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Toxicity of Heavy Metals on Fish(Barbus Luteus) at Euphrates river: Metallothionein Expression as a Biomarker Study on Liver and Gills

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Abstract:

This study was conducted to estimation of water pollution with heavy metals by using biomarker (metalothionein gene expression) ,Metallothionein (MT) is a low molecular weight protein that binds to heavy metals in aquatic organisms and is considered as a biomarker of heavy metal pollution. By RT- PCR assay for the first time in Al-Muthanna province – Iraq, during the period from October-2013 up to March–2014. this study showed increase in metallothionein expression in fish livers which was detected in two stations compared with control and monitoring the fish response to heavy metal pollution.

Key word: Molecular, metallothionein , Heavy metal .

1- Introduction:

Pollution of the aquatic environment with heavy metals has become a worldwide problem during recent years, because they are indestructible and most of them have toxic effects on organisms[1]. Among environmental pollutants, metals are of particular concern, due to their potential toxic effect and ability to bio accumulate in aquaticecosystems[2] in recent years there have been increasing interests regarding heavy metal contaminations in the environments, apparently due to their toxicity and perceived persistency within the aquatic systems[3].Pollutants act by changing the structural or biological functions of biomolecules[4]. Biomarkers for water pollution are early diagnostic tools for biological effect measurement and environmental quality assessment[5].
According to(Connell ,1984)[6] metals are defined as the metallic chemical element that has a relatively high density and are toxic or poisonous at a low concentration. In general, the hazardous effects of these toxic elements depends upon the dietary concentration of element, absorption of the element by the body, homeostatic control of the body for the element and also the species of the animal involved[7] .

One apparently universal method by which eukaryotes cope with heavy metals is by synthesizing a family of proteins known as metallothioneins (MTs)[8].These low molecular weight, thiol-rich proteins are induced at the transcriptional level in response to heavy metals as well as a variety of other agents and environmental stresses[9,10]. It is widely believed that MTs function by binding totoxic metals such as Cd and Hg thus detoxifying thesystem in question[8].

2-Material and methods:

sixty fishes Samples Barbusluteuswere collected in (spring and summer).tow stations were chosen on Euphrates River in Samawa city for this purpose. Station 1 (Al-sibil) and station 2 (Al- atshan). Fresh fish, Barbusluteuswere collected from (1 and 2) stations. In order to minimize the difference in metal accumulation caused by the age of individuals, the body weights were between(80 to130) gm and length were between(9.8 to 23) cm. during the period from October -2013 up to March–2014.These samples were used ton diabetic and measure heavy metal and to genetic study in Al-Muthanna province-Iraq.

2-1- Fish sample:

the current work was done in tow sampling area (contaminated) and one (non- contaminated) as control station, this take from Euphrates river, Fish specimens of each station dissected for gills ,liver and muscle.

2-2-Quantitative Reverse Transcription Real-Time PCR:

Quantitative Reveres Transcription Real-Time PCR technique was performed for estimation of relative quantification (gene expression analysis of metalothionein gene that normalized by 18S rRNA gene). This technique was done according to method described by (Ahmed, *et al*., 2013) as follow:

A- Total RNA extraction:

Total RNA were extracted from fish liver tissue by using (TRIzol® reagent kit) and done according to company instructions

B- Estimation RNA yield and quality:

The extracted total RNA was assessed and measurement by Nanodrop spectrophotometer (THERMO. USA), There are two quality controls were performed on extracted RNA. First one is to determine the quantity of RNA (ng/µL), the second is the purity of RNA by reading the absorbance in spectrophotometer at 260 nm and 280 nm in same Nanodrop machine.

C- DNase I Treatment:

The extracted RNA were treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using samples (DNase I enzyme kit) and done according to method described by promega company, USA instructions as follow in table (3-5):

Table (3-5) DNase I enzyme kit

Mix	Volume
Total RNA 100ng/µl	**10 µl**
DNase I enzyme	**1 µl**
10X buffer	**4 µl**
DEPC water	**5 µl**
Total	**20 µl**

After that, The mixture was incubated at 37C° for 30 minutes. Then, 1µl stop reaction was added and incubated at 65C° for 10 minutes for inactivation of DNase enzyme action.

D- cDNA synthesis:

DNase-I treatment total RNA samples were used in cDNA synthesis step by using AccuPower® RocktScript RT PreMix kit that provided from Bioneer company, Korea and done according to company instructions as following table (3-6):

Table (3-6) RT –PCR master mix

RT master mix	Volume
Total RNA 100ng/ul	10 µl
Random Hexamer primer	1 µl
DEPC water	9 µl
Total	20 µl

This RT PreMix was placed in AccuPowerRocketScript RT PreMix tubes that contains lyophilized Reverse transcription enzyme. Then dissolved completely by vortex and briefly spinning down.

The RNA converted into cDNA in PCR thermocycler under the following thermocycler conditions in table (3-7):

Table (3-7) RT- PCR thermocycler to converted RNA in to cDNA

Step	Temperature	Time
cDNA synthesis (RT step)	50 °C	1 hour
Heat inactivation	95 °C	5 minutes

3.2.4.1.5.Quantitative Real-Time PCR (qPCR) Master Mix Preparation:

qPCR master mix was prepared by using AccuPower^{TM} Green Star Real-Time PCR kit that dependantsyber green dye detection of gene amplification in Real-Time PCR system and include the follow:

After that, these qPCR master mix component that mentioned above Accopwer Green star qPCR premix standard plate tubes that contain the syber green dye and other PCR amplification components, then the plate mixed by Exispin vortex centrifuge for 3 minutes, then placed in Miniopticon Real-Time PCR system.

Table (3-8) qpcr master mix

qPCR master mix	Volume
cDNA template (100ng)	3µL
Forward primer(10pmol)	1 µL
Reverse primer (10pmol)	1 µL
DEPC water	15 µL
Total	20 µL

After that, these qPCR master mix component that mentioned above Accopwer Green star qPCR premix standard plate tubes that contain the syber green dye and other PCR amplification components, then the plate mixed by Exispin vortex centrifuge for 3 minutes, then placed in Real-Time PCR system.

After that, the qPCR plate was loaded and the following thermocycler protocol in the following table(3-9):

Table (3-9) qPCRthermocycler protocol

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	**95 °C**	**5min**	**1**
Denaturation	**95 °C**	**20 sec**	**45**
Annealing\Extention Detection(scan)	**55 °C**	**30 sec**	
Melting	**60-95°C**	**0.5 sec**	**1**

3.2.4.1.6. Data analysis of qRT-PCR :

The data results of q RT-PCR for target and housekeeping gene were analyzed by the relative quantification gene expression levels (fold change) Livak method that described by[11]. The relative quantification method, quantities obtained from q RT-PCR experiment must be normalized in such a way that the data become biologically meaningful. In this method, one of the experimental samples is the calibrator such as (Control samples) each of the normalized target values (CT values) is divided by the calibrator normalized target value to generate the relative expression levels. After that, the ΔCT Method with a Reference Gene was used as following equations:

Gene	Test (Treatment group)	Cal. (Control group)
Target gene	**CT (target, test)**	**CT (target, cal)**
Reference gene	**CT (ref, test)**	**CT (ref, cal)**

First, normalize the CT of the reference (ref) gene to that of the target gene, for calibrator sample:

ΔCT (calibrator) = CT (ref, calibrator)– CT (target, calibrator)

Second, normalize the CT of the reference (ref) gene to that of the target gene, for the test sample:

ΔCT (Test)=CT (ref, test)–CT (target, test)

ΔΔCT= ΔCT (test)–ΔCT (calibrator)

Fold change = 2-ΔΔCT

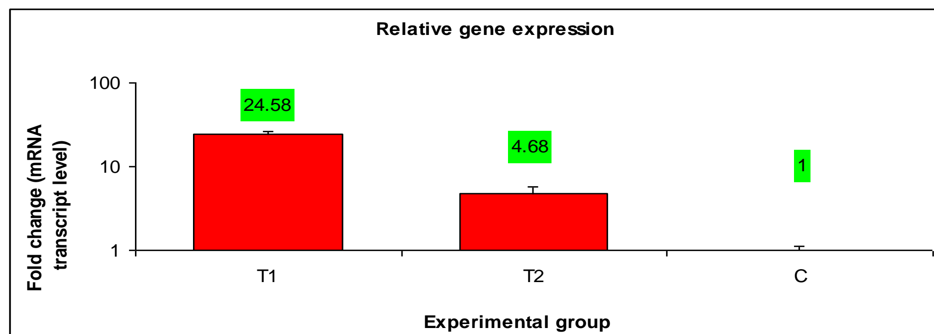
Ratio (reference/target) =2CT (reference) CT (target)

So, the relative expression was divided by the expression value of a chosen calibrator for each expression ratio of test sample.

4. Results

Metalothionine gene expression:

Using real time quantitative PCR assay, to measure gene expression of metalothionein in livers of *Barbusluteus* that collected from two station (AL-Sibil and AL-Atshan),where were taken 18 alive fish(6 fishes from each station and 6 control). The expression data was related to the concentration of some heavy metals in water and fish tissue; our result show in station 1 (Al-Sibil) is high concentration of some heavy metal, and appeared in this station high expression of the MT gene is 24.58 , while Station 2 (Al-Atshan) showed concentrations of heavy metals this was also related to metalothionein expression and resulted of gene expression about 4.68 and this result comparatively with gene expression to control which it was 1.0.



T1:*Barbus luteus* from Sibil station.
T2:*Barbus luteus* from Atshan station.
C:*Cyprinus carpio*as control

Figure (1): Histogram of relative gene expression of metallothionein gene in treatment group relative to normalization control group.

Table (4-6) : The relative gene expression analysis of metallothionein gene by $2^{-\Delta\Delta CT}$ Livak method.

Exp. Group	CT (meta)	CT (18S rRNA)	ΔCT (Test)	ΔCT (control)	ΔΔCT	Fold change (2^-ΔΔCT)	Mean
T1	26.54	32.14	-5.60	-1.27	-4.33	20.11	24.58
T1	26.54	32.36	-5.82	-1.27	-4.55	23.38	
T1	26.14	32.42	-6.28	-1.27	-5.01	32.18	
T1	26.54	32.28	-5.74	-1.27	-4.47	22.16	
T1	26.44	32.45	-6.01	-1.27	-4.74	26.79	
T1	26.54	32.33	-5.79	-1.27	-4.52	22.88	
T2	29.29	32.27	-2.98	-1.27	-1.71	3.28	4.68
T2	29.20	32.12	-2.92	-1.27	-1.65	3.15	
T2	28.17	32.34	-4.17	-1.27	-2.90	7.48	
T2	27.71	32.13	-4.42	-1.27	-3.15	8.86	
T2	30.11	32.32	-2.21	-1.27	-0.94	1.92	
T2	29.24	32.28	-3.04	-1.27	-1.77	3.41	
Mean C	31.19	32.46	-1.27	-1.27	0.00	1.00	1

T1:*Barbus luteus* from Sibil station.
T2:*Barbus luteus* from Atshan station.
C:*Cyprinus carpio*as control

($2^{-\Delta\Delta CT}$ Livak method) as following: First, the CT of the target gene was normalized to that of the reference (ref) 16SrRNAgene, for both the test isolates and the control isolates group.

$\Delta CT(test)=CT(target, test)–CT(ref, test)$

$\Delta CT(control)=CT (target, control)–CT (ref, control)$

Second, the ΔCT of the test isolates were normalized to the ΔCT of the control:

$\Delta\Delta CT=\Delta CT(test)–\Delta CT(calibrator)$

Finally, Fold change of relative gene expression was calculated by following equation =($2^{-\Delta\Delta CT}$): Normalized expression ratio.

5-Discussion:

5.1- Heavy metals in fish:

Local fresh water fish are economically important. Sibil and atshan area is characterized as an important area for fishing and sale of large and small fish. Poor families depend on consuming small fish for low price. Therefore, the study tended to determine the heavy metals concentrationsin economically important fish in the same area.

Many aquatic organism such as fish had high capability for accumulation heavy metals inside their bodies in concentration more highest than found in aquatic system around it[12], these heavy metals inters fish throw skin or gills or food[13].

The heavy metals is heavier than water five times in molecular weight,so its metabolite little inside the organs especially the secretory organs[14] notice the quantum absorbency of this heavy metal depend in direct form on its concentration in water and exposure time to it, the absorption increase with increase all of this.(CET, 1993)[15] (Philips, 1980)[16] shows that we can use this accumulation as biological index on water contamination. The cadmium is not necessary metal and very toxic, the fish as one of the biological organisim sensitively to cadmium. It was found that exposure of fish under leathal concentration of Cd lead to decrease in hemoglobin amount and RBCs and WBCs count and increase blood clotting in fish when exposure to under lethal concentration[17],Pb is one of sever toxic metal to human and other biological organisms [18] its toxicity cause inhibit in work of many enzymes and cause damage in blood system and central nervious system and reproductive function in fish and other different organisms [19].

Zn is the basic metal in various biological reaction it had different effect of in aquatic organism especially fish, turnip attention the researcher found that 0.3 part per million of zn in water become toxic to fish[20].The liver accumulate higher amount of heavy metals in than other organs because a high capability in accumulation of heavy metals inside its tissue to special section in blood cycle system and this led it to receive most metals which absorb and transport from blood [21],the liver can excretion heavy metals from body during formation complex and disposed it [22], the role of liver in protein production,and its importance in attaching the metals with it to transport them to places for excretion out the body [23].

The amount of heavy metals in muscle which lower from liver and this because deficiency of lipid amount which found was in muscle or because food type or metal concentration in the ecology, whenever the concentration of these metals in edible part it was lower dangerousness

on human health and the amount of this metal in dietary material ofconsumer level of metalothionein gene in fish has been used as a biomarker for water pollution with heavy metals[28]. To identify relationships between physiological stressors like heavy metal pollution and MT mRNA expression, characterization of a model system using a sentinel species which must be performed with an evaluation of specific inducers. For utilization in potential environmental monitoring projects, RT-PCR has been shown to provide the ability to sensitively and accurately quantitative levels of MT mRNA expression in target tissues of the fish and various other aquatic species. However, heavy metals in the contaminated water attach to the waterorganisms mainly to the MT protein, which makes the up-regulation of MT gene to be a biological indicator for heavy metal pollution in water [28]. George *et al.*, 1996 in his study observed a significant increase in gill MT mRNA expression in killifish exposed to Cd compared with controls, this result supports our study with *Barbusluteus* that have demonstrated an obvious increase MT mRNA expression in heavy metal polluted areas.

In several species of fish, MT levels have been demonstrated to increase in a dose-responsive and/or time responsive manner after intra-peritoneal administration of heavy metals.

depend on concentration of this metal in dietary material[24] and this in agreement with study [25] who found that the concentrations of metals in muscle is lower from its concentration in other body part, this because deferent in patterns of metal accumulate in different organs which have different ability in accumulation these metal in tissue by different biological function , a study on Zn shows it was more accumulate from other metals , and this indicate the importance of this metal in living aquatic organism , it was necessary metal to perpetuate the cell which inside in structure of enzyme and this result was agreement with[26] showed the higher concentration of Zn in liver more from other organs which study, whereas the Cd have the lower concentration in liver and muscle, this indicate the efficient of fish in excretion this toxic metal from it tissue.

The accumulation and toxicity of heavy metal depend on ecology ,this explain the difference in value concentrations of study metal in this type of organs in this study.

5.2- Metallothionein gene expression:

Metalothionein is a small protein of 60 amino acid residues in fish , as in other vertebrates. Resistance to heavy metal toxicity in fish is related to their ability to over express MT genes after exposure to metal ions[27]. The change in gene expression is used as biomarker for exposure to pollutants. The expression

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