THE EFFECT OF HIGH AMMONIA CONCENTRATION ON GILL STRUCTURE ALTERNATION AND EXPRESSION OF *SOD* AND *HSP*90 GENES IN GRASS CARP, *CTENOPHARYNGODON IDELLA*

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Abstract: Histological and molecular responses of grass carp *Ctenopharyngodon idella* to high concentration ammonia exposure were studied in this study by determining the lethal concentration, gill cellular structure, and expression patterns of *sod* and *hsp*90 genes. Grass carps were treated at five ammonia concentrations (50, 72, 104, 151 and 220 mg/L) for 24h, and then the gill tissues were collected for histological analysis. In addition, three tissues (gill, intestine and liver) were collected to measure the expression of *sod* and *hsp*90 genes. After exposure to high concentration of ammonia, the arrangement and structures of gill cells changed dramatically. Edema and fusion of secondary lamellae were observed, and the edge of cell and nucleus could not be clearly defined. Meanwhile, the two genes (*sod* and *hsp*90) in different tissues were unregulated significantly, indicating that high concentration of ammonia could impair the cellular structures and induce the expression of stress proteins. These results also suggested that *sod* and *hsp*90 were suitable biomarkers for ammonia exposure assessment in grass carp.

Key words: Grass carp; Ammonia exposure; 24h LC_{50}; Histological damage; *sod; hsp*90
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Ammonia is a highly toxic waste product produced by plants and animals[1]. Decomposition mediated by microorganisms also generates this compound. Ammonia is known to be one of the common causes for death in aquatic animals [2—4] since high concentrations of ammonia frequently occur in aquatic environments[5]. It was reported that high ammonia and low dissolved oxygen concentrations during summer and spring seasons were the major factors responsible for mortality in sewage-fed fishponds [6].

In fish, ammonia is produced in white muscles when adenylates are broken down to inosine monophosphate [7] and ammonium (NH_4^+) during anaerobic energy production associated with burst exercise [1, 8]. The toxicity of ammonia is affected by several factors including temperature and pH [9], and ammonia toxicity increases with ammonia concentration, water pH, and temperature [10—12]. Exposure to high concentrations of ammonia reduces survival, inhibits growth, and causes a variety of physiological malfunctions. On the organismal level, ammonia causes hyperventilation [13, 14], hyper-excitability, coma, and convulsion. On the cellular level, ammonia can interfere with energy metabolism through impairing the tricarboxylic acid cycle in fish [15]. In extreme conditions, high concentration of ammonia can finally cause large-scale death.

When an organism is subjected to chemical, physical or biological stress, sudden shortage of oxygen causes abnormal oxidative reactions in the aerobic metabolic pathways, resulting in the formation of excessive amounts of singlet oxygen and other reactive oxygen species (ROS). Superoxide dismutase (SOD) catalyzes a rapid two-step dismutation of the toxic superoxide anion by producing molecular oxygen and H_2O_2 [16, 17], and this reaction provides protection against the damaging effects of oxidant stress in living organisms [18]. It has been proved that SOD is associated with both acute and chronic pathology. There are many biological stimulators that can regulate the expression of the *sod* gene, such as heat shock [19, 20], shear stress [21, 22], and heavy metals [23].

Previous studies have shown that contaminants could induce the gene expression of Phase 2 metabolic enzyme [24] and reactive oxygen species (ROS) as the byproduct could lead to a common pathway toxicity [25, 26]. Meanwhile, *sod* gene expression is also tissue-specific, and the expression pattern and time course may differ indifferent tissues [27].

As molecular chaperones, Heat Shock Proteins assist cells in their recovery from stress and promote cytoprotection [28]. Heat shock proteins consist of several families of highly conserved proteins that play an essential role in a number of cellular processes. As an important member of heat shock protein family, HSP90 responds to a wide variety of physiological and environmental stressors, including thermal shock, heavy metals, free radicals, or almost any sudden changes in the cellular environments that induce protein damage[29—31]. Because of their important roles in protecting organisms from damages, HSPs are regarded as sensitive biomarkers for monitoring the health of aquatic environments[32]

Grass carp *Ctenopharyngodon idella* (Valenciennes 1844) is one of the most widely cultivated freshwater fish species in China. Both acute and chronic toxicities of ammonia have been extensively reviewed for freshwater species [33—36]. However, few studies examined the effect of ammonia exposure on the mRNA expressions of stress proteins in the grass carp. In this study, expression of *hsp*90 and *sod* genes at mRNA levels were determined using semi-quantitative real time PCR (sqRT-PCR) with specific primers and histological changes also measured to determine the damages to the cellular microscopic structure after ammonia exposure. The integration of *hsp* and *sod* as an anti-stress system, along with the help of histological data, should be useful to study the effect of ammonia on the fish and the physiological adaptations of grass carp to ammonia exposure.

1 Materials and methods

1.1 Experiment animals and acclimation conditions

Grass carps with body length of (9.2±0.8) cm (Mean± SD) were obtained from Jiaonan Fish Farm, Qingdao, P. R. China. During the transportation, aeration to the fish containers was provided continuously. After delivery, the fish were maintained in rectangle indoor tanks filled with fully aerated running water and fed with compound feed once a day. Water temperature was controlled at (20± 0.2)℃, and one third of water was replaced daily. Light was controlled on a 12h   12h light/dark cycle.

1.2 Test for the upper limit of ammonia tolerance

Seventy-five grass carps were divided into five groups (15 individuals per group). For each trial, one group of grass carps were placed into an aquarium (45 cm×24 cm×34 cm, L×W×H) and exposed to one of the desig-

nated ammonia concentrations (150, 186, 229, 282, 350 mg/L) for 24h. After the 24h exposure, behavior and mortality were recorded over time. During the whole experimental period, the rearing conditions were similar to those used during the acclimation and aeration was provided continuously. There were three replicates in each treatment.

1.3 Expressions of stress proteins under the ammonia exposure

Ammonia exposure and tissue collection   After a two-week acclimation, different treatment groups of fish were transferred into aquariums (45 cm×24 cm×34 cm, L×W×H). There were 15 fish in each aquarium and the water was exchanged daily as described above. Five ammonia concentrations were controlled (50, 72, 104, 151 and 220 mg/L) using ammonium chloride (Promega, USA). After a 24h exposure, six specimens were randomly chosen from each aquarium and anesthetized with ethyl alcohol (ETOH) before dissection. Liver, intestine, and gill were collected and frozen in liquid nitrogen for total RNA isolation. At different time points (0, 2, 4, and 24h) after the ammonia exposure, water ammonia concentrations were determined using a colorimetric method[37].

Quantification of genes expression   Total RNA was isolated from approximately 60 mg of different tissues using the Trizol reagent (Invitrogen, USA). 1 μg of total RNA was used as the template for synthesis of the first strand of cDNA. Partial *β-actin* gene (480 bp) was selected as an internal control. Four pairs of primers, SOD-F and SOD-R, Hsp90-F and Hsp90-R, and β-actin-F and β-actin-R were designed based on their respective sequences from Genbank (Tab. 1). Semi-quantitative PCR conditions and components for *hsp*90, *sod* and *β-actin* were optimized, especially for the amplification cycles and annealing temperatures. PCR was carried out in 25 μL reaction solution that contained 2.5 μL of 10×PCR buffer, 1.6 μL of $MgCl_2$ (25 mmol/L), 2.0 μL of dNTP (2.5 mmol/L), 1 μL of primer (10 pmol/mL), 15.875 μL of PCR-grade water, 0.125 μL (5 U/μL) of Taq polymerase (Promega, USA), and 1 μL of cDNA reaction mix. The PCR procedure was performed at 95℃ for 5min, followed by 32 cycles for and *hsp90*, 28 cycles for *sod*, and 26 cycles for *β-actin* at 95℃ for 30s, 55℃ for 30s, 72℃ for 1min, and the final extension step was done at 72℃ for 10min. The PCR products were separated usinga 1.2% agarose gel stained with ethidium bromide (EB). To confirm the specificity of RT-PCR amplification, the products were purified usingthe agarosegel and sequenced. Electrophoretic images and the optical densities of amplified bands were analyzed using the software Gene Tools (*Syn* gene, USA).The abundance of mRNA was normalized to corresponding β-

Tab.1 Primer sets designed for semi-quantitative RT-PCR analysis of *sod*, *hsp*70, and *hsp*90 mRNA in grass carp, *Ctenopharyngodon idella*

Primers	Primer Sequences	Purposes	Access No.
SOD-F	GGACCAACCGATAGTGAAAG	sqRT-PCR	FJ458445
SOD-R	GAACAAAGCAGAAATCAGAGG	sqRT-PCR	
Hsp90-F	AGCTGGCGGATCGTTCACTGTC	sqRT-PCR	AF068773
Hsp90-R	AAAACTCGCCGTACTCCTCATTGG	sqRT-PCR	
β-actin-F	CAGAGCTTCTCCTTGATGTC	sqRT-PCR	DQ211096
β-actin-F	GATATGGAGAAGATCTGGCA	sqRT-PCR	

Note: SOD-F and SOD-R were used for amplifying *sod*; Hsp90-F and Hsp90-R were used for amplifying *hsp*90; and β-actin-F and β-actin-R are used for amplifying *β-actin*

actin abundance in all samples and expressed as the optical density ratiosof *hsp* or *sod* and *β-actin* ($C_{hsp}/C_{\beta-actin}$ or $C_{sod}/C_{\beta-actin}$).

1.4 Histological analysis

Gill tissues of five individual fish from each treatment including the control (fish without high concentration ammonia exposure) were collected to determine the ammonia exposure effect on the gill cellular microscopic structure. The tissue samples were fixed in the Bouin solution (70% saturated water solution of picric acid, 25% formalin, 5% glacialacetic acid) for 24h, and then transferred to 70% ethanol for storage. For microscopic examination, the samples were dehydrated by serial dilutions using alcohol and embedded in paraffin. Thick sections (6–8 μm) were cut on a microtome and stained with haematoxylin and eosin. The histological slides were examined using a microscope at 40× magnification. The images of each slide were recorded with an Olympus BX-51 camera and subsequently analyzed using the software Image J.

1.5 Statistics

The data were analyzed using the statistical package SPSS for Windows(Version 16.0; SPSS, Chicago, IL, USA). Briefly, the data were tested for homogeneity of variances using Mauchly's Test of Sphericity. Lethal Concentration 50 (LC_{50}) was calculated using Probit analysis. The inter-treatment differences of expression of the *hsps* and *sod* genes were analyzed by one-way ANOVA, and two-way ANOVA was applied to discern significant differences in expression among different tissues at different ammonia concentrations. The differences were considered significant if $P< 0.05$.

2 Results

2.1 Lethal ammonia concentration for the grass carp

There was no mortalityat the ammonia concentration of 100 mg/L. Mortality occurred at the concentration of 150 mg/L, and increased with the increase of ammonia concentration. The mortality rates of ammonia treated grass carp were 4.4%, 26.7%, 40%, 64.4%, and 88.9% at the ammonia concentration of 150, 186, 229, 282 and 350 mg/L, respectively. Probit analysis showed that the ammonia concentration had clear relationship to fish mortality (Probit value =7.6111 lg LC-13.158, R^2= 0.9757, $P<0.05$) and the LC_{50} of ammonia for 24h was 243 mg/L (Fig. 1).

2.2 Histological damage of ammonia exposure

Without exposure to high concentration of ammonia, gill cells arranged compactly and the edge or nuclei were clearly defined. Thus the stained cells and nuclei were dark and clear (Fig. 2A). After a 24h exposure at the ammonia concentration of 104 mg/L, however, the stained cytoplasm was pale pink while the nuclei remained dark, and blank in cell could also be observed (Fig. 2B). After a 24h exposure at the ammonia concentration of 220 mg/L, all the stained cells were pale in color and the edge of the cells and nuclei could not be clearly defined. The edema and fusion of secondary lamellae could be observed (Fig. 2C). Occurrence and severity of gill tissue damage were directly related to ammonia levels and these histological alternations were more obvious at concentration of 220 mg/L, which was near the 24h LC_{50} (243 mg/L).

2.3 Gene expression at different ammonia concentrations

Sod expression Ammonia exposure can induce up-regulation of *sod* gene expression. A two-way ANOVA analysis showed that the up-regulation was affected by both tissue type (liver, intestine and gill; $F_{2, 10}$= 23.755; $P<0.01$) and ammonia concentration ($F_{5, 10}$= 11.159; $P<0.01$) (Fig. 3). In the intestine and liver, the expression of *sod* gene increased with increasing ammonia concentration, while in liver the maximum *sod* gene expression occurred at the ammonia concentration of 151 mg/L. In the liver, level of *sod* at 151 mg/L ammonia was significantly higher than those at the 50, 72 and 220 mg/L
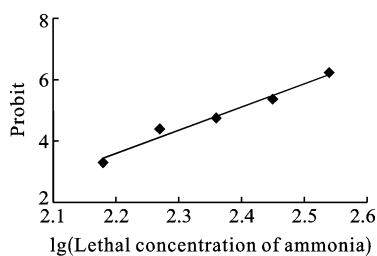

Fig.1 The relationship between the concentration of ammonia-N and the 24h mortality *Ctenopharyngodon idella*

ammonia treatments ($F_{5,12}$=3.460; P<0.05). In the intestine, however, *sod* gene expression at 104, 151, 220 mg/L was significantly higher than the control, 50, and 72 mg/L ammonia treatments ($F_{5,12}$=3.754; P<0.05). In the gill, *sod* level at 220 mg/L ammonia treatment was significantly higher than those of all the other treatments ($F_{5,12}$=11.324; P<0.01).
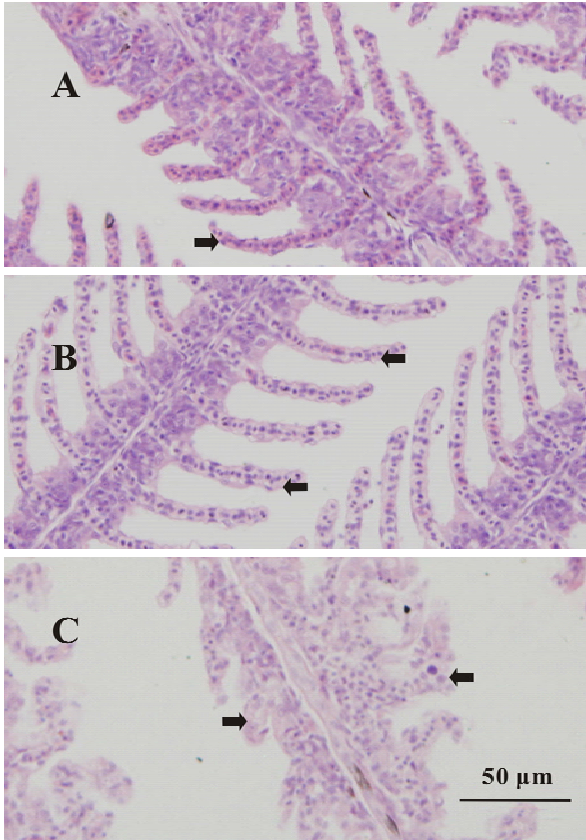

Fig. 2 Gills of grass carp from (A) control without exposure to high concentration of ammonia, (B) exposure to 104 mg/L ammonia, and (C) exposure to 220 mg/L ammonia
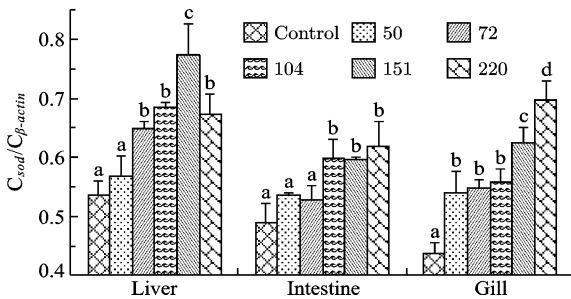

Fig. 3 Effect of ammonia exposure on *sod* mRNA expression in grass carp *Ctenopharyngodon idella*

The control group was the treatment without ammonia exposure. The abundance of *sod* mRNA was normalized to the corresponding β-actin abundance and expressed as the optical densityratio of *sod* and *β-actin* (C*sod*/C*β-actin*).

The values are Mean±SE n = 3). Columns of the same tissue without common superscripts are significantly different (P< 0.05).

Hsp90 gene expression   The responses of *hsp*90 gene expression to ammonia exposure were significantly different at different ammonia concentrations ($F_{5, 10}$= 29.085, P< 0.01). However, no significant difference in the up-regulation of *hsp*90 gene expression was found among the three tissue types (i.e., liver, intestine, and gill) ($F_{2, 10}$=2.704, P = 0.081) (Fig. 4). In the liver, *hsp*90 gene expression reached the maximum value at the concentration of 104 mg/L ammonia and then decreased when ammonia concentration increased. At ammonia concentration of 104 mg/L, *hsp*90 level was significantly higher than both those under other three ammonia concentrations and the control ($F_{5, 12}$=21.699, P< 0.01). In the intestine, *hsp*90 gene expression reached the maximum value at 50 mg/L ammonia exposure and was significantly higher than all other treatments except the 72 mg/L ammonia treatment ($F_{5,12}$=18.147, P<0.01). In the gill, *hsp*90 level increased with increasing ammonia concentration and reached the maximum value at the highest ammonia concentration ($F_{5, 12}$=17.241, P<0.01).
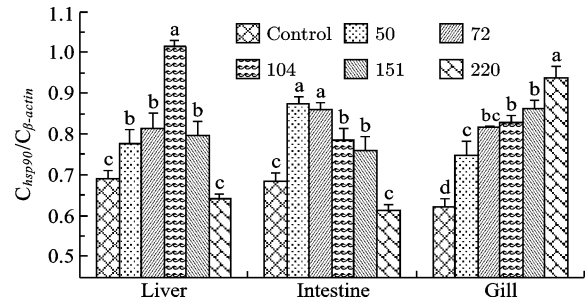

Fig. 4   Effect of ammonia exposure on *hsp*90 mRNA expression

The abundance of *hsp*90 mRNA was normalized to the corresponding β-actin abundance and expressed as the optical density ratio of *hsp*90 and *β-actin* (C*hsp*90/C*β-actin*). The values are Mean±SE n=3   . Columns of the same cluster without common superscripts are significantly different (P<0.05).

3   Discussion

Mortality rates across all the ammonia treatment levels went up in this study. Based on the Probit analysis, the LC50 for 24h was ~243 mg/L at water temperature 20℃ and pH 7.2, the concentration of un-ionized ammonia was ~1.25mg/L. In previous studies, the 24h LC_{50} of un-ionized ammonia for the common carp fry was reported as 1.78 mg/L [38], and LC50 levels for beam, perch, roach, and rudd are 0.29, 0.35, 0.41, and 0.41 mg/L, respectively [39]. Furthermore, tolerance limits of ammonia for different species are different, and common carp and

grass carp showed higher tolerance to ammonia than the other species (add references). However, the lethal concentration of ammonia during chronic exposure for the grass carp was lower than the acute exposure, and the allowable toxicant concentration of un-ionized ammonia for grass carp was far below the 96h LC_{50} as described in previous studies. It has been reported that total ammonia increased directly with culture time, and can reach as high as 46 mg/L in intensive ponds[40]. Therefore, ammonia is one of the major constrains for intensive culture of grass carp and the concentration of ammonia should be monitored regularly during the aquaculture period.

Gill is the most sensitive organ to external stimuli as it is directly exposed to the external environment[41]. Gill damage as a result of exposure to toxicants has beenreported extensively[42—45], and gill damage has also been linked to impaired physiological functions[46]. In the present study, epithelial cell hypertrophy and hyperplasia were observed (Fig.2). The gill cellular structure is very sensitive to ammonia exposure, and thus can be used as an indicator for ammonia stress [47]. Based on the histological data, apparent damages from ammonia stress can be found in the treatments involving high concentration of ammonia, especially in the concentration of 220 mg/L. The damage of gill from ammonia should be a cause for the mortality at high ammonia concentration.

Superoxide dismutase, the major enzyme in phase 1, was used as an indicator to discuss the physiological defense against ammonia exposure in grass carp in this study. The expression of *SOD* mRNA asa protection mechanism against oxidative stress caused by ammonia exposure in gill and digestive gland was determined using semi-quantitative real-time RT-PCR analysis. The expression of *sod* gene is tissue-specific, consistent with are port in sliver carp [48]. In the liver, the highest expression level was observed at 151 mg/L ammonia, and it was significantly higher than other treatments; in both the intestine and the gill, the expression up-regulated at high ammonia concentrations. In previous studies, various types of toxicants significantly induce the expression of SOD [49, 50]. In addition, the decreases in *SOD* mRNA expression after a specific period of exposure may be caused by reduced metabolic capacity of the organism, consistent with the results of several previous studies, indicating that antioxidant enzyme mRNA expression is both time- and dose-dependent and is related to metabolic capacity[51, 52]. The up-regulation of *sod* gene expression further indicated that sod was a useful biomarker to examine ammonia stress in fish.

*Hsp*90 is widely studied recently as an environmental stress indicator and is related to cellular immune functions [53—57]. In grass carp, two isoforms of *hsp*90 have been identified, i.e., *hsp90α* and *hsp90β*. The isoform of *hsp90α* could be induced greatly by environmental stres-

sors [58]. In this study, the expression of *hsp*90 was tissue-specific. In gill, *hsp*90 was up-regulated and in liver the expression reached the maximum at ammonia concentration of 104 mg/L and then decreased. The tissue-specific difference of *hsp*90 gene expression in grass carp indicated that different tissues had different sensitivities to ammonia exposure. The decrease in hsp90 expression in liver could be due to chemical-induced injury [59], and gill is the most sensitive organ to external stimuli as it is directly exposed to the external environment [41], therefore in this study we observed that *hsp*90 expression in gill increased with increasing ammonia concentration. As described in previous studies, *hsp*90 is involved in protein folding, cytoprotection, proteomic degradation, and a number of cellular pathways. Toxicants exposure could surely induce the expression of *hsp*90 gene [60]. It could be inferred from this study that *hsp*90 could be significantly induced by ammonia exposure to cope with the protein damage caused by ammonia exposure, and the protection against ammonia exposure was tissue-specific [61].

In conclusion, ammonia caused large-scale mortality in grass carp and the 24h LC_{50} was about 243 mg/L. The ammonia exposure had dramatic impact on sub- cellular levels, including cellular structure impairments and up-regulation of stressor proteins. The up-regulations of *sod* and *hsp*90 mRNA expressions after ammonia exposure were closely related to ammonia concentration, indicating that these two biomarkers were good indicators to evaluate the effect of ammonia exposure, and ammonia exposure had an apparent impairments to grass carp even in sub-lethal concentration of ammonia.

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sod 和 *hsp*90

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(1.　　　　　　　　　　　　　　　　　　　　　　,　　　266003;

2.　　　　　　　　　　,　　　　　　　　,　　　361005)

:　　　　　　　　　24h　　　　　　,　　　　　　　　　*sod*　　*hsp*90

24h LC_{50}　　243 mg/L

5　　　　　　　　　50　72　104　151　220 mg/L　,　　　　　　　　　　　　　　　　　　　　　,

sod　　*hsp*90　　　　　　　　　　　　　　　　　　,　　　　　　　　　　　　　　　　　　,

sod　　*hsp*90　　　　　　　　　　　　　　,

,　*sod*　　*hsp*90

:　　;　　　　　; 24h　　　　　　;　　　　　; *sod*; *hsp*90