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Acute toxicity of copper sulphate on *Catla catla* larvae and its effect on expression of three commonly used housekeeping genes

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Pisces are commonly used to study the effects of metals, including copper on the environment. However, until now only scant information is available about the responses induced by waterborne copper during early life stages and housekeeping gene expression in fishes. In the present study, we evaluated acute toxicity of copper sulphate on larvae of *Catla catla* and also the stability of expression of three housekeeping genes, beta-actin (β-actin), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and elongation factor 1 alpha (EF1α). The results have shown increased mortality of *Catla catla* larvae with the increased concentration of copper sulphate. The median lethal concentration (LC_{50}) of copper sulphate at the end of 96 h exposure was 1.032 ppm. During the challenge test of copper sulphate, the minimal coefficient of variation (CV) and stability index were observed for GAPDH and maximum for β-actin indicating minimum variation of GAPDH and maximum variation of β-actin. With the results, it can be concluded that GAPDH is most stable during copper sulphate challenge test on *Catla catla* larvae, followed by EF1α and β-actin.

Keywords: β-actin, *Catla catla*, Copper sulphate, EF1α, GAPDH, Housekeeping gene

Different environmental pollutants that affect survival and health of fishes have been reported from various aquatic ecosystems[1,2]. Heavy metals are considered as serious pollutants due to bioaccumulation and biomagnification along the food chain[3]. The heavy metals such as arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), nickel (Ni), lead (Pb), iron (Fe) and zinc (Zn) are common heavy metal pollutants which cause severe toxicity to fishes[4]. Among the listed heavy metals, copper is one of the important heavy metal which is released into the aquatic environment due to different anthropogenic activities such as mining, manufacturing and agricultural runoff. In aquaculture practices, copper sulphate has been widely used to control algae and pathogens[5]. However, this practice can pose acute risks to various organisms, with the greatest risk to aquatic organisms resulting from direct water applications and runoff from fields adjacent to water bodies[6].

At the molecular level, these heavy metal pollutants can alter/regulate gene expression that has an important role in the metabolism of xenobiotic and oxidative phosphorylation pathway of aquatic organisms[7]. Previously various biochemical markers have been used to study the effect of heavy metals on the physiology of aquatic organisms particularly on fishes[8,9]. Recently, Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) technique has been widely used to evaluate the effect of heavy metal pollutants on gene expression pattern of aquatic organisms[10,11]. However, the accuracy of these studies depends on the selection of optimal housekeeping gene (HKG) to normalize biomarkers gene expression for qRT-PCR.

The effect of waterborne copper on the expression of different HKGs has not been studied so far and scant information has been available about its effect on larvae of fishes[12]. With this background the present study was carried out to investigate the acute effect of copper sulphate on Catla (*Catla catla*) larvae and three commonly used HKGs, beta-actin (β-actin), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and elongation factor 1 alpha (EF1α) expression stability. Further, the potential of these three HKGs as an internal control during acute toxicity test of Copper has been validated/tested.

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Materials and Methods

Experimental Animals and Treatments

Four days post-fertilization larvae of Catla were procured from ICAR-CIFE freshwater fish farm, Powarkheda, Madhya Pradesh and acclimatized for 24 h before the beginning of the experiment. The Larvae (6-6.5 mm) were fed with freshly hatched artemia daily.

A static non-renewable acute toxicity bioassay was conducted according to standard method[13,14] to determine LC_{50} (median lethal concentration) of copper sulphate (copper sulphate pentahydrate; CAS Number: 7758-99-8) for Catla larvae following exposure of 96 h. Initially, range finding test was conducted at 0, 0.25, 0.5, 1, 2, 4, 8 and10 ppm concentration to ascertain the range to be followed in the definitive test and subsequently, definitive test was conducted for seven different test media (mortality percentage of 0 and 100%) with 3 replications for each treatment (20 larvae per tank). Percentage mortality was recorded at 24, 48, 72 and 96 h interval. Dead fishes from each tank were removed immediately. Based on LC_{50} for 96 h, a challenge test at 50% of LC_{50} was done. The challenge test was set up in triplicate (100 larvae each tank) and 10 larvae samples were collected at 0, 6, 24, 48, and 96 h post challenge. Data obtained from the experiment were processed by probit analysis using SPSS V.16.

RNA isolation and cDNA synthesis

Total RNA was extracted from pooled samples (10 larvae) using 500 µL of Trizol (Invitrogen, Germany) and potential contamination of genomic DNA was removed by treatment with DNAse I (Fermentas, USA) for 30 min at 37°C. RNA quality and quantity were checked by spectrophotometric measurements (NanoDrop® Technologies, USA) and integrity was confirmed by agarose gel electrophoresis. cDNA was synthesized from 1 µg of total RNA using the Fermentas RevertAid premium reverse transcriptase kit and oligo dTprimer.

Quantitative real time reverse transcription polymerase chain reaction

qRT-PCR was performed to quantify the RNA transcripts of various candidate HKGs and these amplifications were performed with a LightCycler 480 II-real time PCR detection system (Roche, Germany). Gene-specific primers of Catla HKGs earlier designed for qRT-PCR was used in the present study. The sequence, optimum annealing temperature and PCR efficiency for qRT-PCR primers are described in our earlier study[15]. Each 10 µL reaction in a 96-well plate comprised 1 µL of cDNA template, 80 nM of each primer and 5 µL of SYBR Green I master mix. Plates were sealed with adhesive optical film (Roche). The PCR conditions comprised an initial denaturation step at 95°C for 15 min, followed by 40 cycles of 10 sec at 94°C, 15 sec at 60°C, 15 sec at 72°C. Fluorescence data were acquired during extension step. A dissociation protocol with a gradient from 65-97°C was used to investigate the specificity of the qRT-PCR reaction and the presence of primer dimmers. The difference in mRNA transcription levels was detected by comparing cycle threshold (Ct) values. All samples of three HKGs were run in triplicate on the same plate and no template controls were included in all plates.

Data analysis

Results (mean Ct values of the replicate sample at each time point for challenge test) from the LightCycler II 480 thermocycler were analysed in Excel Analysis ToolPak and SPSS V.16. The Ct is defined as the number of cycles needed for the fluorescence to reach a specific threshold level of detection and is inversely correlated with the amount of template nucleic acid present in the reaction. Overall mean Ct, standard deviation (SD) and coefficient of variation (CV) were obtained for all three HKGs across the challenge test. Gene stability across the challenge test at different time point was assessed by Box Plot, CV and stability index following linear regression analysis.

Results and Discussion

Effect of Copper on larvae

Copper, although an essential nutrient may become highly toxic to fish at higher concentration[16]. Among different life stages of fish, larvae are more sensitivity than embryos, and embryos are more sensitive than adults (larvae > embryos > adults). Toxicity data of most vulnerable life stage would offer protection to all life stages in the natural environment[17]. In the present study, acute toxicity test has been done to evaluate the effect of copper sulphate on Catla larvae and results showed a dose-dependent mortality. Most of the fishes died during first 24 h exposure and mortality decreased with time, however, movement of the surviving larvae in the treatment group was reduced as compared to control. Several previous studies have also reported that heavy metals reduce survival and cause behavioural

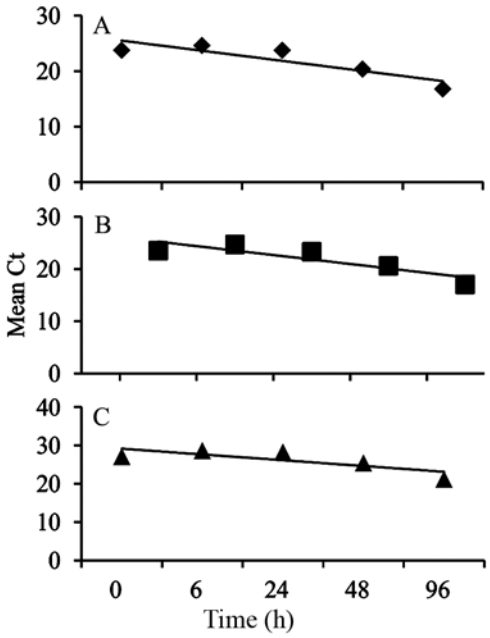

Fig. 1—Linear regression curves of housekeeping genes. [Each symbol on the graph represents the mean Ct value]

anomalies such as impaired locomotors performance resulting in increased susceptibility to predators[18,19]. The median lethal concentration (LC_{50}) of copper sulphate at the end of 96 h exposure in the present study was 1.032 ppm (95% confidence interval 0.889-1.173 ppm), and percentage of normal larvae in the control group was at least 90%. However in Catla fingerling (length: 8-10 cm and weight 5-8 g), 96 h LC_{50} of copper sulphate was 4.8 ppm[20]. The apparent variability in sensitivity between different life stages may be due to several factors; surface area/volume ratio (particularly with young fish); greater uptake of toxicant from the environment; underdeveloped homeostatic mechanism to deal with the toxicants; immature immune systems and underdeveloped organs (liver and kidney) which has an important role in detoxification and elimination of toxicants[17]. Further for other carp species, *Labeo rohita* (5.15-11.40 g), *Cirrhinus mrigala* (1.60±0.10 g), *Hypophthalmichthys molitrix* (200 g), *Ctenopharyngodon idella* (4.3±0.5 g) 96 h LC_{50} of copper sulphate were 3.15, 1.26, 0.98±1.98 and 1.717 respectively[21-24], which differ with the present fish species. It indicates that toxicity varies with species, ages of fishes used, their susceptibility rates and physicochemical characteristics of the test medium[25].

Expression levels of β-actin, EF1α, and GAPDH during challenge test

Transcription profiles (mean Ct) of the three HKGs for each time point of the challenge test from replicate samples are given in Table 1 and Fig. 1A-C. The range of Ct values during challenge test for β-actin, EF1α and GAPDH were 16.79-24.63, 17.04-24.71 and 21.18-28.64, respectively. The lowest mean Ct value was observed in β-actin (16.79) and the highest in GAPDH (28.64). The expression of β-actin, EF1α and GAPDH were highest at 96 h and lowest at 6 h post challenge.

Table 1 — Transcription profiles of the house keeping genes

Sample	β-actin		EF1α		GAPDH	
	Mean Ct	SD	Mean Ct	SD	Mean Ct	SD
0 h	23.77	0.08	23.51	0.04	27.09	0.10
6 h	24.63	0.13	24.71	0.16	28.64	0.18
24 h	23.77	0.16	23.30	0.04	28.26	0.07
48 h	20.37	0.12	20.60	0.23	25.42	0.13
96 h	16.79	0.19	17.04	0.09	21.18	0.09

Table 2 — Summary statistics of the house keeping gene for measuring stability

Gene	Overall Mean Ct[a]	SD[b]	CV %	Slope[d]	Intercept[e]	Stability index[f]
β-actin	21.86	3.27	14.97	−1.82	27.33	27.26
EF1α	21.83	3.07	14.06	−1.70	26.95	23.92
GAPDH	26.12	3.03	11.60	−1.50	30.63	17.41

Overall mean Ct value ranged from 21.83-26.12 across the challenge test (Table 2). During the challenge test highest overall mean Ct values (26.12) was observed for GAPDH than those of other genes, indicating a relatively low level of GAPDH transcription. However, genes encoding EF1α display relatively low overall mean Ct values (21.83) and highest expression.

Variation and transcriptional stability of β-actin, EF1α and GAPDH during challenge test

Biomarker genes expression is being used as an early diagnostic tool to measure the biological effect of pollutants and environmental quality assessment[26,27]. The expression pattern of these biomarker genes would give insight into the effect of pollutants on biological systems. To normalize biomarker genes expressions in qRT-PCR, HKGs have been used which minimizes experimentally induced non-biological variation from the true biological variation. Previous studies have shown that the expression of commonly used reference genes can be altered by toxicological processes[28,29]. However, most of the HKGs are often adopted from the literature and used without proper validation across a variety of experimental conditions assuming its constant level of expression which could result in erroneous conclusions about real biological effects[30]. To select an HKG as a control to normalize the

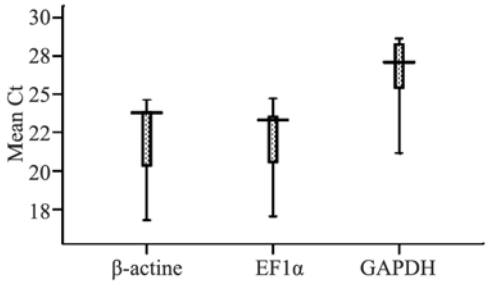

Fig. 2—Box plots representing variation in expression of candidate housekeeping genes. [Each box represents median Ct value, the 25 and 75 percentile, while the whiskers represent the maximum and minimum values from each dataset]

biomarker gene expression it must have the stable expression with in response to heavy metals. For Catla, few HKGs including β-actin, EF1α, GAPDH have been reported and β-actin has been widely used as internal control[15]. To evaluate reference gene stability from a limited number of HKGs, CV[31] along with box plot, and stability index could be used to get a clear picture[15]. In the present study, box Plot (Fig. 2) provided initial measure of variation of candidate HKGs during challenge test. Each box represents median Ct value, the 25 and 75 percentile while the whiskers represent the range from each data set, demonstrating clear differences between the genes. The median Ct values for β-actin, EF1α and GAPDH were 23.77, 23.30 and 27.09 respectively. The 25 and 75 percentiles were 20.37, 20.60, 25.42 and 23.77, 23.51, 28.26 respectively.

CV (ratio of the SD to the mean) is the normalized measure of dispersion of a probability distribution and it also provides a measure of variation and stability. If the CV of the Ct value is low it means that the gene expression is more stable and has less variability[31]. Further, stability index (product of overall CV and slope of linear regression) following linear regression analysis[32] gives a more clear description of stability of gene expression (Fig. 1 A-C). The slope of linear regression indicates the direction and degree of change in transcript abundance over time. A slope of zero or near zero indicates stable expression over time, while a low overall CV indicates minimal variation throughout the time course. Therefore, lower the stability index refers more stable or suitable gene, as a reference gene[33]. In the present study, minimal CV (11.60) and stability index (17.41) was observed for GAPDH and the maximum value of CV (14.97) and stability index (27.26) was for β-actin indicating the minimum variation of GAPDH and maximum variation of β-actin (Table 2). So, GAPDH is the most stable during challenge test followed by EF1α and β-actin genes, however, β-actin was chosen as HKG for Rainbow trout (*Oncorhynchus mykiss*) and Gibel carp (*Carassius auratus gibelio*) but EF1α for Common carp (*Cyprinus carpio*) and Zebrafish (*Danio rerio*), following copper exposure since those genes were not differentially expressed during the entire experiment[34,35]. The difference in these observations may be the result of certain physiological differences between different animals or due to different gene expression patterns in different fish species. However, no single reference gene can be used for different species and experimental conditions and optimal HKG can only be confirmed based on particular cases[36].

From the results obtained, it can be concluded that the copper sulphate is quite toxic to *Catla catla* larvae (96 h LC_{50}: 1.032 ppm). The present investigation provides useful information that can be exploited to formulate the safety levels of copper in water bodies to control algae and pathogens. Further, we validated the use of HKG as an internal control for normalizing the qRT-PCR data during an acute test of copper and found that GAPDH has more stable expression than other two genes.

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