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Research Article

Bioinformatics and mRNA expression of catalase gene and determination of catalase enzyme activity in zebrafish (*Danio rerio*) exposed to the herbicide paraquat

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Abstract

Effects of two different doses, treatment I (0.30 ppm) and treatment II (0.15 ppm) of paraquat, were investigated on mRNA transcript of the *catalase* gene (*cat*) and CAT activity in zebrafish. We determined tissue-specific distribution of zebrafish *cat* and bioinformatics analysis. Muscle, liver, intestine, heart, kidney, brain, eye, gills, swim bladder, skin, ovary and testis tissue samples from three female and three male fish were dissected to detect tissue-spesific distrubition while liver and gill tissues were dissected to evaluate the effect of acute stress in zebrafish. The steady-state level of zebrafish *cat* transcript in gill (311±11.32) was lower than liver (595.53±14.41) but higher than muscle (132.89±9.21), testis (120.03±6.99), kidney (118.96±8.87), ovary (104.31±8.22), intestine (37.28±3.11), brain (19.17±2.01), eye (18.30±2.10), heart (15.79±2.01), spleen (7.7±0.91), swim bladder (6.97±0.21), and skin (4.59±0.31). Expression of *cat* was significantly decreased in liver and gill tissues after exposure to two concentrations of paraquat (p<0.05). Measurement of enzyme activities in zebrafish, exposed to 0.30 and 0.15 ppm of paraquat for 72 h duration, showed significant (p<0.05) variations in CAT activity compared to that of the control. Different steady-state levels among tissues in zebrafish could be explained by duplicated genes in teleost fish that arose through teleost specific-whole genome duplication exhibiting a dissimilar tissue distribution.

Keywords: Bioinformatics, Gene expression, Enzyme activity, Catalase, Zebrafish

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Introduction

Zebrafish (*Danio rerio*) is a member of Cyprinidae family of ray finfish, Actinopteri class. Zebrafish is one of the most studied models in scientific research (Carpio and Estrada, 2006). Because of these features, zebrafish has taken an important place in scientific studies as a research model (Ma, 2004; Lieschke and Currie, 2007).

Oxygen is an all-important compound for many organisms because it is used as a final electron acceptor in biological systems (Buechter, 1988). However, all cells create highly reactive oxygen derivatives (ROS), although they do not have any unpaired electrons with free radicals which have one or more unpaired electrons (Halliwell *et al*., 1992; Lindsay and Astley, 2002; Bartosz, 2003). The ROS created in living systems can lead to changes in normal cells and tissue functions by oxidizing biologically useful molecules. This oxidation is minimized by a highly complex antioxidant defense system (ADS). Antioxidants, produced in the body or imported from outside, act as a protective defense system against oxidative stress caused by reactors cleaning and preventing damages in the organism (Shinde *et al*., 2012). In addition to preventing damage they also perform detoxification (Şener and Yeğen, 2009). Also many antioxidants are known to regulate gene expression and alter antioxidant enzyme activity. Catalase (CAT) is an enzymatic antioxidant (Aydemir and Karadağ-Sarı, 2009) which like all other enzymes has a protein structure and is found in animals, plants and microorganisms (Sen *et al*., 2010). The most important task of this enzyme is to remove hydrogen peroxide from cells (Master and Holmes, 1977; Çimen *et al*., 2005). NADPH molecule is close to the surface and tightly connected in most catalase molecules (Zámocký and Koller, 1999). They are mostly found in peroxisomes, intracellular organelles, partially in mitochondria and in endoplasmic reticulum. The main physiological role of CAT enzyme in biological systems is catalyze, conversion of harmful hydrogen peroxide (H_2O_2) into water and molecular oxygen (Limón-Pacheco and Gonsebatt, 2009), and this transformation is carried out by superoxide dismutase (SOD) radical (Cheung *et al*., 1988). Although there is enough information about the structure of *cat* in mammals (Bryant and Wilson, 1995), bacteria (Storz *et al*., 1990) and plants (McClung, 1997), there is not enough information about bioinformatics and functioning of this gene in other vertebrates, including aquatic organisms as a model.

Pesticides that reach the aquatic ecosystem directly or indirectly through drift, spray, draining, runoff, and discharge from factories and sewage have negative health effects on aquatic organisms (Barlas, 1999; Katagi, 2010). Paraquat ($(C_6H_7N)_2]Cl_2$) 1,1-dimethyl-4,4-bipyridinium ion) is one of the most widely used herbicides which is used in controling both aquatic plants and terrestrial weeds and is known to be found in many freshwaters in the world (Filizadeh, 2002; Ye *et al*., 2002; Gao *et*

al., 2010; Ismail *et al*., 2011; Ayanda *et al*., 2015). In parallel with increasing use of herbicides in agricultural applications, the ecosystem is negatively affected and ecological imbalance occurs. Fishes, which can serve as bioindicators of the environmental condition of their habitat, are crucial indicators of the effects of toxic compounds in aquatic toxicology (Glusczak *et al*., 2011). They are highly preferred for toxicity tests because they respond to low pesticide concentrations too (Davies and Vethaak, 2012; Okechukwu *et al*., 2013).

It is believed that organisms' increasingly complex structure in the process of evolution is the result of duplication of genes and genomes (Venkatachalam *et al*., 2017). In recent years, some theoretical models, such as duplication-degeneration-complementation model, are used to extrapolate the fate of duplicated genes. According to this model, the common destiny of duplicated genes disappears from the genome due to its nonfunctionalization. The presence of duplicated genes in the genome is explained by subfunctionalization or neofunctionalization which are two processes that occur with the loss or gain of regulatory elements in their promoters (Venkatachalam *et al*., 2017). However, research in the ENSEMBL database showed loss of a copy of duplicated *cat* gene in teleost fishes, includes zebrafish, following the teleost specific whole genome duplication, except for pike (*Esox lucius*) and some cichlid fish, such as Zebra mbuna (*Maylandia zebra*), Fairy Cichlid (*Neolamprologus brichardi*), Eastern happy fish (*Astatotilapia calliptera*) which have *cat* duplicated.

Stress responses in fish can occur at multiple levels involving the actions of different gene groups and their products (Velki *et al*., 2017). For molecular studies, it is important to understand the genetic traits associated with the model organism exhibiting a stress response. Because fish have different stress tolerances, identification and characterization of stress-related genes can provide important genetic markers that can be used in aquaculture selection programs to help improve stress tolerance. In addition, these markers can serve as model for humans and other vertebrates (Iwama *et al*., 1999). It is known that oxidative stress causes many diseases in humans or negatively affects many diseases (Halliwell *et al*., 1992; Poljsak *et al*., 2013). For this aim, we characterized *cat* gene in zebrafish that serve as a model organism, and searched the effect of paraquat on *cat* in zebrafish to explain molecular effect of stress response in fish.

This study aimed to detect acute toxicity effects of paraquat in zebrafish, an aquatic model organism, by determining changes in catalase enzyme activity, one of the antioxidant enzymes. The second aim of the study was to complete bioinformatics studies of zebrafish *cat* and confirm the bioinformatics by determining tissue specific distribution of zebrafish tissue. Determining *cat* gene expression and catalase enzyme activity in liver and gill

tissues of zebrafish exposed to this chemical was another aim of this study. Thus, information are provided on acute toxicity of paraquat, which is used extensively in agriculture. This knowledge can be useful in predicting and impeding acute harm to fish in paraquat-contaminated waters.

Materials and methods

Teleost fishes exhibit strong evolutionary conservation for gene structure in the same gene family (Venkatachalam *et al*., 2017). The exon-intron organization of the zebrafish *cat* gene was detected by aligning the longest EST with the genomic sequence in this sudy. The rate of identity-similarity sequence between zebrafish and medaka, tetraodon, stickleback, mouse and human *cat/CAT* gene was evaluated. However, phylogeny analysis showed a high phylogenetic similarity between *cat* genes in zebrafish and its orthologs.

Husbandry and dissection of fish and exposure to paraquat

42 adult zebrafish of wild-type phenotype were used in the study. The fish (1.1±0.1 g weight and 3.3±1.0 cm in length) were obtained from Model Organism Unit of Department of Agricultural Biotechnology, Ataturk University. The fish were maintained in 10 L aerated aquaria for two weeks for acclimation before paraquat treatments (27±1°C and pH=6.6-6.9). The fish were separated into three experimental groups. Two groups of acclimatized fish were exposed to 0.30 and 0.15 ppm of paraquat for 72 h duration. The third group served as the control group which was not exposed to paraquat. All experiment groups were replicated three times. After 72 h of exposure 18 fish were sacrificed by a cephalic concussion for measuring CAT enzyme activity and 24 fish (3 female and 3 male fish used for bioinformatics analysis) were anaesthetized with clove oil 90 μL L^{-1} to evaluate gene expression. Experiments were carried out following the protocol of Atatürk University Animal Studies Local Ethics Committee. Liver and gill tissues were used to determine both CAT activity and gene expression to evaluate the effect of paraquat in zebrafish. Muscle, liver, intestine, heart, kidney, brain, eye, gills, swim bladder, skin, ovary and testis tissues from 3 male and 3 female fish taken from the control group were used to determine tissue specific distribution. Dissecting instruments and working area were sterilized and cleaned with RNase ZAP before dissection. Tissues used for gene expression were transferred into tubes containing RNA later kept in cool 4^{o}C overnight and stored at -80^{o}C freezer until work. Liver and gill tissues (1 g) used for enzyme assay were weighed and homogenized in 10 vol (w/v) 1.15% KCl for 5 min. To obtain supernatants for enzyme activity measurement, the homogenates were centrifuged at 1000 rpm for 30 min at +4^{o}C and a double-beam spectrophotometer (Thermo Evolution 100; Accelonix Ltd; www.accelonix.co.uk) was used for measurements (Parmar *et al*., 2013).

Catalase enzyme assays and lipid peroxidation levels

Beers and Sizer (1952) method was used to measure CAT (EC 1.11.1.16) enzyme activity. Phosphate buffer (0.05 M, pH 7.0), H_2O_2 (0.019 M), liver and gill tissue homogenate (10 µL) comprised the assay mixture. Absorbance changing in the tissues was recorded after reduction of H_2O_2 at 240 nm for 1 minute (Beers and Sizer, 1952). Uchiyama and Mihara (1978) method was used to determine lipid peroxidation level by assessment of malondialdehyde (MDA as a lipid peroxidation marker) as total thiobarbituric acid-reactive products. Liver and gill homogenates were mixed with thiobarbituric acid (0.6% w/v) and phosphoric acid (1% v/v) and the mixture was incubated in boiling water bath for 45 min. N-butanol was added to the tubes after cooling. The samples were centrifuged at 1756 rpm for 10 min to remove the precipitates. Finally, MDA concentration was determined spectrophotometrically at 532 nm using the supernatant. Catalase enzyme activity was defined as enzyme unit (EU)/mg protein, and MDA concentrations were defined as nmol/ mg protein.

Protein content of liver and gill samples

To determine protein content of the samples, bovine serum albumin (BSA) was used as standard and the method of Bradford (1976) was followed.

RNA isolation and reverse transcriptase (RT) and real-time PCR (qPCR) analysis

For total RNA extraction, all tissue samples were homogenized with trizol reagent (Life Technologies) after being removed from RNA later. RNA concentrations were measured by nanodrop 8000 spectrophotometer (Thermo Scientific, Multiskan Go, Japan) and quality of total RNA was determined by agarose gel electrophoresis. For cDNA synthesis (High capacity cDNA kit), 2 µg of RNA from each tissue were used. RNA samples firstly were treated with DNase (DNase I, Amplification Grade, Life Technologies) and converted to cDNA using High-Capacity cDNA Reverse Transcription Kit (Life Technologies). A Rotor-Gene 6000 thermal cycler system (Qiagen GmbH, Düsseldorf, Germany) and a QuantiTect SYBR Green PCR kit (Qiagen) were used for RT-qPCR analyses to determine the expression of *cat* gene and the reference genes (*beta actin (b-actin)* and *elongation factor1 alpha* (*ef1a f)*) (number of copies/µL) in the tissues of zebrafish. Quantitative PCR was run in the final volume of 20 µL (10 µL SYBR Green, 2 µL forward primer, 2 µL reverse primer, 5 µL DNAse/RNAse free water and 1 µL cDNA template) for each tissue. However, negative control for each qPCR reaction tube didn't include cDNA. RT-qPCR conditions were initial denaturation (95.0^{o}C for 15 min), 40 cycles-denaturation (95.0^{o}C for 20 s), primer annealing [optimum temperature for each primer for 30 s] and elongation (72.0^{o}C for 30 s). After the qPCR reaction, expression levels of the *cat* in all tissues were normalized to *b-actin*

and *ef1a* to evaluate tissue specific distribution. However, $2^{-\Delta\Delta Ct}$ method was used to determine expression levels of the *cat* gene in liver and gill tissues (Livingstone, 2001). Level of the gene expression was given consistently to the mean value of the control groups (Torstensen *et al*., 2009; Anderson and Elizur, 2012).

Primer optimisation

Forward and reverse primers for the target (*cat*) and reference genes (*actb* and *ef1α*) were designed according to exon-exon junction model using NCBI Primer-BLAST for real-time qPCR amplification of zebrafish (Table 1). Lyophilized primers were diluted in TE buffer (10mM Tris, 1mM EDTA and pH 8.0) to arrange 100 pmol/µl stock concentration of primers (Venkatachalam *et al*., 2017).

Table 1: Sequences of target and reference genes primers.

	Zebrafish *catalase*	**Zebrafish *ßeta actin***	**Zebrafish *Elongation factor 1-alpha***
Forward primer (5′ → 3)	CAGGGGCGTTTGGCTACTT	ATCAGGGAGTGATGGTTGGC	AGCTCAAACATGGGCTGGT
Reverse primer (5′ → 3′)	CTGCGAAACCACGAGGATCT	GGCTTTGGGATTCAGGGGAG	CACACGACCCACAGGTACAG
Temperature (°C)	60	60	60
Amplicon size (bp)	165	224	189
Ensembl ID	ENSDARG00000104702	ENSDARG00000099197	ENSDARG00000020850

Identification of zebrafish cat gene

Exon-intron organization of the zebrafish *cat* gene was detected by aligning the longest EST with the genomic sequence. Rate of identity-similarity sequence between zebrafish and medaka, tetraodon, stickleback, mouse and human *cat/CAT* gene was designed using BİOEDİT Program (Table 2). For this reason, amino acid sequences of the Cat in these organisms were collected from NCBI database. Teleost fishes exhibit strong evolutionary conservation for gene structure in the same gene family. ENSEMBL ID and NCBI ID of zebrafish *cat* gene were determined as ENSDARG00000104702 and NM_130912, respectively.

Phylogenetic analysis

CLUSTALW at BioEdit software (http://www.mbio.ncsu.edu/bioedit/page2.html) was used for sequence alignment of *cat* gene in zebrafish (*D. rerio*, Thompson *et al*., 1994). Protein sequence of zebrafish Cat was aligned with Cat/CAT protein sequences from horned golden-line barbel, common carp German mirror, common carp hebao red, Mexican tetra, Atlantic salmon, chinook salmon, brown trout, eastern happy, platyfish, Amazon molly, chicken, human, mouse. Rat LCP1 gene was used as distinct group for phylogenetic tree (Fig. 4). A phylogenetic tree with Poisson correction distance model based on amino acid substitution per site was built according to maximum-likelihood

method (Tamura *et al*., 2013) to detect the phylogenetic relationship of these organisms.

Table 2: Nucleotide sequence of zebrafish *cat.*

5′cccacatctatgaaccaaaccattgataaatgttgcctcaagttgttacaaggatttc
attctttttcgtcttgtctgcttccctttaaaatgcgtttgtgagatgttttaagtctaa
atattaagttttaaatccactgaactaacaaatacaaagaaaggggacttaaaaatgaaa
atatcaatcattttacttgtttctgtttatattctgtaactgggaaagatgtcatgtaca
cattgagtgcataattaactagatatcacaacatttttTAATAtgtgaactatccctttа
+1
aaatgacaGAGTGTATGTATATCCATCGAGCTTGGCCAATAAGAAACACCTGTTTGGTCA
CATAACCCCGCCCACTTAAGTGAAACGTTCACGTCCCTTCACTGTGAATATTTAGGTGGA
AACAATCCTCCAGACAGAAAGACCCCGAGTCCAGTGGTTTGGATCCGCAGTAGTTTTGCC
TCGTGTTTTGTCACACTGAAGAGCTGAATA**ATGGCAGACGACAGAGAAAAGTCGACGGAT**
-M--A--D--D--R--E--K--S--T--D-
CAGATGAAACTGTGGAAGGAGGGTCGCGGTTCCCAGgtctg”N1172”ctcag**CGTCCGG**
-Q--M--K--L--W--K--E--G--R--G--S--Q- -R--P--
ATGTCCTGACCACTGGAGCTGGAGTCCCGATAGGGGACAAGTTAAATGCGATGACGGCAG
D--V--L--T--T--G--A--G--V--P--I--G--D--K--L--N--A--M--T--A--
GGCCTCGTGGGCCATTGCTGGTCCAGGATGTGGTGTTTACTGATGAAATGGCCCATTTTG
G--P--R--G--P--L--L--V--Q--D--V--V--F--T--D--E--M--A--H--F--
ACCGAGAGCGGATACCAGAGAGAGTCGTGCATGCTAAAGGAGCAGgtgaa”N723”cacag
D--R--E--R--I--P--E--R--V--V--H--A--K--G--A--
GGGCGTTTGGCTACTTCGAAGTCACTCACGACATCACGCGCTACTCCAAAGCCAAAGTGT
G--A--F--G--Y--F--E--V--T--H--D--I--T--R--Y--S--K--A--K--V--
TTGAGCATATTGGAAAGACGACACCGATCGCTGTCCGCTTTTCCACAGTGGgtatg”N768”
F--E--H--I--G--K--T--T--P--I--A--V--R--F--S--T--V--
tatag**CGGGTGAGGCTGGGTCATCAGATACTGTCCGAGATCCTCGTGGTTTCGCAGTGAA**
A--G--E--A--G--S--S--D--T--V--R--D--P--R--G--F--A--V--K
GTTCTACACCGATGAGGGCAACTGGGATCTTACAGGAAACAACACCCCCATCTTCTTTAT
--F--Y--T--D--E--G--N--W--D--L--T--G--N--N--T--P--I--F--F--I
CAGGGATACGCTTCTGgtaac”N619”tgtag**TTTCCGTCTTTCATCCACTCTCAGAAGC**
--R--D--T--L--L- -F--P--S--F--I--H--S--Q--K--
GCAATCCGCAGACTCACCTGAAGGATCCGGACATGGTTTGGGATTTTTGGAGCTTGCGTC
R--N--P--Q--T--H--L--K--D--P--D--M--V--W--D--F--W--S--L--R--
CTGAATCGCTGCACCAGgtaag”N77”tacag**GTGTCTTTCCTGTTCAGTGATCGAGGGA**
P--E--S--L--H--Q- -V--S--F--L--F--S--D--R--G--
TTCCCGATGGCTACCGTCATATGAACGGATACGGATCGCACACCTTTAAACTGGTCAATG
I--P--D--G--Y--R--H--M--N--G--Y--G--S--H--T--F--K--L--V--N--
CTCAGGGCCAGCCGGTGTACTGCAAGTTCCACTACAAGgtacg”N382”tgcag**ACTAAT**
A--Q--G--Q--P--V--Y--C--K--F--H--Y--K- -T--N-
CAGGGCATTAAGAATATTCCTGTTGAAGAAGCGGATCGTCTGGCTGCCACTGATCCGGAT
-Q--G--I--K--N--I--P--V--E--E--A--D--R--L--A--A--T--D--P--D-
TACTCTATCAGAGACCTTTACAACGCTATCGCCAATGGCAACTTCCCATCCTGGACCTTC
-Y--S--I--R--D--L--Y--N--A--I--A--N--G--N--F--P--S--W--T--F-
TACATCCAGGTTATGACCTTTGAGCAGGCTGAAAACTGGAAGTGGAATCCATTTGATTTG
-Y--I--Q--V--M--T--F--E--Q--A--E--N--W--K--W--N--P--F--D--L-
ACCAAGgtagc”N536”ctcag**GTCTGGTCCCATAAAGAGTTCCCTCTG**ATTCCTGTGGG
-T--K- -V--W--S--H--K--E--F--P--L--I--P--V--G
GCGTTTTGTGTTGAACCGAAACCCCGTTAACTATTTCGCGGAGGTTGAGCAGCTGGCGTT
--R--F--V--L--N--R--N--P--V--N--Y--F--A--E--V--E--Q--L--A--F
TGATCCCAGTAACATGCCACCCGGCATTGAGCCCAGCCCAGACAAGATGCTGCAGgtacg
--D--P--S--N--M--P--P--G--I--E--P--S--P--D--K--M--L--Q-
”N210”tgcag**GGGCGTCTTTTCTCCTACCCAGACACACATCGCCATCGACTCGGAGCTA**

Table 2 (continued):

```
-G--R--L--F--S--Y--P--D--T--H--R--H--R--L--G--A--
TTACACTCCAACTACCAGTCAACTGCCCGTACCGCACCCGTGTGGCAAACTATCAGAGAG
N--Y--L--Q--L--P--V--N--C--P--Y--R--T--R--V--A--N--Y--Q--R--
ATGGACCCATGTGCATGCATGACAACCAGGgtgag"N1507"tgtagGTGGAGCTCCAAA
D--G--P--M--C--M--H--D--N--Q--                 G--G--A--P--N
CTACTACCCCAACAGCTTCAGTGCTCCTGACGTCCAGCCACGCTTCCTTGAGTCAAAGTG
--Y--Y--P--N--S--F--S--A--P--D--V--Q--P--R--F--L--E--S--K--C
TAAAGTGTCTCCTGATGTGGCCCGATACAACAGCGCAGACGATGACAACGTGACCCAAgt
--K--V--S--P--D--V--A--R--Y--N--S--A--D--D--D--N--V--T--Q-
gtg"N73"tgcagGTGCGCACGTTCTTCACTCAGGTGCTAAATGAAGCCGAGAGAGAGCG
-V--R--T--F--F--T--Q--V--L--N--E--A--E--R--E--R
TCTGTGCCAGAATATGGCCGGGCATCTGAAAGGAGCTCAACTCTTCATCCAGAAACGCAT
--L--C--Q--N--M--A--G--H--L--K--G--A--Q--L--F--I--Q--K--R--M
Ggtatg"N106"tacagGTGCAAAACCTGATGGCTGTTCACTCTGATTATGGCAACCGAG
-                -V--Q--N--L--M--A--V--H--S--D--Y--G--N--R--
TTCAGGCTCTCCTGGATAAACACAACGCTGAAGGGAAAAAGgtaga"N1074"tccagAA
V--Q--A--L--L--D--K--H--N--A--E--G--K--K-                -N
CACTGTTCATGTTTATTCACGTGGTGGAGCGTCTGCTGTGGCTGCAGCTTCTAAGATGTG
--T--V--H--V--Y--S--R--G--G--A--S--A--V--A--A--A--S--K--M--*
Agacgtcccactacacacacacacacacacacacacagattcattctcttatagagtg
-
tgatatagtg ttataatgcctaattcattccatatatggtttagaaatgcagacatatc
taatatggccaattgacaacacaagtgatcttagcaaatgcaacactgactccttaatct
tgatttatgccttcaagcaaaatgggggcctttgcatacaaagatttgagctaatccaaa
ctaaatcagcctatttcagtatctttctgcattttgatttctgtgaaatgtagaatagcc
aataataatctgagaatcaaagcaactgtttagtttaatgctgtatgacacttgattttt
tttctccacaatttctaaaaaccttggctacttatgcagtttttaagctgaattaagatg
gaatgaatgtttgccgtcctttctgccttgactgaatataattcggttctcataatatga
atgtaactcgttttgtcattttgtaattAATAAAtgataatatttaatctcagtgac  3'
```

Conserved gene synteny

Conserved gene synteny was arranged manually using the region conceptus selection of Ensembl database to recognize co-localized genes for creating conserved gene synteny of zebrafish *cat* gene with the *cat/CAT* genes of medaka and human (Fig. 3).

Statistical data analysis

SPSS version 10.0 for Windows (SPSS, www.spss.com) was used for the statistical analyses. Data were given as mean±standard deviation and analysed by one-way Analysis of Variance (ANOVA). significant differences (p<0.05) were determined with Duncan's multiple range post hoc test (IBM Corp., 2011).

Results

Effect of paraquat on catalase activity and MDA level

CAT levels were increased in liver and gill of paraquat two concentrations-exposed zebrafish (Table 3). Differences among control group and treatment groups were statistically significant (p<0.05). However, two concentrations of exposure of paraquat caused no significant change in MDA levels in liver and gill (Table 4).

Molecular studies

Tissue specific distribution of zebrafish *cat* gene and change of liver and gill mRNA expression of zebrafish exposed to paraquat was determined by RT-qPCR.

Table 3: Identity and similarity rate of *cat* in zebrafish (Zf) and medaka (Me), mouse (Mo), stickleback (St), human (Hu) and tetraodon (Te).

```
Zf Cat   1   MADDREKSTDQMKLWKEGRGSQRPDVLTTGAGVPIGDKLNAMTAGPRGPLLVQDVVFTDE
Me Cat   1   ..EN.D.T.....T...N...RK.........H.......LQ..................
Mo Cat   1   .S.S.DPAS....Q...Q.A..........G.N.......I....S..............
St Cat   1   ...S.D.A.....T...N.AR....T......H.......LQ...L..............
Hu Cat   1   ...S.DPAS...QH...Q.AA.KA.........N.V.....VI.V................
Te Cat   1   ...K...T.........S..K.Q.......G.H.......LQ....K.............

Zf Cat  61   MAHFDRERIPERVVHAKGAGAFGYFEVTHDITRYSKAKVFEHIGKTTPIAVRFSTVAGEA
Me Cat  61   .................................C.........................S
Mo Cat  61   ............................................R..........T..S
St Cat  61   ..................DAGAFGYFEVTHDITRFCKAKVFEHVGK.TPIAVRFSTVAGE
Hu Cat  61   ................................K............K.............S
Te Cat  61   ................................C.......V................S

Zf Cat  121  GSSDTVRDPRGFAVKFYTDEGNWDLTGNNTPIFFIRDTLLFPSFIHSQKRNPQTHLKDPD
Me Cat  121  ..A.............................V.....A......V.T........M....
Mo Cat  121  ..A.............ED.....V............AI.....................
St Cat  121  SG.ADTVRDPRGFAVKFYS.EGNWDLTG.NTPI.FIRDSMLFPSFVHSQKRNPQTHMKDP
Hu Cat  121  ..A.............ED.....V............PI.....................
Te Cat  121  .KKE.YVNVGRYNICGRPNSVVFISSQ.VYFFVLHVFERSSIDCLQVSFLFSDRG.P.GH

Zf Cat  181  MVWDFWSLRPESLHQVSFLFSDRGIPDGYRHMNGYGSHTFKLVNAQGQPVYCKFHYKTNQ
Me Cat  181  ..........C............L.....................D.DR.........D.
Mo Cat  181  ............................H.................D.EA.........D.
St Cat  181  DMVWDFWSLRPESLHQVSFLFSDRGLPDGFRHMNGYGSHTFKLVNADGEPLYCKFHFKTD
Hu Cat  181  ............................H.................N.EA.........D.
Te Cat  181  RHMNGYGSHTFK.VNAKGECVYCKFHYKTDQGIKNL.VEEAERL.STD.D.AIGDLFNAI

Zf Cat  241  GIKNIPVEEADRLAATDPDYSIRDLYNAIANGNFPSWTFYIQVMTFEQAENWKWNPFDLT
Me Cat  241  ....LS....EH..S.N...A.G..F.......Y................KFQF......
Mo Cat  241  ....L..G..G...QE....GL...F.......Y............KE..TFPF......
St Cat  241  QGIKNLSV.EADRL.STNPDYAIGDLFNAIANGNFPSWTFFIQVMTFEQAERVGVHPLSS
Hu Cat  241  ....LS..D.A..SQE....G....F....T.KY............N...TFPF......
Te Cat  241  ANG.Y.SWTFYIQVM.FEQAEKFQFNPFDLTKIW.HKEYPLIPVGKMVLNRNPF.Y.AEV

Zf Cat  301  KVWSHKEFPLIPVGRFVLNRNPVNYFAEVEQLAFDPSNMPPGIEPSPDKMLQGRLFSYPD
Me Cat  301  ..............M.............................................
Mo Cat  301  ...P..DY......KL...K...........M.......................A...
St Cat  301  QHVQVWSHKEY.LIPVGKLVLNR.PVNYFAEVEQLAFDPSNMPPGIEASPDKMLQGRLFS
Hu Cat  301  ...P..DY......KL..............I............A...........A...
Te Cat  301  EQMAFDPSNMP.GIEPSPDKMLQGRLFSYPDTHRHRLGANYLQI.VNCPYRARVANYQR.

Zf Cat  361  THRHRLGANYLQLPVNCPYRTRVANYQRDGPMCMHDNQGGAPNYYPNSFSAPDVQPRFLE
Me Cat  361  ............I.....F..............F................ET..Q.V.
Mo Cat  361  .......P....I.......A.............................EQ.RSA..
St Cat  361  YPDTHRHRLGANYLQIPVNCPFRTRVANYQRDGPMCMFDNQGGAPNYYPNSFSAPEDQPR
Hu Cat  361  .......P...HI.......A.............Q..............G..EQ..SA..
Te Cat  361  GPMCMSDNQGGAPNYYPNSFSAPEIQSHCVESKFKVHPDV.RYNSSDEDNVTQ.RTFYTQ

Zf Cat  421  SKCKVSPDVARYNSADDDNVTQVRTFFTQVLNEAERERLCQNMAGHLKGAQLFIQKRMVQ
Me Cat  421  ..FQ............E......C....K....D..Q........S..E.........E
Mo Cat  421  HSVQCAV..K.F...NE.........Y.K....E..K...E.I.....D........KA.K
St Cat  421  FVESKFKVSPDVGRYNSADEDNFTQVR.FYTDVLNE.ERQRLCQNMAGALKGAQLFIQKR
Hu Cat  421  HSIQY.GE.R.F.T.N........A.YVN....EQ.K...E.I.....D..I....KA.K
Te Cat  421  VLNEEQRQRLCQ.M.GSLKGA.LFIQKRM.E.LKAVHSDYASRVQA.LDKFNEEADKNAS
```

			Identity (%)	Similarity (%)
Zf Cat	481	NLMAVHSDYGNRVQALLDKHNAEGKKNTVHVYSRGGASAVAAASKM	100	100
Me Cat	481	..K...P.......T....Y..QA..SASVHVYNRPGASAI...SKM	86	94
Mo Cat	481	.FTD..P...A.I......Y...KP..AI.T.TQA.SHMA.KGKANL	80	89
St Cat	481	MVENLKAVHADYGNRVQTLL.QNNAVEKKVLKDIMKFCLQSTTRLLI	80	88
Hu Cat	481	.FTE..P...SHI......Y...KP..AI.TFVQS.SHLA.REKANL	77	87
Te Cat	481	VHVYTRPGASTIAASKKCNI	73	82

Table 4: Effect paraquat on CAT activity and MDA level in zebrafish. Notes: The data shown are mean of three replicates±standard deviation. Different letters in a column show statistically different groups.

	CAT (EU/ mg protein)		MIDA (n mol/ mg protein)	
	Liver	Gill	Liver	Gill
Treatment I	279.62±21.87[a]	152.33±11.23[a]	0.0082±0.0011[a]	0.0058±0.0011[a]
Treatment II	231.22±6.00[b]	115.41±8.16[b]	0.0079±0.0013[b]	0.0055±0.0013[b]
Control	64.00±21.91[c]	50.73±5.86[c]	0.0077±0.0013[c]	0.0056±0.0013[c]

The highest level of *cat* gene expression in zebrafish was found in liver (595.53±14.41). Transcription of gill (311±11.32) was found significantly lower than that of liver and other tissues (p<0.05). Expression of muscle (132.89±9.21), kidney (118.96±8.87), testis (120.03±6.99) and ovary (104.31±8,22) were not different although they were higher than that of intestine (37.28±3.11), brain (19.17±2.01), eye (18.30±2.10), heart (15.79±2.01), spleen (77.7±0.91), swim bladder (6.97±021) and skin (4.59±0.31). Although there was no difference in levels of *cat* gene transcripts between male and female zebrafish tissues, it was observed that *cat* gene was highly expressed in liver, muscle and gill tissues (Fig. 1).

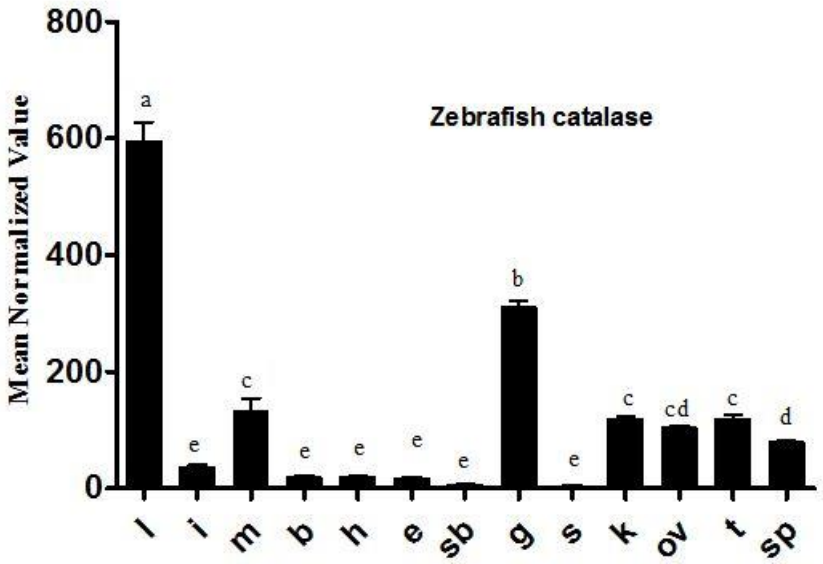

Figure 1: Steady-state levels of zebrafish *cat* transcripts in liver (l), intestine (i), muscle (m), brain (b), heart (h), eye (e), swim bladder (sb), gill (g), skin (s), kidney (k), ovary (ov), testis (t) and spleen (sp). The values are presented as mean of six fish (three males and three females) except three for ovary and testis where three fish of each sex were used. Error bars show standard deviation.

Moreover, mRNA expression of *cat* was significantly decreased in liver and gill tissues after exposure to two different paraquat concentrations (p<0.05). Liver mRNA expressions were 811.51, 597.14 and 628.24 in Control, treatments I and II, respectively. Gill mRNA expressions were 604.97, 311.35 and 417.50 in

Control, treatments I and II, respectively. Difference among groups was found to be significant (p<0.05) (Fig. 2).

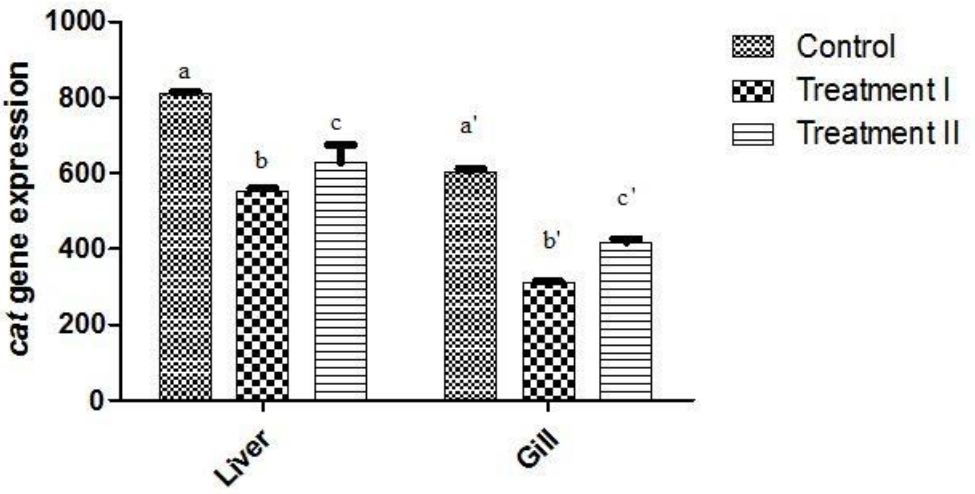

Figure 2: The *cat* mRNA expression of liver and gill tissues in zebrafish exposed to paraquat. The data shown are mean of six replicates. Different letters in a column show statistically different groups in liver and gill tissues. Error bars show standard deviation.

In silico analysis of zebrafish cat gene

In this study, some bioinformatics analysis on the zebrafish *cat* gene was given. For this reason, we retrieved bioinformatics data using some databases (ENSEMBL, UNIPROT and NCBI), and programs (BioEdit software, pairwise alignment of BLOSUM62, MEGA6). The transcript of the cDNA sequence (ENSDARG00000104702) was acquired from the Ensembl genomic database to detect the gene structure of the zebrafish *cat* gene. The zebrafish *cat* gene has 13 exons and 12 introns. All the introns follow gt-ag rule. This gene has putative TATA (Table 2).

The identity and similarity sequence of zebrafish *cat* gene with medaka, tetraodon, stickleback, mouse and human *cat/CAT* gene was given in Table 3. The highest identity and similarity rates for zebrafish *cat* gene was determined with its orthologs which are medaka (86 and 94%), mouse (80 and 89%), stickleback (80 and 88%), human (77 and 87%) and tetraodon (73 and 82%).

Conserved gene synteny was an obvious fact between *cat* gene of zebrafish and the orthologous *cat/CAT* genes of other teleost fishes and human (Fig. 3). The syntenic genes of zebrafish (*D. rerio*) *cat* located on chromosome 25 exhibit conserved gene synteny with human (*Homo sapiens*) *CAT* gene located on chromosomes 11 and 15, medaka (*Oryzias latipes*) *cat* gene located on chromosome 15, respectively. The results clearly showed that *cat* gene in zebrafish exhibits a highly conserved gene structure.

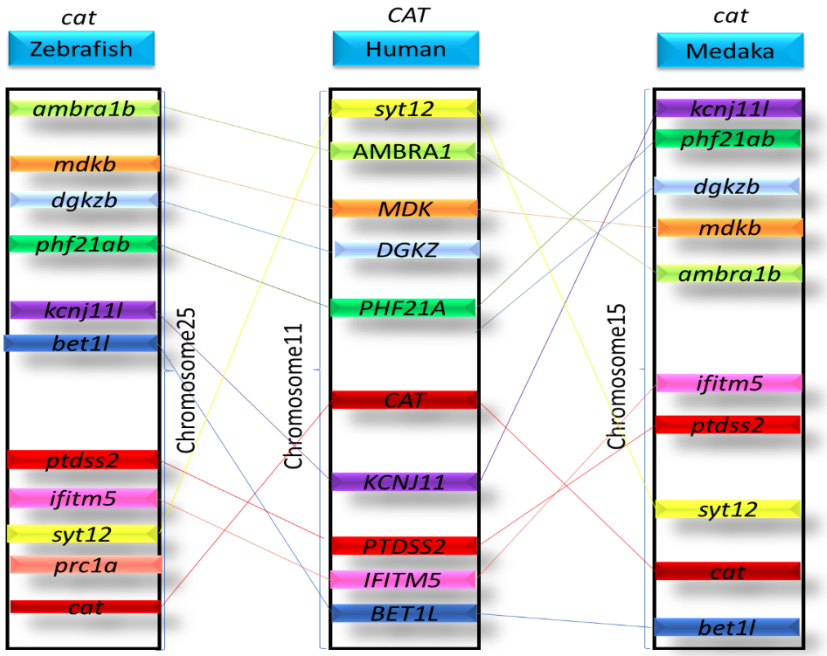

Figure 3: Conserved gene syntheny.

Identity and similarity results were used for the phylogenetic tree (Fig. 4).

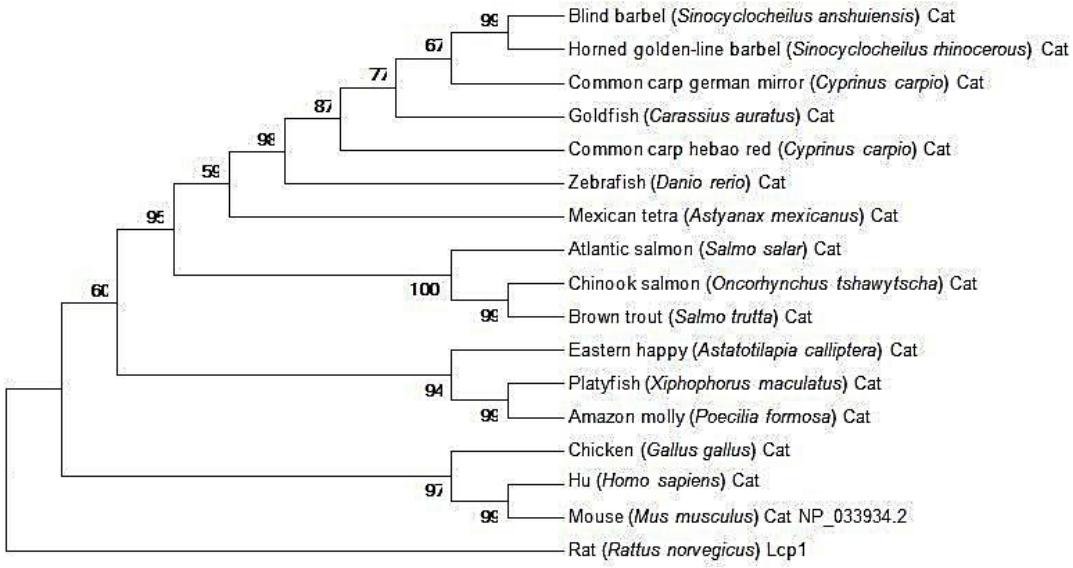

Figure 4: Phylogenetic tree of zebrafish *cat*.

Phylogeny analysis showed a high phylogenetic similarity between *cat* genes in zebrafish and its orthologs. Phylogenetic relationships between *cat* sequences from zebrafish and *cat* sequences from other vertebrates. The tree was produced using Maximum Likelihood Method (Kumar *et al.*, 2016). Accession numbers of the sequences used for the phylogenetic tree are the following: Horned golden-line barbel Cat: 107740891, Common carp German

mirror Cat:30068, Goldfish Cat:113066695, Common carp hebao red Cat: 30068, Zebrafish Cat: NP_570987.2, Mexican tetra Cat: 103032916, Atlantic salmon Cat:106564824, Chinook salmon Cat:112218377, Brown trout Cat:115197208, Eastern happy Cat: 30068, Platyfish Cat: XP_005815336.1, Amazon molly Cat: XP_007546846.1, Chicken Cat: NP_001026386.2, Hu Cat, Mouse Cat: NP_033934.2, Rat Lcp1:1308288.

Discussion

In the present study, effects of paraquat on zebrafish were investigated by assessing responses at molecular (gene expression changes) and biochemical (enzymatic activity) levels. It was observed that exposure of zebrafish to two concentrations of paraquat (0.30 ppm and 0.15 ppm) lead to changes in CAT enzyme activity as well as *cat* gene expression while didn't change MDA level in gill and liver.

Importance of pesticide-induced oxidative stress studies, as a possible toxicity mechanism, gained importance (Sayeed *et al*., 2003). For a complete understanding of cellular and molecular events these studies are required for a full understanding of pesticide-induced stress in fish. Antioxidant enzymes have always been important in aquaculture studies. Antioxidant levels and ROS production, which are in balance under normal condition in organisms, deteriorate due to oxidative stress caused by harmful effect of free radicals and various diseases (Pizzino *et al*., 2017).

Paraquat which is highly toxic to both human and animals (Williams *et al*., 2016) is widely accepted as a model of oxidant damage (Pruett *et al*., 2010). Toxic effects of paraquat cause gill, kidney and liver changes in fish (Di Marzio and Tortorelli, 1994). Considering the antioxidant defenses, CAT activity was increased in both doses (0.30 and 0.15 mg/L) of paraquat while no significant change in MDA levels in liver and gill were observed. Although toxic effect of paraquat is reported to be associated with increased ROS (Parvez and Rausiddin, 2006), there are still some discussions about the relationship between paraquat and lipid peroxidation. Though there are studies reporting a tendency to decrease lipid peroxidation in all tissues of fish exposed to paraquat (Grim *et al*., 2010; Vinagre *et al*., 2012), the change in MDA level was observed to be insignificant in our study. Changes in redox parameters and mitochondrial function in zebrafish liver and gill, as well as behavioral changes, should serve as main tools for better understanding molecular mechanisms induced by paraquat exposure. Parvez and Raisuddin (2006) reported similar results and declared that 24-hour exposure to paraquat did not cause significant change in LPO values in tissues of zebrafish. However, the result of our study coincides with Gaaied *et al*. (2019) who reported that CAT activity increased in zebrafish larvae exposed to 0.8 mg/L of herbicide. Similar results reported that paraquat affected some antioxidant enzyme activity, for

example it changed lactate dehydrogenase (LDH) levels and increased peroxidase activity, inhibiting acetylcholinisterase activity in carp (Gabryelak and Klekot, 1985). Previous studies showed that different pesticides have similar acute toxicity on zebrafish and cause changes in antioxidant enzyme activities (Yang *et al*., 2016; Jiang *et al*., 2019; da Silva Barreto *et al*., 2020). Beside CAT activity, changes of the gene expression in zebrafish gill and liver were also assessed by applying the two concentrations of paraquat exposure scenario. When RT-PCR results were evaluated, it was seen that *cat* expression in zebrafish exposed to paraquat could be an immediate response mechanism to stress caused by chemical application. Expression of *cat* decreased in liver and gill tissues while CAT activity increased after exposure to paraquat treatment. Decrease in expression of *cat* indicates involvement of oxidative stress in toxicity mechanism of paraquat. It's known that different factors affect gene expression and many factors may be responsible for the observed inconsistency between the detected enzyme activity levels and gene expression (Glanemann *et al*., 2003). For this reason, the disparity between mRNA abundance and enzyme activity should not be surprising. That is a possible reason for observed changes in expression of the measured *cat*, which is one of the stress response related genes. As regards to the observed down regulation of *catalase* in zebrafish, similar trends were obtained in a previous study (Mu *et al*., 2015).

The *cat* gene in zebrafish genome was found to have a highly conserved exon number with 13 exons and 12 introns (Table 2). Zebrafish Cat sequence showed a higher identity/similarity ratio with its orthologous. Phylogenetic analysis showed a strong evolutionary relationship between zebrafish Cat and Cat/CATs from vertebrates and zebrafish *cat* were orthologs of *cat*/*CAT*s from other teleost fishes and tetrapods. Evidence was found for a conserved gene synteny for zebrafish *cat* with human *CAT* and medaka *cat.* Zebrafish genome has one copy of the *cat* gene. Nonfunctionalization is one of the most common situations in evolution of duplicated genes (Glasauer and Neuhauss 2014; Bayır 2021) and is thought to be the cause of copy loss of *cat* gene in zebrafish genome. Different steady-state levels among tissues in zebrafish could be explained that duplicated genes in teleost fish arose through WGD exhibit a dissimilar tissue distribution. The reason for this difference is thought to be due to the difference in regulatory elements in promoters that control the transcriptional regulation of *cat* gene in zebrafish due to neofunctionalization (Belliveau *et al*., 2010; Parmar *et al*., 2013; Bayır 2021).

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