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Adverse effects and metabolic impairment in liver of fresh water fish, *C. punctata* exposed to mercuric chloride and cold stress

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Abstract

Response to environmental stress is an important aspect in metabolism of organisms. For this purpose, *C. punctata,* a variety of fish was used and examined the effect of cold acclimation on the regulation of protein, cholesterol and triglyceride in liver induced by $HgCl_2$ for 1h. The stimulatory effects on protein content were demonstrated whenever exposed to $HgCl_2$ (1 and 10 µM) (10.63 *vs* 31.25, 38.62). Cholesterol and triglyceride levels in excised liver were enhanced in response to $HgCl_2$ when compared to respective controls (145.73 *vs* 1678.42; 16.46 *vs* 102.24). To find interaction with cold acclimation, fish were treated with $HgCl_2$ (1 and 10 µM) and exposed to 4 ^{o}C. The stimulatory effects on protein synthesis in cold stress were observed (10.63 *vs* 36.49, $HgCl_2$ 1 µM; 10.63 *vs* 67.0, $HgCl_2$ 10 µM) compared to control and the effects were potential whenever exposed to mercury (10 µM) in cold. The cholesterol level in response to cold along with $HgCl_2$ (1 and 10 µM) was found similarly to increase (145.73 *vs* 1086.13, 1838.48) respectively and the results were assumed to be higher than that of $HgCl_2$ alone. Cold acclimation also affects triglyceride in presence of different concentrations of $HgCl_2$ and increased level of triglyceride in liver exposed to $HgCl_2$ (1 and 10 µM) and cold (16.46 *vs* 34.22, 42.47) was demonstrated. Collectively, both these stimuli cause severe stress to the organism, involved in diverse metabolic regulation which may contribute for survival of species in the environment.

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Introduction

Channa punctata (Taki fish) is strong and survive in adverse environment however the survival of these fish is impaired by the environment. Therefore, it is important issue to determine the strategy for the prevention of the toxic effects of mercury (Hg) which causes environmental pollution and impairment of metabolic regulation in fish and other aquatic organisms. It is assumed that the higher energy content of this fish is caused by the increased activity of the sympathetic nerves. Peripheral tissue metabolism is affected by both environmental and chemical stimuli; however, endogenous auto regulation of metabolic processes of all species is a common biological process. The abiotic and biotic stresses cause diverse metabolic alterations and the response to environment is an important aspect for this species although not clarified well. Among the peripheral tissues, liver plays a great role in metabolic regulation. The metabolic functions in this tissue are influenced by both environmental and chemical stimuli. Cold acclimation has been recognized as a major sympathetic and environmental stimulus affecting metabolic activities (Dos Santos *et al.*, 2013). It has been demonstrated that cold acclimation is involved in lipolysis process and is augmented by environmental stimulus (Grim *et al.*, 2010). Moreover, liver glycogenolysis is one of the biological processes yielding energy for doing mechanical work and the process is enhanced upon activation of the sympathetic nervous system. Therefore, it is speculated that cold exposure may affect on the regulation of metabolic functions through activation of these nerves. Although fish are exposed to various environmental stimuli, the species wants to maintain the homeostasis of the body. Adaptive thermogenesis, the dissipation of energy in the form of heat in response to external stimuli, has been implicated in the regulation of energy balance and body temperature. Moreover, oxidative stress along with adverse effects in response to cold acclimation has been observed (Kammer *et al.*, 2011). Recent investigations reveal that chilling induced injury is associated with the formation of reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and singlet oxygen ($^1O_2$) (Lee and Lee, 2000; Basra, 2001). Several lines of evidences reveal that anti oxidative enzymes and anti oxidant molecules can neutralize ROS (Oidaira *et al.*, 2000; Lee and Lee, 2000). Therefore, it is assumed that liver metabolism might be affected by cold stress although the exact mechanism of this environmental stimulus is not clarified.

Hg is a toxic and heavy element available in water and other biological samples. The incorporation of Hg from the natural resources to human and other aquatic organisms impairs the metabolic activities and therefore causes severe adverse effects. Severe exposure of these elements cause metabolic imbalance and pathological syndromes and alternatively causes death. It may impair the glycolysis as well as the oxidative processes (Mieiro *et al.*, 2015) and causes different types of pathogenic syndromes in rodents, fishes and other organisms. However, the mechanism underlying the effects of acute mercury exposure on the regulation of oxidative and glycolytic processes in tissues of fish exposed to cold acclimation is not known. Mercury is classified as a human carcinogen based on several epidemiological studies showing an association of mercury exposure with cancers in lung, bladder, kidney and liver (Sharma *et al.*, 2014). Moreover, fish have long been used as sentinels for biomonitoring of aquatic environmental pollutants and are good indicators of mercury toxicity (Carvan *et al.*, 2000; Ung *et al.*, 2010). Both cold acclimation and toxic mercury make a critical environment where the fish survive however the mechanism underlying the survival process and the sensitivity to stimuli are not clarified. Therefore, the present study has been undertaken to analyze the role of cold acclimation on mercury induced metabolic regulation in liver of *C. punctata*.

Materials and methods

Fish

Channa punctatus weighing 50 g to 60 g were used and maintained in normal water with ambient temperature (25.0 ± 1°C). In the day of experiment,

exposure of mercuric chloride and cold acclimation was given to the different groups of fish in small plastic pots for 1h period with full aeration and with free access of water. After the treatment, fish were quickly decapitated and liver was sampled carefully and taken weight by digital balance (Chyo, JL-180, China) and kept at –20 °C. Control fish were similarly used for sampling of tissue except giving mercuric chloride or cold acclimation exposure.

Cold acclimation and mercury treatment

To examine the role of cold acclimation on the regulation of metabolic activity involving the amount of protein, triglyceride and cholesterol in liver of *C. punctatus* induced by mercuric chloride, fish were exposed to mercuric chloride (1 and 10 µM $HgCl_2$, BDH Chemical Ltd.) in water (500 mL) for 1h along with cold exposure (4 °C). The different groups of fish were treated with mercuric chloride (1 and 10 µM $HgCl_2$) in water (500 mL) while other groups of fish were exposed to cold acclimation for 1h only. Cold exposure was given to fish from the refrigerator (Pioneer, Tejgaon, Dhaka-1215) equipped in the laboratory. The tissue was sampled after the treatment similarly as mentioned above and analyzed for different metabolites.

Assay of tissue protein content

Tissues were homogenized with pre-cooled water and were centrifuged at 8000 rpm for 10 min. The supernatants from each tissue homogenate were used as crude extract for assay of protein by using 50 µL extract. The protein content in tissue was determined by the procedure of Lowry *et al.* (1951). Briefly, alkaline solution was prepared by mixing 50 mL of alkaline Na_2CO_3 solution (2% Na_2CO_3 in 0.1N NaOH) and 1.0 mL of copper-sodium potassium tartarate solution (1 g sodium potassium tartarate and 0.5 g $CuSO_4$. $5H_2O$ were dissolved in 100 mL distilled water). Fifty micro liters of tissue extract was taken to the test tube and made up to 1 mL with distilled water. For blank, 1 ml water was used in place of tissue extract. Five milliliters of alkaline solution was added to each tube and mixed well. The tubes were allowed to stand for 10 min at room temperature and 0.5 mL of diluted FCR (Commercial FCR was diluted with equal volume of water) was added and mixed well. After 30 min, the absorbance was taken at 650 nm against the blank. The protein content in each tissue was calculated from the standard graph of bovine albumin (1 mg mL^{-1}) and is expressed as mg/100 g of tissue weight.

Assay of tissue triglyceride content

Triglyceride content in liver of different groups of fish was measured quantitatively by LABKIT (Triglycerides kits), Crest Biosystems, Bambolim Complex Post Office, Goa - 403 202, INDIA. *For assay of triglyceride, 100* µL of crude liver sample were used.

Assay of tissue cholesterol content

Cholesterol content in liver was determined by using the method of Liebermann-Barchard reaction (Kenny, 1952). For assay of cholesterol, 0.5 mL of crude extract was taken to test tubes and 10 mL of ethanol-ether mixture (3:1) were added. The test tubes were shaken vigorously and the contents were taken to centrifuge tubes and were centrifuged for 15 min at 8000 rpm. The supernatants were transferred to new glass tubes and evaporated to dryness in a water bath. After evaporation, 5 mL of chloroform were added to dissolve the residue and 2 mL of acetic anhydride-H_2SO_4 mixture (20 mL of acetic anhydride and 1 mL of concentrated H_2SO_4) were given, mixed and allowed to stand in dark at 25 °C for 20 min to develop the color. The spectrophotometer reading was taken at 680 nm against the blank. Cholesterol content was measured with the help of standard solution of cholesterol (20 mg/100 mL in chloroform) where 2.5 mL of standard solution was taken in test tubes and 2.5 mL of chloroform were mixed and followed the same procedure. For blank, only 5 mL of chloroform and 2 mL of acetic anhydride-H_2SO_4 mixture were used. The amount of cholesterol was expressed as mg/100 g of tissue weight.

Statistical analysis

Results of the experiments were expressed as meanand standard error of different groups. The differences between the mean values were evaluated by ANOVA followed by paired *t*-test using SPSS software.

Results

Mercuric chloride induced metabolic regulation in liver

Protein content

As shown in Table 1, the average protein content in liver in response to $HgCl_2$ (1 μM) was 31.25 ± 2.47 g/100 g of tissue whereas for the control liver, the amount of protein was determined as 10.63 ± 0.72 g. A significant (2.9-folds, $p< 0.05$) increased protein level was observed after 1h when compared to the liver of control fish.

Table. 1. Effects of $HgCl_2$ on protein content in liver of *C. punctata*. Fish were treated with mercuric chloride (1 and 10 μM) for 1h. Control fish were similarly used except giving mercuric chloride.

Treatments	Protein content (g/100 g of tissue weight)
Control	10.63 ± 0.72
$HgCl_2$ (1 μM)	31.25 ± 2.47 [A]
$HgCl_2$ (10 μM)	38.62 ± 3.26 [B]

The data are means ± SE for 5 fish in each group. [A]$p< 0.05$ and [B]$p< 0.05$ versus control for 1h.

Another group of fish were exposed to 10 μM concentration of $HgCl_2$ and the amount of protein was recorded as 38.62 ± 3.26 g/100 g of tissue after 1h. The results demonstrated that the protein content in liver had been enhanced significantly ($p< 0.05$) (3.6-folds) when they were exposed to 10 μM concentrations of mercuric chloride, compared to the control fish. The increased protein content was found to be higher for 10 μM than that of 1 μM concentration (Fig. 4A).

Cholesterol content

The uptake and detoxification of mercury in liver is an important aspect in liver metabolism. The cellular uptake of mercury may impair lipid metabolism. To clarify whether $HgCl_2$ affects cholesterol level in liver, groups of fish (*C. punctata*) were exposed to $HgCl_2$ (1 and 10 μM). After the treatment, liver was excised and cholesterol content in liver was determined. Control fish were similarly used except $HgCl_2$ treatment. As shown in Table 2, the average cholesterol content in liver of fish exposed to 1 μM concentration of $HgCl_2$ was 1678.42 ± 288.44 mg while for the control fish, the value was 145.73 ± 17.95 mg/100 g of tissue weight.

Table 2. Effects of $HgCl_2$ on cholesterol content in liver of *C. punctata*. Fish were treated with mercuric chloride (1 and 10 μM) for 1h. Control fish were similarly used except giving mercuric chloride.

Treatments	Cholesterol content (mg/100 g of tissue weight)
Control	145.73 ± 17.95
$HgCl_2$ (1 μM)	1678.42 ± 288.44 [A]
$HgCl_2$ (10 μM)	911.14 ± 194.75 [B]

The data are means ± SE for 5 fish in each group. [A]$p< 0.001$ and [B]$p< 0.001$ versus control for 1h.

The results show that cholesterol content in liver was increased significantly (11.5-folds) ($p< 0.001$) when compared to the liver of control fish. On the contrary, fish exposed to different concentrations of $HgCl_2$ (10 μM) for 1h had 911.14 ± 194.75 mg of cholesterol. Mercuric chloride causes the synthesis of cholesterol significantly ($p< 0.001$) (6.2-folds) when compared to the liver of control fish, however 1 μM concentration of mercuric chloride was found to be involved in higher synthesis of cholesterol than 10 μM concentration (Fig. 4B). The results appear that $HgCl_2$ is toxic compound and might be involved in

causing the chemical and environmental stresses seemed to cause cholesterol biosynthesis and induce lipogenesis in liver.

Triglyceride content

To clarify whether $HgCl_2$ is involved in inducing triglyceride biosynthesis, groups of fish were exposed to different concentrations of $HgCl_2$ (1 and 10 μM) to examine the role of $HgCl_2$ on the changes of triglyceride in liver.

As shown in Table 3, the amount of triglyceride in liver of fish in response to $HgCl_2$ (1 μM) for 1h was 102.24 ± 16.12 mg while for 10 μM concentration, the value was found to 92.87 ± 14.47 mg/g of tissue weight. On the contrary, the amount of triglyceride in livers of group of fish (control) was recorded as 16.46 ± 3.07 mg/g of tissue weight.

Table 3. Effects of $HgCl_2$ on triglyceride content in liver of *C. punctata*. Fish were treated with mercuric chloride (1 and 10 μM) for 1h. Control fish were similarly used except giving mercuric chloride.

Treatments	Triglyceride content (mg/g of tissue weight)
Control	16.46 ± 3.07
$HgCl_2$ (1 μM)	102.24 ± 16.12 ^{A}
$HgCl_2$ (10 μM)	92.87 ± 14.47 ^{B}

The data are means ± SE for 5 fish in each group. $^{A}p< 0.001$ and $^{B}p< 0.001$ versus control for 1h.

A significant increased (6.6-folds) ($p< 0.001$) response on triglyceride synthesis in liver was observed for fish exposed to $HgCl_2$ (1 μM). Similar stimulatory effects (5.6-folds) ($p< 0.001$) on triglyceride synthesis in liver were observed whenever fish were exposed to 10 μM concentrations; however the effects were assumed to be potential for the fish exposed to 1 μM concentrations of $HgCl_2$ (Fig. 4C). The results demonstrated that mercury had been involved in impairment of triglyceride in liver inducing lipogenesis and would suggest that this heavy element create an adverse environment and the increased triglyceride in liver may play the critical role to survive in this circumstance.

Table 4. Role of cold acclimation on $HgCl_2$ induced metabolic regulation of protein. Fish were treated with mercuric chloride (10 μM) and mercuric chloride (10 μM) + cold acclimation for 1h. Control fish were similarly used except giving mercuric chloride.

Treatments	Protein content (g/100 g of tissue weight)
Control	10.63 ± 0.72
$HgCl_2$ (10 μM)	38.62 ± 3.26 ^{A}
Cold	10.20 ± 0.55
$HgCl_2$ (10 μM) + Cold	67.00 ± 3.21 ^{B}

The data are means ± SE for 5 fish in each group. $^{A}p< 0.05$ and $^{B}p< 0.01$ versus control and $HgCl_2$ (10 μM) respectively for 1h.

Interaction of cold acclimation with mercuric chloride and its metabolic effects

Protein content

Cold acclimation is an adaptive response, recognized to be a major environmental stimulus and was given to fish for 1h from the refrigerator to clarify its role on metabolic regulation in liver of *C. punctata* induced with $HgCl_2$. To examine the role of cold acclimation on protein content in liver, groups of fish were treated with 1 and 10 μM concentrations of $HgCl_2$ in cold environment (4 ^{o} C) for 1h and the respective control livers were also examined with similar concentration of $HgCl_2$ only (Fig. 1, Table 4). The amount of protein in livers of groups of fish in response to $HgCl_2$ (1 μM)

and in cold were 36.49 ± 3.82 g while for control and cold exposed livers, the values were 10.63 ± 0.72 g and 10.20 ± 0.55 g/100 g of tissue respectively. For 10 μM concentrations of $HgCl_2$ and cold, the protein contents were recorded as 67.0 ± 3.21 g/100 g of tissue.

Table 5. Role of cold acclimation on $HgCl_2$ induced metabolic regulation of cholesterol. Fish were treated with mercuric chloride (10 μM) and mercuric chloride (10 μM) + cold acclimation for 1h. Control fish were similarly used except giving mercuric chloride or calcium carbonate.

Treatments	Cholesterol content (mg/100 g of tissue weight)
Control	145.73 ± 17.95
$HgCl_2$ (10 μM)	911.14 ± 194.75 ^{A}
Cold	264.92 ± 63.93
$HgCl_2$ (10 μM) + Cold	1838.48 ± 326.48 ^{B}

The data are means ± SE for 4~5 fish in each group. ^{A}p< 0.001 and ^{B}p< 0.01 versus control and $HgCl_2$ (10 μM) respectively for 1h.

A significant increased response on protein level was observed for fish exposed to mercury (193.9%, 2.9-folds, p< 0.05) and 263.3%, 3.6-folds, p < 0.05) respectively and also even in cold (243.2%, 3.4-folds, p < 0.05, $HgCl_2$ 1 μM and 530.2%, 6.3-folds, p < 0.05, $HgCl_2$ 10 μM) when the groups were compared to control. On the other hand, cold exposed liver had no significant effects on protein contents when compared to control and the effects of cold exposure and Hg are much higher than cold exposed fish only. However, the effects were assumed to be potential whenever the fishes were exposed to mercury in cold.

Table 6. Role of cold acclimation on $HgCl_2$ induced metabolic regulation of triglyceride. Fish were treated with mercuric chloride (10 μM) and mercuric chloride (10 μM) + cold acclimation for 1h. Control fish were similarly used except giving mercuric chloride.

Treatments	Triglyceride content (mg/g of tissue weight)
Control	16.46 ± 3.07
$HgCl_2$ (10 μM)	92.87 ± 14.47 ^{A}
Cold	63.78 ± 6.36
$HgCl_2$ (10 μM) + Cold	42.47 ± 7.59 ^{B}

The data are means ± SE for 5 fish in each group. ^{A}p< 0.001 and ^{B}p< 0.01 versus control and $HgCl_2$ (10 μM) respectively for 1h.

The results indicated that cold acclimation might be involved in inducing the synthesis of protein in liver exposed to $HgCl_2$ and would suggest that both mercury and cold environment create an adverse environment and the increased protein in liver may play the critical role to survive in these situations.

Cholesterol content

Fig. 2 shows the effect of cold acclimation on cholesterol level in liver of *C. punctata* exposed to $HgCl_2$.

Groups of fish were used to examine the role of cold acclimation on the changes of cholesterol in liver. The amounts of cholesterol of mercury-treated fish (1 and 10 μM) for 1h were 1678.42 ± 288.44 mg and 911.14 ± 194.75 mg/100 g of tissue respectively where as for control fish; the value was 145.73 ± 17.95 mg/100 g of tissue. The cholesterol contents in response to mercury were found to be increased significantly (11.5-folds, p < 0.001; 6.2-folds, p < 0.001 respectively) when the values were compared to the control (Table 2).

Groups of fish were exposed to cold with 1 μM concentrations of mercuric chloride solution and the cholesterol content in liver was recorded as 1086.13 ± 309.06 mg/100 g of tissue and for the cold exposed fish, the value was only 264.92 ± 63.93 mg.

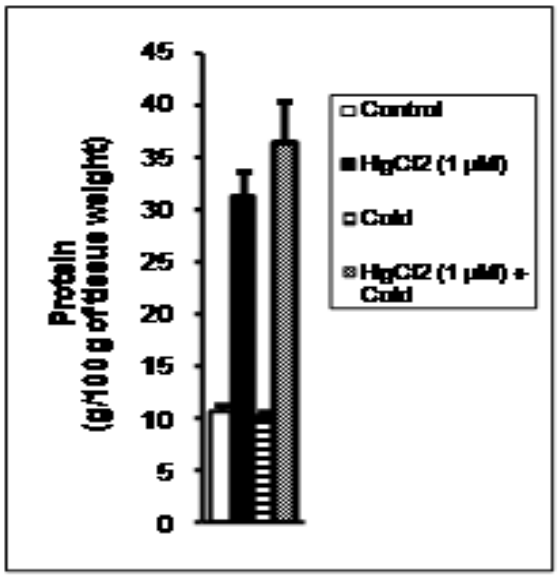

Fig. 1. Effects of $HgCl_2$ and cold acclimation on protein level in liver of *C. punctata*. The groups of fish were treated with $HgCl_2$ (1 μM) and $HgCl_2$ (1 μM) + cold exposure for 1h. After the treatment, the fish were immediately decapitated and sampling of tissue was performed. Control fish were similarly used except giving $HgCl_2$ or cold acclimation. The data are ± SEM for 4~5 fish in each group.

The cholesterol level in response to cold exposure and $HgCl_2$ was found similarly to be increased when compared to control (7.4-folds, 645.3%, $p < 0.001$) and the respective cold exposed fish (309.9%, 4-folds, $p < 0.001$).

The amount of cholesterol was recorded as 1838.48 ± 326.48 mg/100 g of tissue whenever fish were exposed to $HgCl_2$ (10 μM) and cold. The cholesterol content was similarly increased (12.6-folds, $p < 0.001$) in response to cold exposure when compared to control fish (Table 5) and the results are much potential than that of 1 μM of $HgCl_2$ and cold exposure (Fig. 2, Table 5). The results would suggest that the increased cholesterol in liver in response to mercury was enhanced by cold acclimation however cold acclimation might be involved in the regulation of its amount even in presence of toxic mercury since these environmental stimuli cause the adverse situation where the species survive. The results shows that toxic Hg causes the higher cholesterol synthesis in liver and cold acclimation might be involved in interaction with the effect and produces the higher cholesterol level.

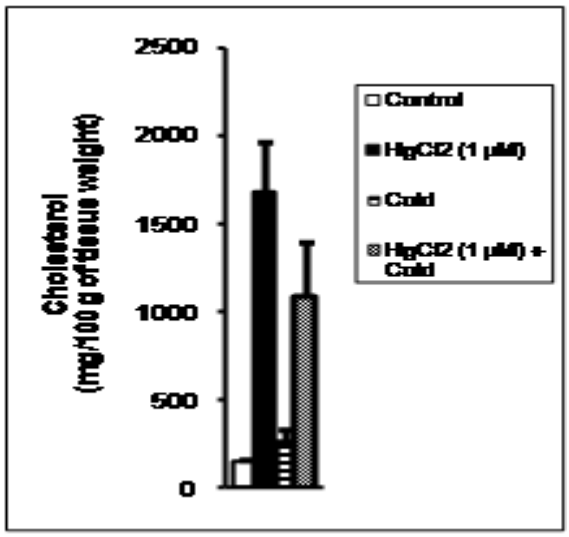

Fig. 2. Effects of $HgCl_2$ and cold acclimation on cholesterol level in liver of *C. punctata*. The groups of fish were treated with $HgCl_2$ (1 μM) and $HgCl_2$ (1 μM) + cold exposure for 1h. After the treatment, the fish were immediately decapitated and sampling of tissue was performed. Control fish were similarly used except giving $HgCl_2$ or cold acclimation. The data are ± SEM for 4~5 fish in each group.

Triglyceride content

As shown in Table 3 and Fig. 3, triglyceride content in liver of fish exposed to $HgCl_2$ (1 and 10 μM) were recorded as 102.24 ± 16.12 mg and 92.87 ±14.47 mg/g of tissue respectively where as for fish exposed to $HgCl_2$ (1 μM) as well as cold for 1h, the amount of triglyceride was determined as 34.22 ± 12.63 mg and for the control fish kept in water, the value was found to 16.46 ± 3.07 mg/g of tissue. The amount of triglyceride in response to $HgCl_2$ was increased significantly (Fig. 4C) compared to the control fish (6.6-folds, $p< 0.001$ and 5.6-folds, $p< 0.001$ respectively). Fish exposed to $HgCl_2$ (1 μM) and cold had also increased level (107.8%, 2.0-folds, $p< 0.05$) of triglyceride when compared to control fish. Similar stimulatory effects (158.0%, 2.6-folds, $p< 0.05$) were

observed whenever fish were treated with $HgCl_2$ (10 µM) and cold for 1h compared to control fish where the values were recorded as 42.47 ± 7.59 mg/g of tissue. However, the results are much lower than that of 10 µM concentrations of $HgCl_2$ alone (Table 6, Fig. 3).

For cold exposed fish, the triglyceride content in liver was recorded as 63.78 ± 6.36 mg/g of tissue and cold acclimation causes the synthesis of triglyceride (287.4%, 3.9-folds, $p < 0.01$) when compared to control, however, the increased triglyceride in presence of 1 or 10 µM of $HgCl_2$ in cold exposure were lower than that of cold exposed fish.

The results conclude that both cold acclimation and toxic mercury show the higher triglyceride in liver and the effect of Hg was assumed to be enhanced in response to cold and therefore, is an essential parameter for characterization of adaptive response to adverse environment particularly low temperature and heavy metal toxicity.

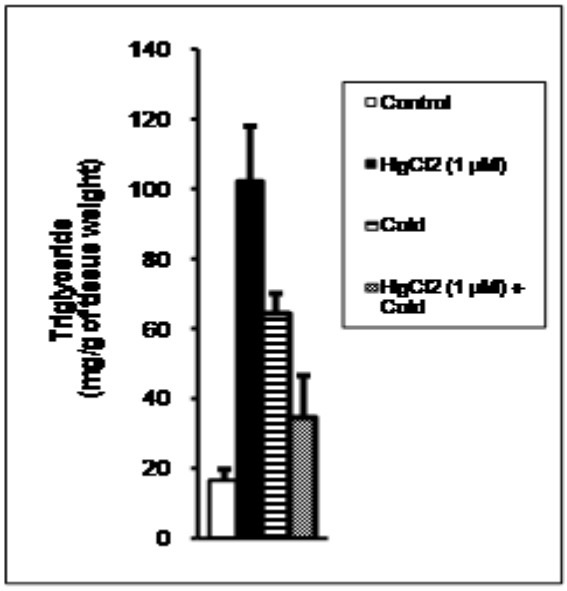

Fig. 3. Effects of $HgCl_2$ and cold acclimation on triglyceride level in liver of *C. punctata*. The groups of fish were treated with $HgCl_2$ (1 µM) and $HgCl_2$ (1 µM) + cold exposure for 1h. After the treatment, the fish were immediately decapitated and sampling of tissue was performed. Control fish were similarly used except giving $HgCl_2$ or cold acclimation. The data are ± SEM for 4~5 fish in each group.

Discussion

Mercuric chloride induced metabolic regulation in liver

Protein content

In the current study, $HgCl_2$ was found to enhance protein in liver for this species of fish. Fish are considered as suitable biomonitors for environmental pollution and they are exposed to the heavy metals *in vitro* to study the effects of heavy metals in aquatic ecosystems (Padmini *et al*., 2004). Up- regulation of heat shock protein genes, oxidative stress-inducible genes and genes coding for proteins associated with antioxidant activity suggests increased oxidative stress and reactive oxygen species (ROS) in liver of mercury treated fish (Li *et al*., 2014).

The results are good agreement with the present investigation showing the increased protein in liver of *C. punctata* treated with $HgCl_2$. The increased synthesis of protein in liver in presence of toxic environment induced by mercury (Hg) might be involved in the regulation of metabolic functions of this species of fish. The alteration of protein concentration in liver in response to mercury is an index for characterization of the sensitivity to the environmental stress. The increased protein in liver may contribute to survival of the species of fish in the toxic environment created by $HgCl_2$.

Cholesterol content

Cholesterol is another molecule available in liver however the amount of cholesterol in response to $HgCl_2$ was increased in this study showing the higher lipogenesis in liver. The increased synthesis of cholesterol also may induce higher liver weight and fatty liver as demonstrated by Ung *et al.* (2010). Mercuric chloride ($HgCl_2$) is highly toxic to the living organisms and its exposure with higher concentration in water causes severe effects in fish and might be involved in the impairment of metabolic activities in cellular level. Treatment with $HgCl_2$ may cause severe oxidative stress and stimulate the synthesis of cholesterol in liver.

Triglyceride content

Triglyceride turnover is a metabolic and biological process and represents a characteristic feature for the organisms so that they can survive in the environment. The synthesis and degradation of triglyceride are essential biochemical process and are influenced by alteration of the environmental stimulation. The toxic effects of mercury may impair the synthesis of triglyceride in liver. Therefore, to clarify whether $HgCl_2$ is involved in inducing triglyceride biosynthesis, groups of fish were exposed to different concentrations of $HgCl_2$ (1 and 10 µM) to examine the role of $HgCl_2$ on the changes of triglyceride in liver.

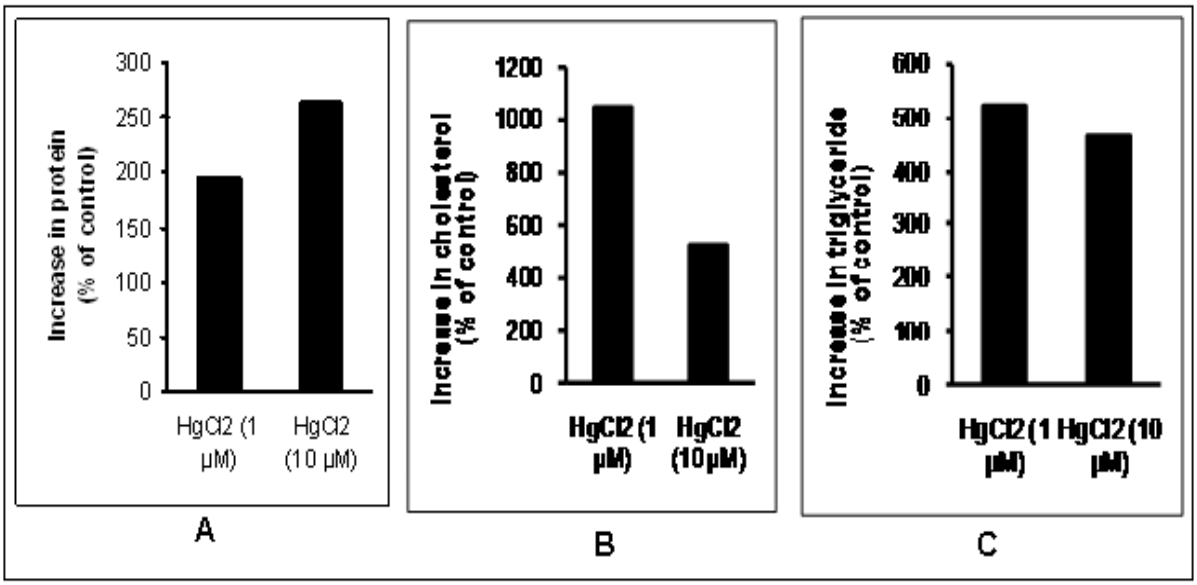

Fig. 4. Increase in metabolites (protein, cholesterol and triglyceride) (Fig. 4A, 4B and 4C respectively) in liver of *C. punctata* in response to different concentrations of $HgCl_2$. The results are expressed as the percentage of control.

Although much evidence were not observed in presence of $HgCl_2$ on the enhancement of triglyceride, the previous study reveals that arsenic, a potent toxic and heavy element similar to Hg causes the similar effects and produces the fatty liver with increasing liver weight (Roy and Haque, 2009). Dosage-dependent lipid accumulation, as indicated by the increased number and size of red-stained lipid vesicles, was detected in the liver of $HgCl_2$-treated fish (Ung *et al.*, 2010). The histological phenotype was thus consistent with the transcriptome analysis where up-regulation of fatty acid synthesis and down-regulation of mitochondrial fatty acid β-oxidation were found in the liver of $HgCl_2$-treated zebrafish. Accumulation of lipids can lead to adipogenesis, steatostasis or non-alcoholic fatty liver diseases. The transcription factors CCAAT/enhancer-binding proteins (C/ebps) are known to modulate gene expression leading to adipogenesis (Rangwala and Lazar, 2000; Rosen *et al.*, 2000). The results of the present investigation therefore, are good agreement with their findings.

Both chemical and environmental factors were believed to cause stress to the organism on metabolic alteration in liver however they survive in the adverse environment by alteration of some of the metabolic functions. *C. punctata* is very strong fresh water fish, survives in the adverse environment and therefore, sensitivity of chemical and environmental stress is of particular interest for this species.

As an environmental toxicant, mercuric chloride has been considered to be potent compound causing adversity to the environment particularly its severe toxic effects in the biological system where the diverse metabolic functions are impaired. In the current investigation, different concentrations of $HgCl_2$ were used

In this study where 1 μM rather than 10 μM concentrations were found to be potentially involved in enhancing the metabolic activities particularly cholesterol, triglyceride and protein contents in liver. It has been demonstrated that this heavy element (Hg) is involved in causing the toxic effects through formations of the reactive oxygen species (ROS) (Verlecar *et al.*, 2007). In aquatic ecosystems, inorganic Hg can be methylated by bacterial processes to form a more toxic substance, methylmercury (MeHg). However, because less than 10% of total Hg exists in the MeHg form, inorganic Hg is believed to have a more significant effect on aquatic animals (Zhang and Wong, 2007). Aquatic animals such as fish take up Hg either by direct exposure through their body or by ingestion. Mercury can then bioaccumulate and biomagnify through the food chain (Alvarez *et al.*, 2006). The uptake and elimination pathways differ substantially among tissues (e.g., liver, kidney, gills, and muscle), thus, Hg-accumulation is tissue-specific (Rothschild and Duffy, 2005). The accumulation of Hg above certain levels in fish can result in serious biological disturbances or individual death. Giudetti *et al.* (2013) demonstrated that reactive oxygen species might be involved in lipogenesis in liver through impairment of the lipid biosynthesis.

Cold acclimation and its Interaction with mercuric chloride on metabolic effects

Cold acclimation as well as its interaction with mercury on metabolic regulation has been demonstrated in the present findings. Cold acclimation is an adaptive response involving diverse metabolic alterations in the organisms (Jeon and Kim, 2013; Dos Santos *et al.*, 2013) and has been shown to be involved in causing oxidative stress (Kammer *et al.*, 2011). Fish treated with $HgCl_2$ (1 and 10 μM) and cold stress shows higher protein contents in liver than that of $HgCl_2$ alone. Both chemical and environmental stimuli cause higher oxidative effects therefore, it is reasonable and rational that the protein contents synthesized in liver were found to be higher.

The previous study reveals that cold sensitive stress proteins were synthesized in liver of individuals in response to environmental stimuli (Ibarz *et al.*, 2010). Their findings are compatible and correlated with the present investigations. The results are appreciably because of the additive effects of both Hg and cold acclimation or the influencing effect of cold stress on mercuric chloride effect.

In separate experiments, groups of fish were exposed to both $HgCl_2$ and cold acclimation for 1h and cholesterol contents in liver were recorded. The enhanced cholesterol levels were observed in response to 1 or 10 μM concentration of $HgCl_2$ when compared to control fish exposed to water only.

The increased cholesterol might be due to the higher oxidative effects as these stimuli have been believed to be involved in augmentation of causing of oxidative stress. Moreover, it has been shown that cold acclimation to fish potentially stimulates the liver cells and increases the total liver mass and lipid content (Johnston and Dunn, 1987; Kent *et al.*, 1988).

Cholesterol is a sterol compound synthesized in liver and is used to produce hormones and cell membranes. It is transported in the blood plasma of all mammals and is required to establish proper membrane permeability and fluidity. Therefore, synthesis of cholesterol in liver may contribute to other functions of the organisms.

Triglyceride contents in liver were affected in presence of cold acclimation and demonstrated to be enhanced however reduced when compared to the effects of $HgCl_2$ alone. The lipogenesis is a metabolic process in liver and has been demonstrated to be influenced by the environmental stimulus (Das *et al.*, 2013).

Their findings indicate that environmental stress causes the enhancement of gluconeogenesis which might be linked to lipogenesis in liver. The augmented triglyceride in liver of *C. punctata* in the current investigation when exposed to environmental stress might be also because of the higher oxidative effects.

The previous investigations reveal that exposure to low temperature influences fish liver, enhances lipid content and total liver mass (Johnston and Dunn, 1987; Kent *et al.*, 1988).

It is therefore, assumed from the above evidence that the increased triglyceride in presence of cold and mercury is because of the increased lipogenesis process. Both $HgCl_2$ and cold acclimation is a major adaptive and oxidative stress and have been shown to be involved in regulation of the biochemical processes in liver of *C. punctata*.

The present investigations suggest that cold acclimation may cause interaction with the effects of mercuric chloride and affect the diverse metabolic and biological processes in liver.

Conclusion

In summary, a liver is metabolically important for energy consumption and energy expenditure and is considered to be the major area where detoxification of foreign toxic substances occurs. Mercuric chloride and cold acclimation have been found to be involved in enhancement of metabolic activities particularly protein, triglyceride and cholesterol in the liver.

These abiotic and biotic stresses seem to be involved in oxidative stresses affecting metabolic activities in the liver so that they survive in the adverse environment.

The diverse metabolite regulation in response to low temperature is an index for the survival of the species and is a useful biological process while cold acclimation might be involved in producing interaction of the effects of mercuric chloride.

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