subsequently, expression profiling of *Mt*, *p53*, and superoxide dismutase genes was carried out in brain, heart and liver tissues through qPCR analysis. Overall, the obtained results showed notable expression level of As₂O₃ treated fish heart shows *Mt* expression at 42 days while in NaAsO₂ expression fold was higher in 28 days. Similarly, As₂O₃ treated fish brain displays *Mt* expression at 28 days. In NaAsO₂ group *Mt* lacks expression in brain. 14 days of exposure to As₂O₃ drastically increase the expression of *p53* gene compared with control brain tissue, whereas significantly elevated expression was found in the NaAsO₂ exposed heart and liver. Higher level of *p53* gene expression was observed in brine by As₂O₃ while compared with NaAsO₂ exposed heart and liver on the day 14. Henceforward, the present study proposed that expression profiling and stress analysis of As contamination significantly impact the gene expression throw light on detecting the heavy metals contamination in freshwater ecosystem for its forthright application and analysis.

1. INTRODUCTION

Arsenic is leading cause of poisoning in health concern worldwide and about 6.8% reported by U.S. Geological Survey [1]. According to the water survey reported that the groundwater has significant concentration of As or F in India and Asian countries. Chronic exposure of arsenic ingestion through water and food leads to skin disorders, high blood pressure, increased incidence of cancer and diabetes in humans [2]. As per the recent research report, the level of arsenic high in aquatic field has been attained by the manmade activities, such as agricultural and industrial effluence. In the aquatic environment, the redox and methylation reactions are inter-converted inorganic arsenic (arsenite (As³⁺) and arsenate (As⁵⁺)) [3].

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Arsenic, lead, cadmium, and chromium compounds lead to the expression of gene specific such as heat shockprotein 70 and metallothionein [4]. Activation of intracellular Ca²⁺ production and hypersensitivity, modulate release of lymphokines that altered the delayed exposure in mammals [5]. Metallothionein (*Mt*) is well-known metalloprotein and metal binding protein, which response in ion homeost and detoxification, moreover it plays an important role in *fortification against metalotoxicity and oxidative stress* [6]. Furthermore, the expression profile of *Nrf2* (cellular resistance to oxidants regulator) depend on *XMEs* like glutathione peroxidase catalase, superoxide dismutase (SOD), and NAD (P) H: quinone oxidoreductase 1 which is responsible for maintaining cellular redox homeostasis. SODs are one of the major cellular defenses against oxidative stress knocked down superoxide radicals (O²⁻), and it is precursor in reactive O₂ Radicals [7]. The activity of SOD was defined by its ability to inhibit the oxidation reaction of SOD2 hydroxylamine hydrochloride with superoxide to nitrite. In addition, SOD is one of the significant metalloenzymes and impied for the degradation of free radicals O₂⁻ to O₂ and H₂O₂. Arsenic derivatives induce oxidase stress by reacting with dithiol

groups [8]. The protein *p53* has an essential role in the transcriptional regulation of metallothionein, which shows an unusual regulatory role for *p53*. Besides, *p53* - intracellular Ca^{2+} levels increased by arsenic in lymphocytes. Response to level of arsenic changes in intercellular Ca^{2+} regulates the formation of *p53*-DNA complex. Instead, different mechanisms are involved in the activation of kinases in *p53* responses to arsenite in the cell system [9].

In aquaculture system, the numbers of fishes are found to impairment of immune and physiological function following to exposure of heavy metals. In brown trouts (*Salmo trutta*), whole transcriptome analysis revealed that the down regulation of numerous factors of the complements systems are following exposure of Ni, As, Cu, Cd, Pb, Fe, and Zn [10]. Arsenic causes perturbation of defense systems in *Danio rerio*, *Clarius batrachus*, and *Sparus aurata* [11,12].

However, gene expression profiling of fish species in water system following As exposure has not been reported. *Dawkinsia tambraparniei* (Silas, 1953) is an endangered cyprinid only found in Tamiraparani River in southern Western Ghats, India [13]. Recently, *D. tambraparniei* population have declined due to anthropogenic activities such as mining, alterations of habitat, brooders collection for aquarium trade and environmental cruises. Due to these threats, *D. tambraparniei* announced as endangered in IUCN red list [14]. Effective conservation of wild stocks requires information on growth and condition. Hence, *D. tambraparniei* as a suitable model that we used for current experiment to investigate the alteration of the gene expression and regulate stress response by arsenic exposure using RT-PCR.

2. MATERIALS AND METHODS

2.1. Fish

D. tambraparniei (weight 10 ± 0.5 g, length 12.8 ± 0.5 cm SL) were collected from Tamirabarani River basin in Tamil Nadu, India. Fish were acclimated under standardized laboratory conditions. Commercial diet was supplemented in consistent temperature ($28 \pm 0.5^\circ\text{C}$), oxygen above 6.0 mg/L and 12 h light and dark photoperiod. The experiment followed by the guidelines of animal ethics committee, Government of India.

2.2. Chemicals Required

Arsenic (III) oxide (As_2O_3) (CAS Number 1327-53-3) and sodium arsenate (NaAsO_2) (CAS Number 10048-95-0) purchased from Sigma-Aldrich. The gene specific primer, RNase free DNase I, cDNA synthesis PCR master mixture kit procured from ThermoFisher Scientific, India. Analytical grade chemical was used for experiment.

2.3. Experimental Setup

The experimental fishes divided into control (without test solution) and treatment group, each group contains 08 fishes. As_2O_3 (22.60 ppm) and NaAsO_2 (31.65 ppm) dissolved in distilled water as test solution. In the experimental study, the final LC_{50} concentrations used based on previous experiment [15]. The test solutions added in treatment group and the experiment were followed for 42 days. Three experimental samples were collected in each group, anaesthetized for 5 min by clove oil and the brain, heart, and liver tissues were stored at -80°C for future use.

2.4. Synthesis of cDNA

Initially, RNA extraction performed by Trizol (HiMedia, India) according to manufacturer's procedure. Quality of RNA samples assessed by agarose-formaldehyde gel electrophoresis method (agarose

1% w/v; formaldehyde 16%). Quantity and quality of experimental RNA samples analyzed by Nano-drop Spectrophotometer (ThermoFisher, USA) absorbance at A 260/280 ratio of 1.8–2.0. 1 μg of RNA subjected to cDNA synthesis conducted in 20 μl reactions, containing 1 μg of RNA, 2 μl of oligo dT primers, 2 μl of dNTPs, 400 mL of $2\times$ HiScript mix, 0.5 μl of reverse transcriptase. The cDNA synthesis following to the manufacturer's procedure, samples were stored at -20°C .

2.5. Gene Expression Analysis by Semi-Quantitative PCR

The primers *Mt*, *p53*, SOD, and β -actin [Table 1] used for amplification of gene expression by PCR analyzer. PCR reaction conducted in 20 μl volume having of master mix (10 μl), cDNA (1 μl) and primer (0.5 μl of forward and reverse primers). Amplification was carried out following PCR program: 3 min at 95°C then 30–35 cycles of 95°C for 1 min, annealing 55 – 58°C for 30 s and extension at 72°C for 1 min followed by a final extension at 72°C for 7–10 min.

2.6. Statistical Analysis

The expression patterns mentioned in graphical representation by GelAnalyzer software. Expression values analysis carried out by Image J 1.8 software and statistical significance established at $P < 0.05$.

3. RESULTS AND DISCUSSION

Genes such as SOD, *p53*, *Mt*, and β -Actin were analyzed for expression studies. Fragments of 230bp, 180bp, 190bp, and 250bp were amplified for SOD, *p53*, *Mt*, and β -actin, respectively. When compared with expression pattern, in As_2O_3 ide treated fish the heart shows *Mt* expressed laterally at 42 days while in NaAsO_2 group the expression fold was higher in 28 days itself. The case was happened vice versa in brain where the As_2O_3 treated group displays *Mt* expression at 28 days and prolonged to 42 days. In NaAsO_2 group *Mt* lacks expression in brain. Significantly, no changes found in liver for most genes after 7 days of arsenic exposure. While high concentrations of As_2O_3 and NaAsO_2 accumulated in the liver, along with the gills, only the *Mt* gene was upregulated after 28 days of exposure [Figures 1 and 2].

Our study depicted that after 14 days of exposure to As_2O_3 drastically increase the expression of mRNA of *p53* gene in comparison with control brain tissue; whereas the significantly elevated expression of mRNA of *p53* gene was found in the NaAsO_2 exposed heart and liver in comparison with the As_2O_3 group [Figure 3]. There are no significant expression observed in SOD after 7 days of brain exposure to both As_2O_3 and NaAsO_2 . Up-regulated *p53* and *Mt* genes only expressed at 28 days by predictable marker of heavy metal pollution,

Table 1: Oligonucleotide primers used to analysis of expression by semi-quantitative PCR.

| Primer name | Primers usage | Nucleotide sequences | Size |
|---------------------------------|---------------|-----------------------------|------|
| <i>β-actin</i> | Sense | 5' TCATCACCATTGGCAATGAGC 3' | 250 |
| <i>β-actin</i> | Antisense | 5' CACTGTGTGGCGTACAGGT 3' | |
| <i>Mt</i> | Sense | 5' ATGGATCCNTGCGAWTGC 3' | 190 |
| <i>Mt</i> | Antisense | 5' CANTGACAGCAGCTGGAGC 3' | |
| <i>p53</i> | Sense | 5' GGGCAATCAGCGAGCAAA 3' | 180 |
| <i>p53</i> | Antisense | 5' GGGCAATCAGCGAGCAAA 3' | |
| SOD | Sense | 5' TGAGACACGTCGGAGACC 3' | 230 |
| SOD | Antisense | 5' TGCCGATCACTCCACAGG 3' | |

SOD: Superoxide dismutase.

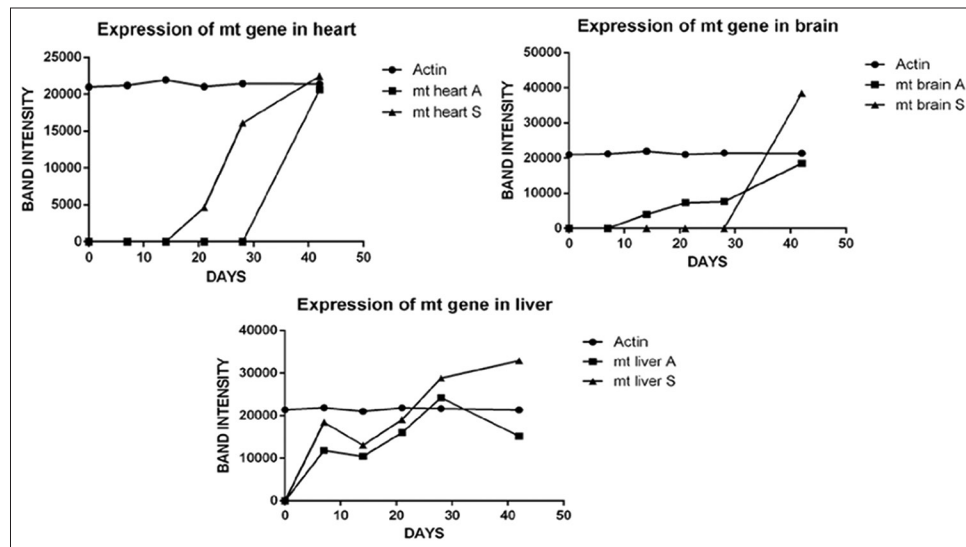


Figure 1: Expression of genes coding for *Mt* isoforms in various organs of *Dawkinsia tambraparniei* relative to different days with sodium arsenite (NaAsO_2) and arsenic trioxide (As_2O_3).

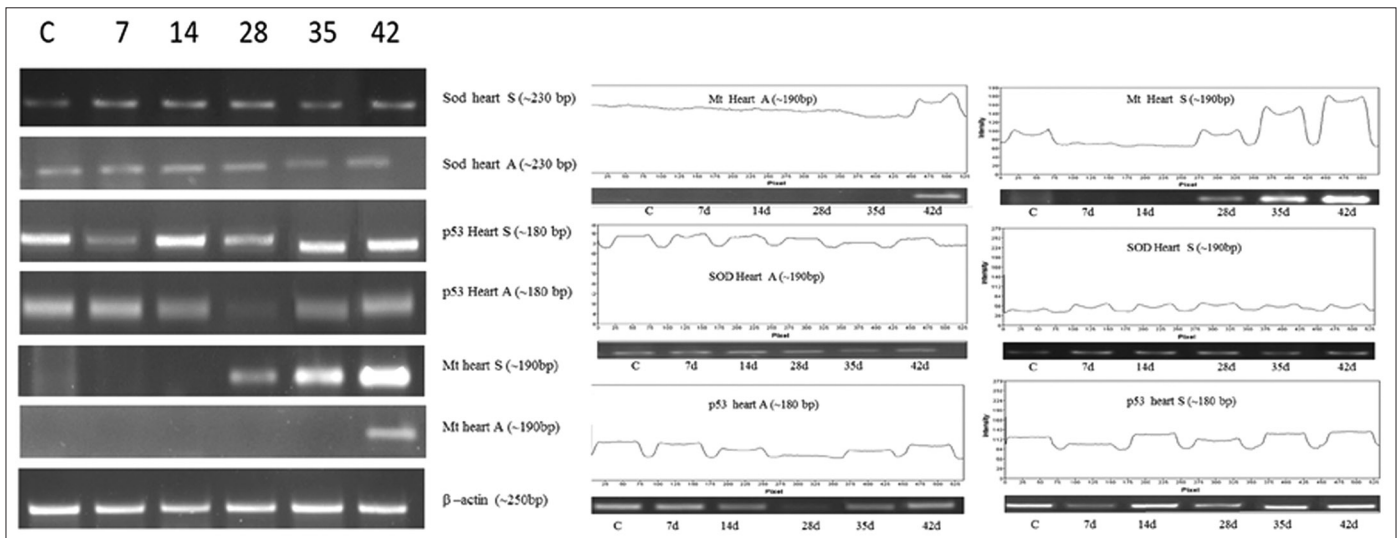


Figure 2: Effect of As_2O_3 (A) and NaAsO_2 (S) on heart. Analysis of *Mt*, *p53*, *SOD* genes expression was performed on heart by means of semi quantitative reverse transcriptase PCR. Amplification was done for 7 days to 42 days samples with respective genes (C for control).

but there were no significant differences observed in between the two arsenical exposure environments. Nevertheless, genes that respond to oxidative stress SOD not affected by these arsenic derivatives within brain, heart, and liver cells [Figure 4].

Our results show that *p53* expression is substantially similar in heart, liver, and brain exposed to both As_2O_3 and NaAsO_2 . Thus, it indicates that *Mt* gene plays an important role in elimination of arsenical derivatives. In brain at 28th day of exposure with As_2O_3 , there is increase expression of *p53* was seen. Individually delayed division of cells as well as decreases the time taken for G1-cell cycle arrested and damage of DNA/mitochondrial membrane have been generally stated that elevated expressions of *p53* in exposed cells to As_2O_3 as an agents for genotoxicity [Figure 3] [16].

Tissue specific *Mt* transcript was highest in liver, followed by brain and heart but displaying relatively low expression in heart and brain depending on the dose days. The tissue distribution patterns at the basal level of *Mt*

in *D. tambraparniei* were similar to that of river puffer fish *Mt*, which exhibits higher expression in the liver, and minimum expression in the kidney and brain and in the arsenic induced cases the same happens *i.e.*, expression at maximum in liver and minimum in heart and brain [17].

In rainbow trout similar accumulation pattern was observed in gills and kidney when cadmium was induced in time course manner [18]. Likewise, higher expression of *Mt* observed in liver compared to gills and kidney in carp and rainbow trout [19,20]. In the case of *Kryptolebias marmoratus* (mangrove rivulus), the highest *Mt* mRNA expression was found on brain region [21]. These reports showed that the tissue distribution of *Mt* mRNA expression has species specificity. In this report, the liver was found to be the major site of *Mt* expression in *D. tambraparniei*. Taken together, the true mode of action of arsenic derivatives in heart and brain cannot predicted with partial series expression patterns of mRNA, but we hypothesize that aqueous arsenic is a strong modulator of *Mt* transcripts in both heart and brain [Figures 2 and 5].

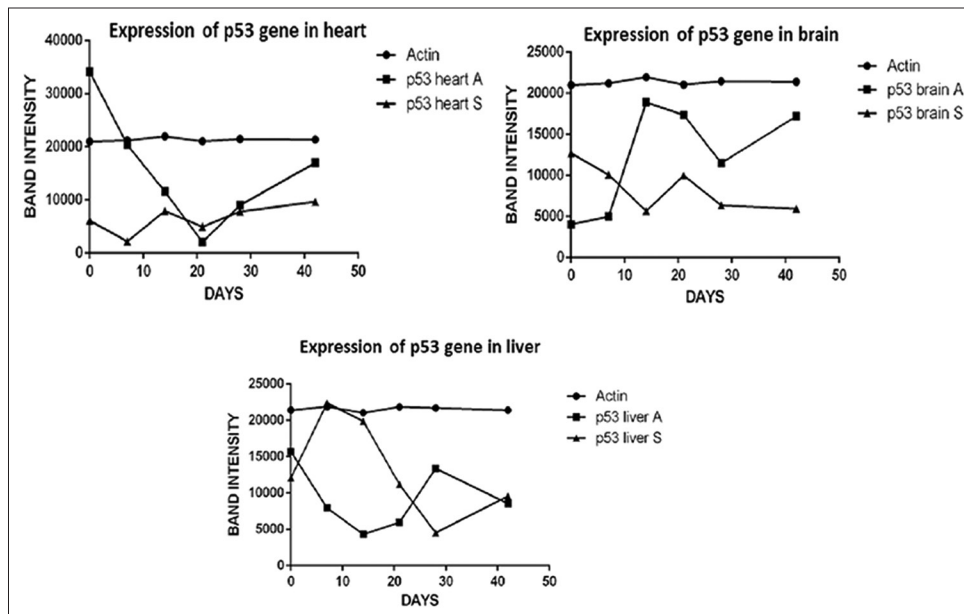


Figure 3: Expression of genes coding for *p53* in various organs of *D. tambraparaniei* relative to different days with NaAsO_2 and As_2O_3 .

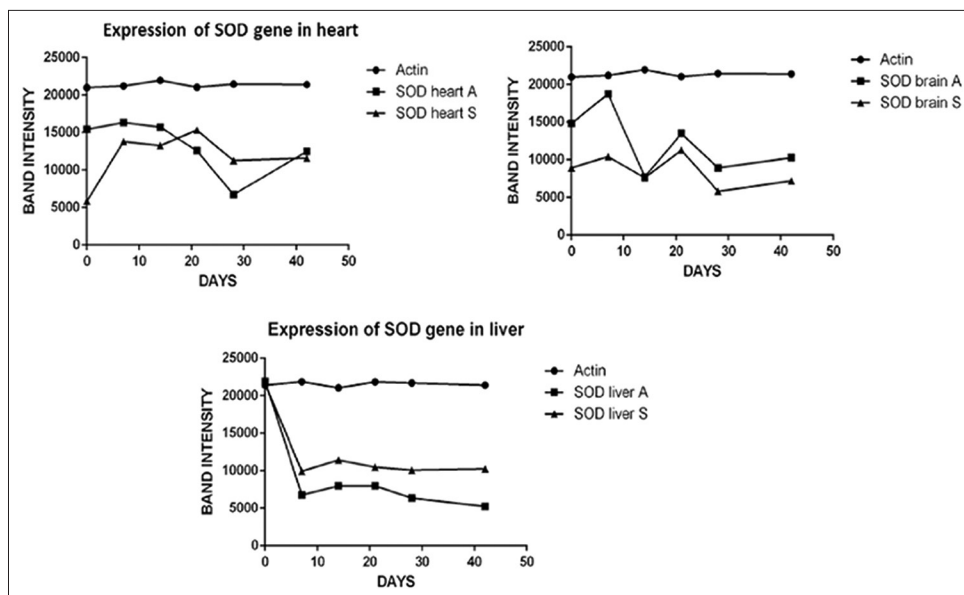


Figure 4: Expression of genes coding for superoxide dismutase (*SOD*) isoforms in various organs of *D. tambraparaniei* relative to different days with NaAsO_2 and As_2O_3 .

Based on these findings it is well known that the expression of *Mt* depends on arsenic accumulation on different organs of fish. Harrison and Klaverkamp (1989) observed that Cd was maximally accumulated in organs such as kidney, and later gills similar to the liver but not as evident in the muscles of rainbow trout [22]. *Mt* expression induction by these arsenic derivatives has been noted in other fish species such as mullet, carp, and rainbow trout, not only in liver but also in other tissues such as brain and heart [23-25].

It is known that when fishes were exposed to arsenic compound results in increase *p53* expression due to damage in mitochondrial membrane and damage in DNA, finally ROS. According to early report, PLHC-1 and primary trout cells that *p53* was not involved in apoptosis when it was triggered with model chemotherapeutic agents [26]. Coincidentally, they concluded that there was some non-specific *p53* pathway in fish.

The same happened in As_2O_3 and sodium arsenite (NaAsO_2) triggered toxicity that no increased expression occurs. Normally, *p53* was activated it resulting in either growth arrest or apoptosis [Figure 6].

Any aquatic organisms exposed with heavy metals such as arsenic, the inside reaction on cell apparently end up with damage to DNA, ROS production results in either upregulation or activation of *p53* pathway and thus arrests G1 phase in cell division got arrested. In such belongings, the activated *p53* signaling pathway permits those affected cells to do DNA editing and repairing. It was either followed by apoptosis or by usual cell division [27-29]. Even the cell was got arrested on G1 phase the damaged cells was repaired by DNA repair mechanism and allow them to survive either for certain period or permanently [30]. Conversely, the amounts of arsenic actually accumulated were not directly proportional to SODs mRNA levels in tissues such as brain,

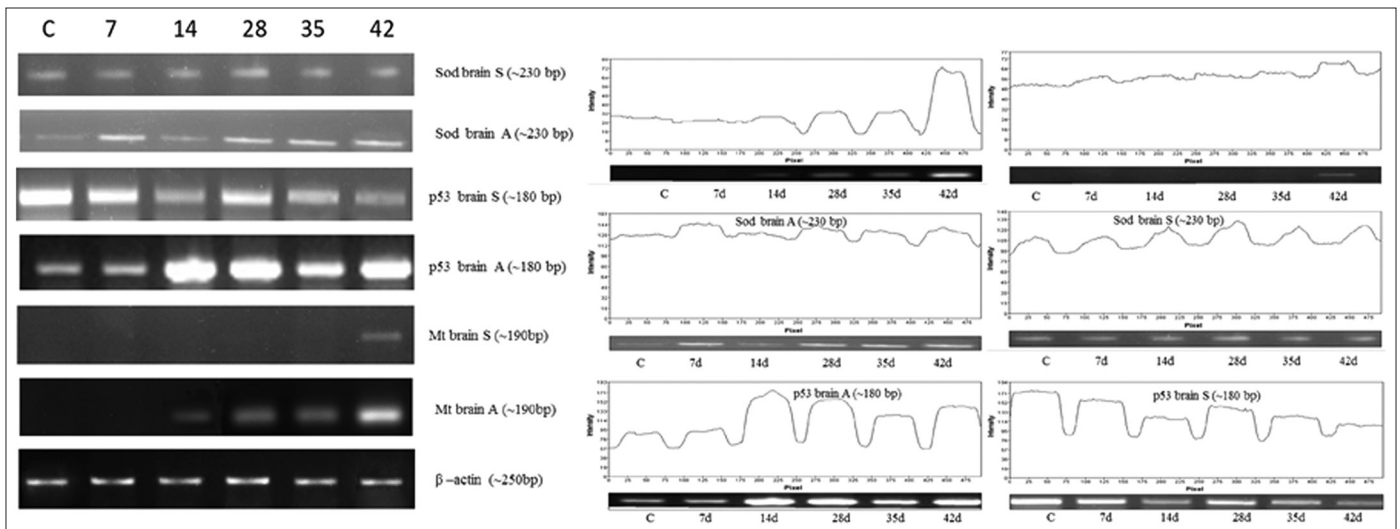


Figure 5: Effect of As_2O_3 (A) and $NaAsO_2$ (S) on brain. Analysis of *Mt*, *p53*, *SOD* genes expression was performed on heart by means of semi quantitative reverse transcriptase PCR. Amplification was done for 7 days to 42 days samples with respective genes (C for control).

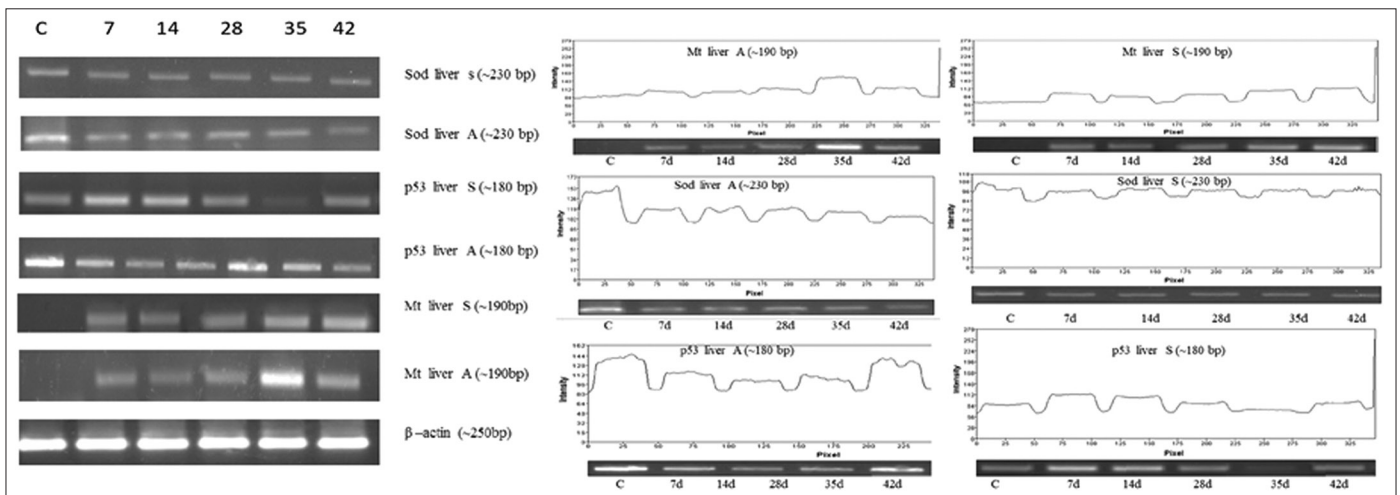


Figure 6: Effect of As_2O_3 (A) and $NaAsO_2$ (S) on liver. Analysis of *Mt*, *p53*, *SOD* genes expression was performed on heart by means of semi quantitative reverse transcriptase PCR. Amplification was done for 7 days to 42 days samples with respective genes (C for control).

heart, and liver in fishes: the highest arsenic accumulation occurred in liver, followed by the brain and heart. Comparable conclusion of metallothionein (*Mt*) gene expression was observed in common carp exposed to another heavy metal like cadmium [31,32].

4. CONCLUSION

The conclusion of the current study reported that arsenic exposure to promoted oxidative stress activation and apoptosis in an endemic fish *D. tambraparniei*. Consequently, the expression profiling analysis of selected marker genes such as *SOD*, *p53* and *Mt* indicates that impact of stress analysis by concentration and time specific manner. Stress-specific *SOD* excess and imbalance of oxidative stress could be considered as an early sign of cellular damage. Therefore, the results of the present study strongly suggested biomonitoring as a valuable technique for detecting heavy metal pollution in freshwater ecosystems for its direct application and analysis.

5. AUTHORS' CONTRIBUTIONS

Dr. Selvakumar Sakthivel has initiated conception, design, analysis and interpretation of data; Dr. Anand Raj Dhanapal and Dr. Vijay Velu has continued interpretation of data and reviewing the primary draft; Mr. Venkataa Suresh Munisamy, Mr. Mohammed Parvez Nasirudeen and Mr. Varatharaji Selvaraj has continued acquisition of data and formatting; Dr. Annadurai Gurusamy has conceptualized the primary draft construction following final drafting, editing, reviewing, all correspondence, and supervision.

6. FUNDING

There is no funding to report.

7. CONFLICT OF INTEREST

The authors declare no conflicts of interest.

8. ETHICAL APPROVALS

Principles of laboratory animal care were followed and all procedures were conducted according to the guidelines established by the National Institutes of Health (Guidelines for the use of fishes, 2004), and every effort was made to minimize suffering. This study was approved by the institutional ethical committee of Manonmaniam Sundaranar University [Ethical Clearance (EC), Biosafety and Animal Welfare Committee].

9. DATA AVAILABILITY

All data generated and analyzed are included within this research article.

10. PUBLISHER'S NOTE

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