SVU- International Journal of Veterinary Sciences, 5(4): 114-124, 2022
Print ISSN: 2535-1826 **Online ISSN: 2535-1877**

Research Article Open Access

Expression profiles of catalase gene in common carp exposed to ammonia

Mahmoud Nasr[1], Ahmad A.A. Elkamel[2,*], Mohamed Youssef[3], Walaa F.A. Emeish[1]

[1]Department of Fish Diseases, Faculty of Veterinary Medicine, South Valley University, Qena 83523, Egypt, [2]Department of Aquatic Animal Medicine and Management, Faculty of Veterinary Medicine, Assiut University, [3]Department of Animal physiology, Faculty of Veterinary Medicine, South Valley University, Qena 83523, Egypt

Abstract

Ammonia is one of the most harmful water quality parameters restricting the growth and survival of aquatic living organisms. As a result, fish must adapt to this stressor by modifying physiological processes that are governed by gene expression regulation. The aim of the present study was to investigate the expression profiles of antioxidant related gene, catalase (*CAT*) in common carp (*Cyprinus carpio*) fingerlings after exposure to 0.7 mg/l of unionized ammonia (UIA) in water. The relative gene expression was measured in liver, gills, and brain tissues at four time points (12 h, 2 d, 4 d, and 7 d post exposure). The expression level of *CAT* gene in the liver and brain peaked after 7 d of ammonia exposure by 13.3 and 5.2-folds, respectively, but in gills it upregulated only after 2 d (2.7-folds) and downregulated at the other time points. This study proved that exposure to ammonia affects the antioxidant status of common carp as indicated by the altered levels of expression of *CAT* gene.

Keywords: common carp, ammonia, gene expression, antioxidant-related genes, *CAT*

DOI: 10.21608/svu.2022.155871.1223 Received: August 12, 2022 Accepted: November 17, 2022 Published: December 24, 2022

*Corresponding Author: Ahmad A.A. Elkamel E-mail: aelkamel@aun.edu.eg

Citation: Nasr et al., Expression profiles of catalase gene in common carp exposed to ammonia**.** SVU-IJVS 2022, 5(4): 114-124.

Competing interest: The authors have declared that no competing interest exists.

Introduction

Common carp (*Cyprinus carpio*) has a high production rate worldwide and is considered as one of the freshwater fish with the highest commercial value (Fisheries, 2018). It is a good candidate for intensive aquaculture systems; however, the intensive rearing strategies are one of the major causes of immune system dysfunction; resulting in increasing the susceptibility of fish to diseases leading to greater economic loss (Dawood et al., 2020). In Egypt, common carps are important for aquaculture as partial solution for the increasing demand for protein (Abbas, 2006).

Ammonia toxicity is a major fish environmental concern worldwide, one of the most harmful water quality problems for fish besides hypoxia (Dosdat et al., 2003), and among the most important factors restricting the growth and survival of aquatic living resources (Russo and Thurston, 1991; Tomasso, 1994). It has been extensively investigated in different fish species such as common carp (Salah El-Deen, 1999; Sampaio et al., 2002; Wicks and Randall, 2002; Chew et al., 2003).

Most teleost fish are ammoniotelic and excrete ammonia as their principal waste product (WOOD, 1993). The most of ammonia is excreted mainly by gills (Evans et al., 2005) and the remaining part by kidneys (WOOD, 1993) and skin (Morii et al., 1978). The direct toxicity of ammonia on fish including oxidative stress and tissue damage (Ackerman et al., 2006; Gonçalves et al., 2012). Oxidative damage caused by ammonia is due to increase the release of significant quantities of reactive nitrogen species (RNS) and reactive oxygen species (ROS) (Ching et al., 2009; Zhu et al., 2020). After the overproduction of ROS, a major defense mechanism for reducing the production of ROS is achieved by raising the levels of antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and glutathione reductase (GR). Major antioxidant elements such as CAT enzyme is among the immune responses involved in protection of the fish body from oxidative stress-induced damage via degenerating the free radicals (Guemouri et al., 1991; Morel and Barouki, 1999). *CAT* gene expression was studied in the liver of pufferfish (Cheng et al., 2015) and crucian carp (*Carassius auratus*) (Qi et al., 2017).

Therefore, this study aimed to elucidate the effects of ammonia intoxication on the oxidative status of common carp through analysis the expression level of *CAT* gene in fingerling exposed to 0.7 mg/l of unionized ammonia. The expression dynamic process was analyzed at four time points (12 h and 2 d, 4 d and 7 d) post exposure in liver, gills and brain tissues.

Materials and methods

Experimental system and animals

One hundred and fifty Common carp fingerlings (2 ± 0.5 g) were obtained from a fish hatchery and transported to the aquatic lab of the Faculty of Veterinary Medicine, South Valley University, Qena, Egypt. The fish were examined for any abnormalities, then acclimated to the laboratory environment in tanks (500 litter) for three weeks in accordance with the instructions for the maintenance of fish bioassay provided by (Ellsaesser and Clem, 1986). Before starting the experiment, the ethical approval was obtained from the, University of South

Valley's Laboratory Animal Ethics Committee (No. 7/13.12.2020).

Experimental groups and ammonia exposure

Fish (n=150) were randomly divided into two groups control and treated (75 fish / group) in 50 L tanks with three replicates / group, each replicate has 25 fish. The treated group was exposed to 0.7 mg/L of UIA for one weak (the dose was chosen after preliminary experiment), ammonium chloride (NH4Cl) (Merck Company) was used as a source of UIA, while the control group remained unexposed. The total ammonia nitrogen, pH, and temperature of the aquariums were taken into account while calculating the NH3 values (Crist, 2007). The tanks were cleaned regularly and aerated continually with air stones. In addition, replacing 25 % of the water per day with new dechlorinated water containing the selected ammonia concentration, measuring and adjusting the ammonia in the tanks to maintain the ammonia level in the treated groups throughout the experiment.

Sample collection

Samples were obtained at 12 h, 2 d, 4 d, and 7 d post ammonia exposure from both treated and control groups. Fish were euthanized with eugenol and then liver, gills, and brain were sampled and preserved in RNAlater (QIAGEN). Tissue samples from 3 fish per replicate per time point were pooled and used as one sample for RNA extraction.

RNA isolation and cDNA synthesis

The extraction of RNA was done using RNeasy® Mini kit (QIAGEN, Germany) as per manufacturer's instructions. RNA samples were maintained at -80 °C till usage, after that the purity and concentration of the RNA were determined using a Nanodrop (NanoDropTM LITE Spectrophotometer, Thermo Scientific, USA). RNA (1 ug) was reverse transcribed using the RevertAid first strand cDNA Synthesis Kit (Thermo scientific), cDNA was used for quantitative real-time PCR.

Quantitative real-time PCR (RT-qPCR)

RT-qPCR was carried out utilizing the CFX 96TM Real-Time PCR detection equipment (BIO RAD, USA) and the HERAPLUS SYBR® Green QPCR kit (Willowfort, England). The primers specific for genes under study listed in (table 1). The reactions were performed in 20 µl - total volume as in (table 2). Triplicate samples were used in each reaction in duplication. Fluorescent data were collected during the extension process. For normalization of the target gene expression level, housekeeping genes: Beta actin (*β-actin*) and Elongation Factor 1-alpha (*EF1α*) were employed. cycles thermal profiles were mentioned in (table 3). At the end of each PCR, a melting curve was run to ensure that only one PCR product had been amplified and identified. This yielded a single product with the following melting temperatures: *CAT* = 84.5°C, *β-actin* = 85°C, and *EF1α* = 81.5°C. The expression level of *CAT* gene was measured by the delta-delta Ct ($2^{-\Delta\Delta Ct}$) according to (Livak and Schmittgen, 2001).

Table 1: The Primers of the genes under study

Gene	primer (5′-3′)	References
CAT	**F: AGACGACACCCATCGCTGTTCG** **R: AAGGTCCCAGTTGCCCTCATCG**	(Ghelichpour et al., 2019)
β-actin	**F: GATTCGCTGGAGATGATGCT** **R: GATGGGGTACTTCAGGGTCA**	(Mráz, 2012)
EF1α	**F: GGAGCCCAGCACAAACATG** **R: TTACCCTCCTTGCGCTCAAT**	(Schyth et al., 2006)

Table 2: The components of Quantitative real-time PCR for 20 µl-end volume reaction:

Components	Amount in µl
2× SYBR® Green	**10 µl**
primer forward (10pmol/ µl)	**1 µl**
primer reverse (10pmol/ µl)	**1 µl**
cDNA template	**2 µl**
sterile nucleases free PCR grade water	**6 µl**

Table 3: Cycling and melting thermal profiles of the genes used in this study

Steps	Temperature	Duration
Initial denaturation	**95°C**	**3 min**
Number of cycles	**40 cycles**	
Denaturation	**95°C**	**10 s**
Annealing and extension	**60°C**	**1min**
Melting thermal profile	**65°C**	**5 s**

Statistical analysis

Data were shown as mean ± SEM (standard error of the mean). Two-way ANOVA (analysis of variance) was used to assess the significant differences in observations between the experimental and control groups; $P<0.05$ was considered significant. The statistical analysis was performed using Graph-Pad Prism (GraphPad 8.0.1 Software, San Diego, CA, USA). The pairwise comparison of the means of the different treatments was done using the Tukey multiple range test.

Results

Clinical and PM examination of common carp

During the ammonia exposure, control group fish showed no lesions (Figure 1), Fish exposed to UIA exhibited slight external congestion (Figure 2A) followed by fin rot (Figure 2B) till complete fin sloughing and damaged skin with dark coloration (Figure 2C and D) and erratic swimming.

Additionally, the gills showed hyperemia, dark redness then became pale

Figure 1: Common carp (*Cyprinus carpio*) fish from control group without any clinical signs.

a few days later, swelling, and damage to the gill margins, as well as excessive mucus production (Figure 3) and almost empty gut. The signs and lesions were observed during the early days of the ammonia exposure then disappeared.

Expression pattern of the gene under study

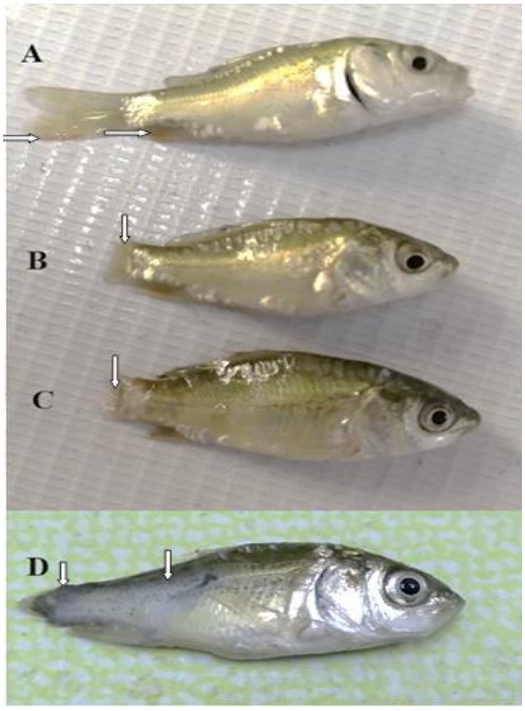

Figure 2: Clinical and PM examination of common carp fingerlings exposed to 0.7 mg/l of unionized ammonia exhibited a slight external congestion (A), fin rot (B), fin sloughing and damaged skin (C and D).

Figure 3: Clinical and PM examination of the gills of common carp fingerlings exposed to 0.7 mg/l of unionized ammonia showed abnormalities such as hyperemia, dark red, swelling, and damage to the gill margins and dark coloration in the skin.

The expression levels of *CAT* gene in liver, gills and brain were shown in (Figures 4, 5 and 6) respectively. In the liver, *CAT* gene significantly increased gradually after 12 h of ammonia exposure reaching the peak after 7 d (Figure 4). While in the gills, it was significantly downregulated after 12 h then upregulated only after 2 d, then downregulated at 4 d and 7 d post ammonia exposure (Figure 5). Regarding its expression in the brain, it upregulated gradually from 12 h till peaked at 7 d (Figure 6). The expression patterns of *CAT* in the selected organs (Figures 7) indicated that the liver and brain had the same expression profile of *CAT* gene, additionally the liver had the highest expression level.

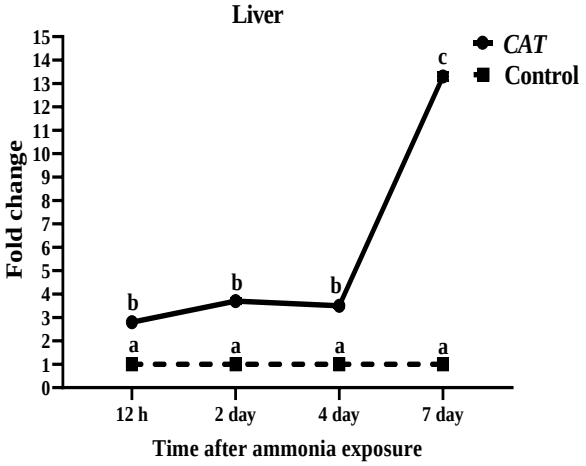

Figure 4: *CAT* gene expression relative to *β-actin* and *EF1α* as housekeeping genes determined by real-time PCR in the liver of common carp fingerlings throughout one week of 0.7 mg/l of unionized ammonia exposure. Values are mean ± standard error of the mean. The bars are standard error of the mean and assigned with different letter denotes significant difference (two-way ANOVA test; $P < 0.05$) with respective untreated controls (value of 1.0).

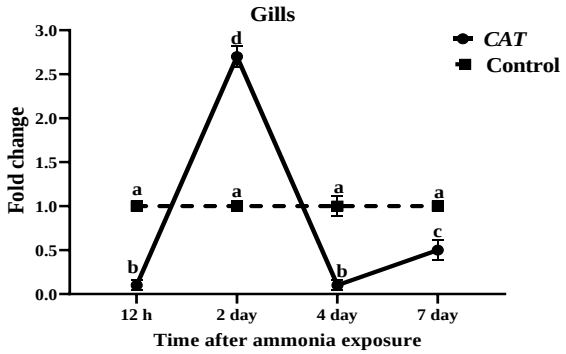

Figure 5: *CAT* gene expression relative to *β-actin* and *EF1α* as housekeeping genes determined by real-time PCR in the gills of common carp fingerlings throughout one week of 0.7 mg/l of unionized ammonia exposure. Values are mean ± standard error of the mean. The bars are standard error of the mean and assigned with different letter denotes significant difference (two-way ANOVA test; $P < 0.05$) with respective untreated controls (value of 1.0).

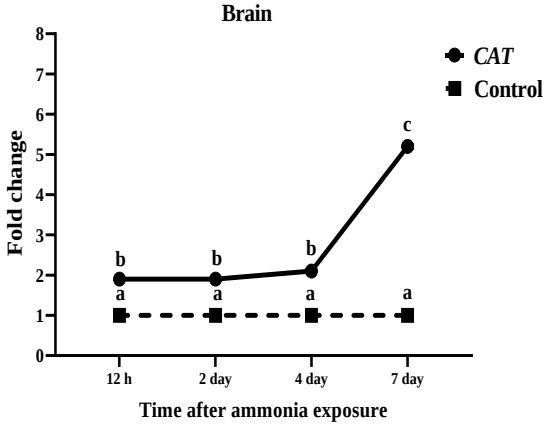

Figure 6: *CAT* gene expression relative to *β-actin* and *EF1α* as housekeeping genes determined by real-time PCR in the brain of common carp fingerlings throughout one week of 0.7 mg/l of unionized ammonia exposure. Values are mean ± standard error of the mean. The bars are standard error of the mean and assigned with different letter denotes significant difference (two-way ANOVA test; $P < 0.05$) with respective untreated controls (value of 1.0).

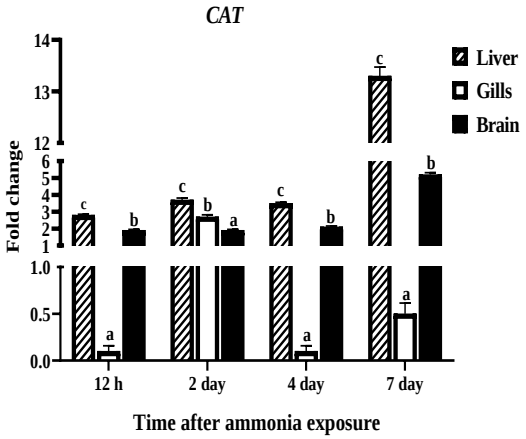

Figure 7: collective comparison of *CAT* gene expression relative to *β-actin* and *EF1α* as housekeeping genes determined by real-time PCR in liver, gills and brain of common carp fingerlings throughout one week of 0.7 mg/l of unionized ammonia exposure. Values are mean ± standard error of the mean. The bars are standard error of the mean and assigned with different letter denotes significant difference (two-way ANOVA test; $P < 0.05$) within each time point.

Discussion

Although the effect of ammonia exposure was intensively studied in various fish species and can affect their biological activities, the molecular study is still lacking. In the present study, ammonia nitrogen stress causes upregulation of *CAT* gene expression. Oxidative damage occurs through stimulation of the releasing of large amounts of reactive nitrogen species (RNS) and reactive oxygen species (ROS) (Ching et al., 2009). ROS including hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), superoxide (O_2^-), and singlet oxygen (O_2) (Choi et al., 2012). Superoxide dismutase (*SOD)* and *CAT* provide the first line of defense to intercept ROS (Farombi et al., 2007). The primary form of intracellular reactive oxygen species is O_2^-, SOD has the ability

to catalyze it into O_2 and H_2O_2 (Ni et al., 2007). The primary role of catalase is to split hydrogen peroxide into molecular water and oxygen by the reaction: $2H_2O_2 \rightarrow 2H_2O+O_2$. (Fahimi and Cajaraville, 1995), so that the cells are protected from H_2O_2 toxicity (Li et al., 2008).

In this study, the liver showed upregulation of *CAT* expression and the liver showed the highest significant expression rate of *CAT* gene after ammonia exposure whereas, in fishes, the liver is the primary organ for detoxification and degradation of exogenous and endogenous free radicals (Lee et al., 2015). This suggests that ammonia exposure causes oxidative stress in common carp.

Gills as an active respiratory and osmoregulatory organ, have a high capacity to produce ROS, in this study the downregulation of *CAT* gene in the gills after 12 h of ammonia exposure, may be due to excessive superoxide radicals production, which could not be detoxified by *SOD*, and directly inhibit *CAT* activity (Kono and Fridovich, 1982; Pandey et al., 2003), another explanation is that ammonia increase resulting in upregulation of *SOD* gene expression and the overproduction of hydrogen peroxide, produced from *SOD* stimulation of kinase cascades in signal transduction pathways (Suzuki et al., 1997), inhibiting specific transcription factors (Franco et al., 1999), and consequently inhibiting *CAT* gene transcription (Sun et al., 2014). A possible explanation about changing patterns of SOD and CAT activities in grass carp is that the grass carp could not scavenge the redundant ROS which accumulated in the cells and inhibited the activities of SOD and CAT, thus caused the significant increase of ROS in 2h and 4h group (Jin et al., 2017). As well, away from gene expression, in another study, the gills of mudskipper (*Boleophthalmus boddarti*) after exposure to 8 mmol l−1 NH_4Cl for 12 h resulted in significant decreases in activities of catalase (Ching et al., 2009).

The transient upregulation of *CAT* gene transcripts after 2d of ammonia exposure may be critical for the maintenance of low gill ROS levels. However, maintenance of the gill antioxidant activity, despite lower *CAT* expression abundance after 4d of ammonia exposure, suggests other molecular mechanisms, including post-transcriptional and/or post-translational modifications regulating the antioxidant status. Since the balance between levels of total antioxidant enzymes activity plays an important role in the processes of antioxidant profile formation during stress exposure. Significant downregulation of *CAT* gene in gills after 4 days of ammonia exposure doesn't indicated that antioxidant defenses are insufficient to keep the levels of ROS below a toxic threshold. This very well explained by its significant upregulation in the liver and brain tissues and moreover stop of mortality in the last two days of the experiment.

Ammonia nitrogen stress also causes over-activation of N-methyl-D-aspartate (NMDA) receptor and Ca_2^+ dependent nitric oxide synthase pathway in the brain, releasing of large amounts of reactive nitrogen species (RNS) and reactive oxygen species (ROS) (Ching et al., 2009) subsequently, *CAT* activated to intercept ROS (Farombi et al., 2007) and this explain the upregulation of *CAT* gene expression in the brain.

Conclusion

Ammonia is a known stress-inducing substance, caused changes in the expression of antioxidant gene in various organs. In general, it stimulated *CAT* gene expression in the liver and brain to act against oxidative conditions caused by inflammation and/or detoxification, this may indicate the oxidative stress. However, it was significantly downregulated in the gills, which might be due to direct ammonia contact and severe oxidative stress.

Acknowledgements

We would like to thank the members of Cell and molecular physiology research center in the Faculty of Veterinary Medicine, South Valley University, Qena, Egypt for their cooperation and supplying the facilities for RT- qPCR.

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