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SHORT COMMUNICATION

Effect of arsenic and lead on glucose level and expression of ERK in air-breathing catfish, *Heteropneustes fossilis*

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Abstract Heavy metal contaminations may have devastating effects on the ecological balance of the environment and diversity of aquatic organisms. Furthermore, they cause great threat to the Indian aquatic ecosystem. In this study, we analyzed the effect of arsenic (As) and lead (Pb) on the blood glucose level and their possible involvement in the mitogen-activated protein kinase (MAPK) pathway in liver and muscle tissues of *Heteropneustes fossilis*. The catfish, *H. fossilis,* were exposed to an acute (35.09 ppm As_2O_3 and 66.20 ppm $PbCl_2$ for ~96 h) and chronic (LC_{50}/20th ppm of both As_2O_3 and $PbCl_2$ for ~30 d) concentration of As and Pb. Thereafter, the blood glucose level and the extracellular signal-regulated protein kinase (ERK) expression level in liver and muscle tissues of the fish were analyzed. It was found that As and Pb caused hyperglycemia in *H. fossilis*. Both on acute and chronic treatment with As and Pb, no significant change in p-ERK1/2 expression level was found in the muscle tissue of *H. fossilis*, whereas, in the liver tissue, the p-ERK1/2 expression level showed a significant increase in both acute (96 h) and chronic treatment (10 d and 30 d) of As and Pb. Therefore, it can be concluded that As and Pb could be highly toxic to the aquatic fauna, which could be a potential threat to human health as well.

Keywords Aquatic pollution . Heavy metals . Carbohydrate metabolism . MAPK pathway

Introduction

The aquatic systems may extensively be contaminated with heavy metals released from domestic, industrial and other man-made activities (Conacher et al. 1993; Velez and Montoro 1998). Heavy metals have been recognized as strong biological poisons because of their persistent-nature toxicity and have a tendency to accumulate in organisms (Kamble and Muley 2000). These pollutants cause severe damage to aquatic life, especially fish (Karbassi et al. 2006). Therefore, fish are widely used to evaluate the health of aquatic ecosystems because the pollutants that build up in the food chain are responsible for having adverse effects on the aquatic ecosystem including death of fish (Vinodhini and Narayanan 2008). Hence, fish can serve as models for studying human diseases as well as health of the ecosystem. This is especially true in addressing the effects of chronic and low-dose exposures of the aquatic contaminants (Carlson and Van Beneden 2014). Arsenic (As) and lead (Pb) are recognized as highly toxic worldwide (Akhtar et al. 2013) and the permissible limit of both the toxicants in drinking water is 0.01 mg/L (BIS 2012). However, higher concentration of As and Pb are observed in Indian groundwater (Adhikary and Mandal 2017; Sridhar et al. 2017). In fish, these heavy metals are accumulated in both soft tissues (e.g., kidney) and hard tissues (e.g., bone) (Bone and Morgan 2006).

Different authors report different 96 h median lethal concentration (LC_{50}) values of As and Pb in different fish species, but in our previous study, the 96 h LC_{50} values for As_2O_3 and $PbCl_2$ on *Heteropneustes fossilis* are found to be 35.097 ppm and 66.205 ppm, respectively (Tariang et al. 2019).

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Heavy metals cause stress and increase the blood glucose level in fishes (Vinodhini and Narayanan 2009). Blood glucose level is increased in spiny damselfish, *Acanthochromis polyacanthus*, in response to any environmental stress (Begg and Pankhurst 2004). Arsenic trioxide leads to hyperglycaemia in *Clarius batrachus* (Kumari and Ahsan 2011). Pb also increases the blood glucose level in different fishes, *Prochilodus lineatus* (Ribeiro et al. 2014), *C. gariepinus* (Mahmoud et al. 2013), *Carassius auratus* (Eslamloo et al. 2014) as well as in starry flounder (*Platichthys stellatus*) (Hwang et al. 2016). However, a previous study shows that on chronic exposure to As, the glucose level is reduced in *Catla catla* (Lavanya et al. 2011). Cu and Pb exposed fish (*Oreochromis niloticus*) show an increase in cortisol and glucose levels at 4 d of exposure (Firat et al. 2011). Sodium arsenate also elevates blood glucose level and declines glycogen in rat (Kumar et al. 2014a).

Mitogen-activated protein kinases (MAPKs) are a family of highly conserved enzymes which comprise ubiquitous groups of signalling proteins that play regulatory roles in cell physiology (Chang and Karin 2001; Chen et al. 2001). Members of each major MAPK subfamily, the extracellular signal-regulated protein kinases (ERK1/2), c-Jun N-terminal kinases (JNK) and $p38^{MAPK}$, have been implicated in the modulation of cell survival or death, cell proliferation, differentiation and adaptation (Chen et al. 2001). MAPK signalling pathway corresponds to every change in physiology of the cell (Gehart et al. 2010). The stimulation of glycogen synthesis is postulated *via* MAPK-dependent phosphorylation of p90rsk (Dent et al. 1990). The signalling proteins affected by the metals include growth factor receptors, G-proteins, MAPK and transcription factors (Flora et al. 2008). As, especially AsO_3^{3-}, is a potent stimulator of AP-1 transcriptional activity and an efficient inducer of c-fos and c-jun gene expression (Cavigelli et al. 1996). MAPK signalling (p38, ERK and JNK) pathways display elevated phosphorylation levels in arsenite-exposed heart tissues of *Cyprinus carpio* (Zhao et al. 2019). As also increases the expression of ERK and calpain-2 in *C. batrachus* (Banerjee et al. 2011). Induction of c-jun and c-fos by AsO_3^{3-} correlates with activation of JNKs and p38/MAPK2, which phosphorylate the transcription factors thus activating c-jun and c-fos genes (Cavigelli et al. 1996). The effects of Pb on MAPKs are widely described in mammalian cultured cells (Zhang et al. 2003). Despite the crucial neurophysiologic roles of ERK1/2 and $p38^{MAPK}$, modulation of these signalling proteins in the fish brain by Pb^{2+} is scarce (Scott and Sloman 2004). Pb^{2+}, an important environmental pollutant, activates ERK1/2 and $p38^{MAPK}$ *in vitro* and *in vivo* in Brazilian catfish, *Rhamdia quelen* (Leal et al. 2006). Therefore, in this study, besides blood glucose level, the pattern of expression of one of the key proteins of the MAPK signaling pathway, ERK1/2, was studied under heavy metals (As and Pb) stress in the catfish *H. fossilis*.

Materials and methods

Materials

$KMnO_4$ (code no. GRM702) was purchased from Himedia, India. As_2O_3 (code no. A1010), $PbCl_2$ (code no. 268690), glycerol (Code no. G5516), β-mercaptoethanol (Code no. M3148), acrylamide (Code no. A3553), methylbisacrylamide (Code no. M7279), ammonium persulfate (Code no. A3678), TEMED (Code no. T9281), glycine (Code no. G8898), CBB R-250 (Code no. B7920), Tween-20 (Code no. 93773), Tris (Code no. 79420), EDTA (Code no. 43272), bromophenol blue (Code no. B0126), agarose (Code no. A9539), BSA (Code no. A2153), were purchased from Sigma-Aldrich, USA. Antibodies against ERK1/2 (Code no. 4695), phospho-ERK1/2 (Code no. 4370) and horseradish peroxidase (HRP)-linked anti-rabbit IgG, (Code no. 7074) were purchased from Cell Signaling Technology, U.S.A. Gluco One Glucometer (Model no. BG-03) was purchased from Morepen Laboratories Ltd., India.

The experimental fish, *H. fossilis*, with an average weight of 25±2 g and length of 15±2 cm were procured from local commercial sources. After reaching the laboratory, the fish were treated with 0.05% $KMnO_4$ solution for 5 min to avoid any dermal infection. They were acclimatized to laboratory conditions in dechlorinated tap water for 15 d in a plastic tank and fed with chicken liver and rice cake. The water was changed daily to reduce the ammonia content in the water. All necessary precautions for maintaining the fish were followed as per the recommendations of the Organization for Economic Co-operation and Development (OECD 1992). The fish were exposed to natural light 12-14 h photoperiod daily. The physicochemical properties of the test water measured during the experiment are given in Table 1. The

Table 1 Physicochemical properties of test water

Physicochemical properties	Values
pH	6.5±0.5
Dissolved oxygen	6.58-7.82 mgL^{-1}
Water temperature	20.0±1.0 °C
Specific conductivity	0.13±0.01 mS
Total hardness	33±3 mgL^{-1} as $CaCO_3$
Total alkalinity	36±2 mgL^{-1} as $CaCO_3$

Values are expressed as mean±SEM (n=3).

water quality was maintained throughout the experimental periods.

Methods

The LC_{50} values of As_2O_3 (35.09 ppm) and $PbCl_2$ (66.20 ppm) on *H. fossilis* were taken as the acute concentration and $LC_{50}/20^{th}$ values as the chronic concentration for the test study (Tariang et al. 2019). The fish were treated with acute concentrations up to 96 h and chronic concentrations up to 30 d. After the exposure periods, the fish were euthanized in an ice-slurry to achieve death by hypothermia (Poli et al. 2005), followed by estimation of blood glucose level and evaluation of ERK1/2 phosphorylation status in liver and muscle tissues of *H. fossilis*. The experimental groups were divided into control, As treated, and Pb treated for both acute and chronic test, keeping 10±2 g fish per litre water as a loading capacity.

Blood glucose level estimation

The blood glucose level of *H. fossilis* was estimated using Dr. Morepen BG-03 Gluco One Glucometer (Model No. BG-03), where a drop of fresh blood was put on a glucose strip inserted in a standard glucometer.

Evaluation of ERK1/2 expression level

Western blot analysis of ERK1/2 was performed following the method described by Laemmli (1970) with minor modifications. In brief, a 10% tissue homogenate (1 g in 10 mL of buffer) was prepared in a homogenizing buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 2 mM 2-mercaptoethanol and 0.01 mM PMFS. The homogenate was treated with 0.5% Triton X-100 in a 1:1 ratio for 30 min followed by sonication for 3 cycles of 30 sec ON and 30 sec OFF using Q500 sonicator. The homogenate was centrifuged at 10,000 rpm for 10 min followed by 14,000 rpm for 30 min at 4 °C, and the resultant supernatant was used for analysis of ERK1/2. The protein content in the tissue sample was quantified following the Bradford's method (1976).

A Mini-PROTEAN casting frame (Bio-Rad) was used to cast 1 mm SDS-PAGE gel, consisting of a 5% stacking gel and 10% resolving gel. The polymerised gel was then assembled and submerged in the gel tank with running buffer (~750 mL). The tissue extract was mixed with an appropriate gel loading buffer containing 2% 2-mercaptoethanol and denatured by boiling at 95 °C for 5 min. The samples (30 µg/ lane) were loaded into the wells and 1 µl of protein ladder was also loaded. The gels were run in a Bio-Rad electrophoresis unit containing running buffer at 75 V for 30 min followed by 100 V until the dye reached the bottom of the gel. Then, the gel was removed from its glass casing and equilibrated in cold transfer buffer for 5-10 min. The proteins were then electro-transferred by Bio-Rad semidry transfer cell onto polyvinylidene difluoride membrane for 1.5 h at 10 V. The membrane was then incubated in 5% non-fat dried milk solution in Tris-buffered saline, 0.1% Tween® 20 detergent (TBST) for 1 h at room temperature to block the non-specific protein. The membrane was then probed with primary antibodies for t-ERK1/2 and p-ERK1/2 diluted to 1:1000 in non-fat dried milk solution overnight at 4 °C. Following primary antibody incubation, the membrane was rinsed and washed 3 times in TBST for 15 min each. The membrane was then transferred to a solution of anti-rabbit IgG HRP-conjugated secondary antibodies (diluted to 1:2500) in non-fat dried milk in TBST for 2 h at room temperature. Following secondary antibody incubation, the membrane was rinsed and washed 3 times in TBST for 15 min each to remove unbound antibodies. The protein bands were developed using an enhanced chemiluminescence reagent (Bio-Rad)

Table 2 Evaluation of blood glucose level (mg/dL) of *H. fossilis* upon acute (35.09 ppm of As_2O_3; 66.20 ppm of $PbCl_2$) and chronic ($LC_{50}/20^{th}$ ppm of both As and Pb) exposure of As and Pb. Values are expressed as mean±SEM (*n*=3).

Treatment	**Exposure Period**	**Control** (mg/dL)	**As_2O_3 treated** (mg/dL)	**$PbCl_2$ Treated** (mg/dL)
Acute	48 h	69.00±3.21	87.00±5.19[a] (+26.09)	77.33±5.17 (+12.07)
	96 h	68.00±2.65	101.67±6.06[a] (+49.51)	102.67±11.21[a] (+50.98)
Chronic	10 d	64.00±4.04	106.00±5.29[a] (+65.62)	102.33±9.61[a] (+59.89)
	20 d	70.33±5.23	135.33±6.88[a] (+92.42)	138.33±8.95[a] (+96.69)
	30 d	66.67±4.91	79.33±4.63 (+18.98)	76.33±5.23 (+14.49)

[a] P-value significant at <0.05 in As and Pb treated fish with respect to their controls.
Percentage of decrease (-) or increase (+) in glycogen content of the fish exposed to As and Pb with respect to their controls is given in parentheses.

and visualized using the Image Quant LAS 500 imaging system (GE Healthcare). Immunoreactive bands were then quantified using ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

The data obtained were statistically analyzed using the SPSS package (version 17.0) software. One way analysis of variance (ANOVA) was applied to compare the means obtained for different parameters among different tissues, and duration, control values considered as 100% when compared with exposed concentrations of As and Pb, respectively. The values for all data were expressed as mean ± SEM (*n*=3). Significant differences were defined at $P < 0.05$.

Results

Blood glucose level

The blood glucose level of *H. fossilis* was estimated for both acute and chronic treatment of As and Pb and presented in Table 2. Following the acute treatment of both As and Pb, the blood glucose level showed a gradual increase in the blood of *H. fossilis* with increase metal concentration. There was a significant increase in the blood glucose level up to 49.51% and 50.98% within 96 h acute treatment of As and Pb, respectively, as compared to their respective controls. However, on exposure to the chronic treatment of both As and Pb, the blood glucose level was increased up to 92.42% and 96.69% till 20 d, but there was no significant increase in the blood glucose level at 30 d of exposure of both As and Pb as compared to their respective controls.

Effect of As and Pb on ERK1/2

The p-ERK1/2 status in liver and muscle tissues of *H. fossilis* was evaluated under the exposure of both acute and chronic treatment of As and Pb. Following the 48 h acute treatment with As and Pb, the p-ERK1/2 status did not show any significant change compared to their respective controls in both liver and muscle tissues of *H. fossilis* (Fig. 1 [a] and [b]). However, after 96 h acute treatment, the p-ERK1/2 status showed a significant increase (of about 2-fold) in liver tissue when treated with As but did not show any change when treated with Pb (Fig. 1 [c]). Whereas, in the muscle tissue of *H. fossilis,* the p-ERK1/2 status did not show any significant change after 96 h acute treatment of As and Pb (Fig. 1 [d]).

After 10 d of chronic treatment, the p-ERK1/2 status in liver tissue of *H. fossilis* showed a significant increase when treated with both As and Pb. The p-ERK1/2 status was observed to increase by 1.9 folds and 1.6 folds when treated with As and Pb, respectively, as compared to their respective controls (Fig. 2 [a]).

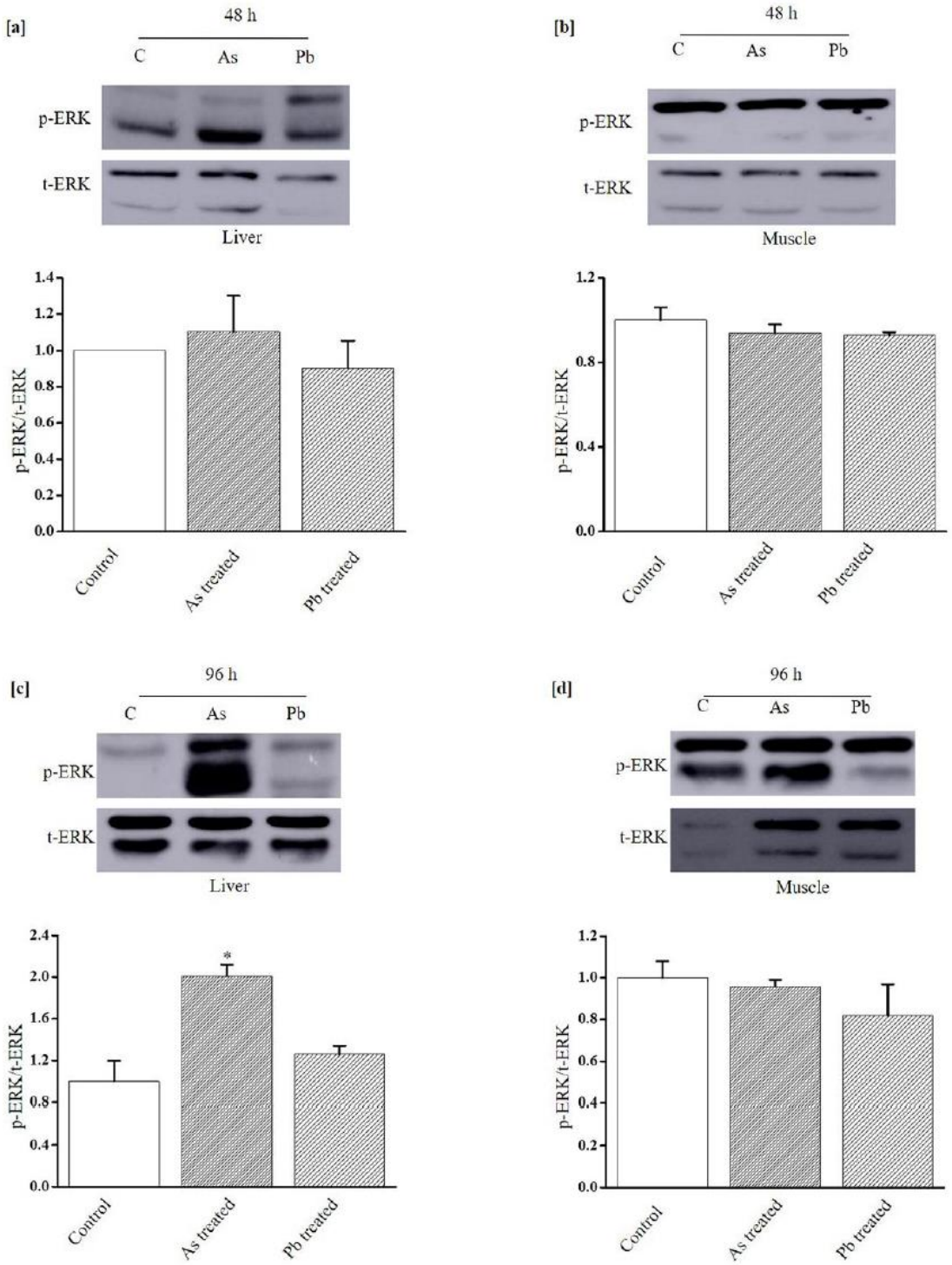

Fig. 1 p-ERK1/2 expression in liver and muscle tissues of *H. fossilis* on acute exposure to As and Pb. **[a]** p-ERK1/2 expression in the liver after 48 h exposure period, **[b]** p-ERK1/2 expression in the muscle after 48 h exposure period, **[c]** p-ERK1/2 expression in the liver after 96 h exposure period, **[d]** p-ERK1/2 expression in the muscle after 96 h exposure period. The values are expressed as mean±S.E.M. (*P <0.05; *n*=3).

Whereas the p-ERK1/2 status in the muscle tissue of *H. fossilis* did not show any significant change after 10 d treatment with the chronic concentration of As and Pb as compared to their respective controls (Fig. 2 [b]). Similar results were observed after 30 d of chronic treatment, where the p-ERK1/2 status in the liver tissue of *H. fossilis* showed a significant increase when treated with both As and Pb. The p-ERK1/2 status was observed to be 1.7 fold and 1.5 fold increase when treated with As and Pb, respectively, as compared

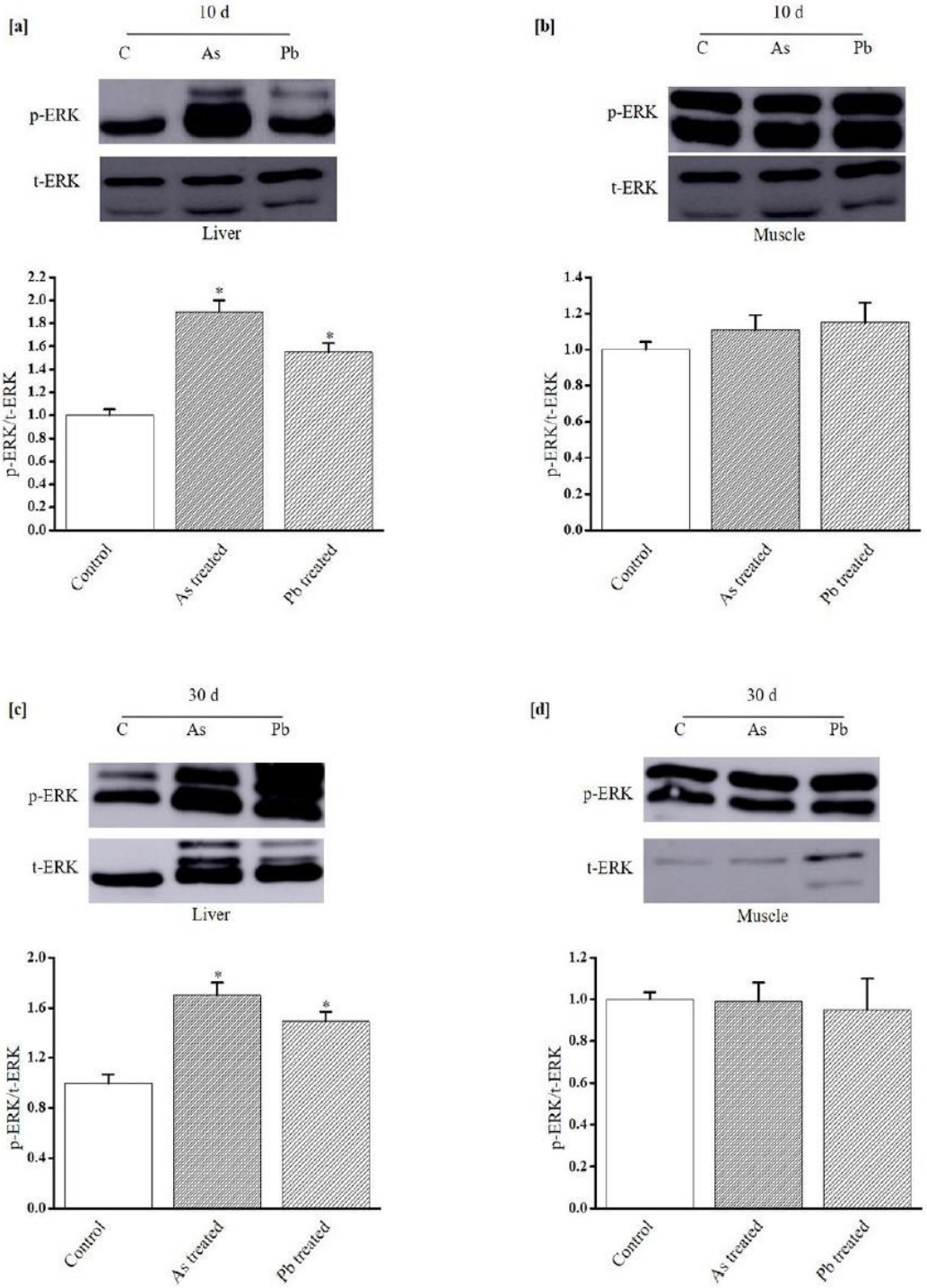

Fig. 2 p-ERK1/2 expression in liver and muscle tissues of *H. fossilis* on chronic exposure to As and Pb. **[a]** p-ERK1/2 expression in the liver after 10 d exposure period, **[b]** p-ERK1/2 expression in the muscle after 10 d exposure period, **[c]** p-ERK1/2 expression in the liver after 30 d exposure period, **[d]** p-ERK1/2 expression in the muscle after 30 d exposure period. The values are expressed as mean±S.E.M. (*P <0.05; *n*=3).

to their respective controls (Fig. 2 [c]). Whereas, in the muscle tissue of *H. fossilis*, the p-ERK1/2 status did not show any significant change after 30 d treatment with the chronic concentration of As and Pb as compared to their respective controls (Fig. 2 [d]).

Discussion

In the present study, a significant increase in the blood glucose level was observed when exposed to an acute concentration of As and Pb. Increased blood glucose level might be related to the stress induced on the fish by these heavy metals (Vinodhini and Narayanan 2009). Previous reports show that As and Pb are associated with altering and increase in blood glucose level (Ettinger et al. 2014; Kumar et al. 2019). Similar results have also been reported in *C. batrachus* (Kumar et al. 2014b), *C. catla* (Lavanya et al. 2011) and *P. hypophthalmus* (Kumar et al. 2020) when exposed to As. Previous studies also show that acute concentration of Pb elevates the blood glucose level in different fishes. Hyperglycemia is reported in *Tilapia zilli* (Zaki et al. 2009), *C. carpio* (Zare et al. 2007), *P. stellatus* (Hwang et al. 2016), *L. rohita* (Vaseem and Banerjee 2013), *O. mykiss* (Patel et al. 2006), and *P. lineatus* (Ribeiro et al. 2014). However, in contrast, a decline in blood glucose level is reported in *C. gariepinus* when treated with Pb (Ubani-Rex et al. 2017), as well as in rats treated with As (Singh et al. 2017).

In this study, chronic treatment of both As and Pb also increased the blood glucose level at the beginning of the exposure period, but there was a sign of recovery in the blood glucose level at the end of the exposure period. Vinodhini and Narayanan (2009) also report a significant increase in blood glucose level in *C. carpio* when treated with a sublethal concentration of the metal solution (Cd+Pb+Cr+Ni). Chronic treatment of Pb increases the blood glucose level in *O. niloticus* (Atli et al. 2015). However, Firat et al. (2011) and Hwang et al. (2016) report similar results, as observed in this study, when *O. Niloticus* and *P. stellatus* are exposed to Pb. In contrast, a previous study reports a decline in blood glucose level when treated with a sublethal concentration of As in *C. catla* (Lavanya et al. 2011).

In the present study, the liver tissue of *H. fossilis* showed a significant increase in p-ERK1/2 status in both acute and chronic treatment with As and Pb. However, in muscle tissue, the p-ERK1/2 status did not show any significant change. Previous studies show that As increases the p-ERK status in heart tissues of *C. carpio* (Zhao et al. 2019) and induces apoptosis in Zebra fish (Seok et al. 2007). Liu et al. (2011) suggest that As induces reactive oxygen species (ROS) generation, which activates ERK1/2 signalling pathways and increases the expression of hypoxia-inducible factor-1. It is also reported that As increases the expression of p-ERK in mice (Kimura et al. 2016), in rats (Khuman et al. 2016), as well as in human (Duan et al. 2020).

In contrast, long-term exposure to As inhibits the expression of p-ERK and short term exposure triggers the expression of p-ERK (Li et al. 2018). ERK and p-ERK levels in the hippocampus and cerebral cortex of rats are also decreased when exposed to fluoride and As (Zhu et al. 2017); whereas, Cavigelli et al. (1996) observe that As does not affect ERK expression level.

ERK1/2 and $p38^{MAPK}$ status are also increased in Brazilian catfish, *R. quelen*, when exposed to Pb (Leal et al. 2006). Pb poisoning also is shown to induce the production of ROS and increase the expression levels of p38, ERK, and JNK in carp (Jing et al. 2020), in chicken (Jiayong et al. 2020), in the rat (Zhang et al. 2007), as well as in human (Wang et al. 2012). However, in contrast to our results, previous reports also show that Pb reduces the expression of ERK in rats (Wen et al. 2018), and in chicken (Yin et al. 2019).

Conclusion

The results of the present study indicated that As and Pb caused hyperglycemia and upregulated p-ERK1/2 expression level in the liver tissue of *H. fossilis*. Hence, it can be concluded that As and Pb induced stress in *H. fossilis*, which could be a potential threat to the aquatic fauna, and thereby, human health as well.

Authors' contributions B.D. conceived the study and participated in its design and coordination. K.T. carried out the experiments and performed the data analysis. K.K. participated in the data analysis and discussion. All the authors participated in writing the manuscript and approved the final version of the manuscript.

Conflict of interest declaration The authors declare that there are no conflicts of interest.

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