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Dietary nano cerium oxide promotes growth, relieves ammonia nitrogen stress, and improves immunity in crab (*Eriocheir sinensis*)

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ABSTRACT

Oxidative stress plays a crucial role in ammonia nitrogen toxicity. In this study, the beneficial effects of dietary nano cerium oxide (nano CeO_2) as a potent antioxidant were examined in the Chinese mitten crab (*Eriocheir sinensis*). Crabs were fed a diet supplemented with 0, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, or 12.8 mg/kg nano CeO_2 for 60 d. The optimum supplementation level of nano CeO_2 that significantly increased weight gain rate and decreased feed coefficient was 0.8 mg/kg. This level also offered immune protection when crabs were kept under ammonia nitrogen stress and/or exposed to pathogen infection (*Aeromonas hydrophila*). Supplementation with 0.8 mg/kg of CeO_2 (i) relieved pathological damage to the hepatopancreas; (ii) increased hemocyte counts, including total number of hemocytes, granulocytes, and hyalinocytes; (iii) decreased malondialdehyde content and increased antioxidant enzyme activities of superoxide dismutase and catalase in the hemolymph; (iv) increased the activities of lysozyme, acid phosphatase, and alkaline phosphatase in the hemolymph; and (v) increased gene and protein expression of cathepsin L in the hepatopancreas. Mortality increased when crabs were injected with bacteria under ammonia nitrogen stress, but dietary supplementation with 0.8 mg/kg nano CeO_2 decreased the mortality rate. Thus, the results of this study suggested that dietary supplementation with nano CeO_2 in crabs promoted growth and up-regulated immunity to bacterial infection under ammonia nitrogen stress.

1. Introduction

Urban development and human activities are increasing the ammonia nitrogen (NH_3–N) content in freshwater and marine water, which can influence aquatic ecosystems and the quality of life of aquatic inhabitants [1–3]. NH_3–N is a major end product of protein catabolism, and its buildup in water becomes a limiting factor for health of aquatic animal in high density intensive breeding systems where aquaculture species live in most of their lives [4]. Thus, high NH_3–N content in water has negative consequences for the aquatic animal breeding industry. Studies have shown that long-term exposure to high concentrations of NH_3–N in water can hinder the growth of animals, decrease their immunity, and increase their susceptibility to pathogens [5,6]. Therefore, studies of the toxicity of NH_3–N in water and protection measures have become a hot topic in the aquaculture all over the world.

Native freshwater crabs have special economic value in China. The Chinese mitten crab (*Eriocheir sinensis*) is widely cultured throughout the eastern part of mainland China [7]. Since the early 1990s, the crab-breeding industry has made considerable progress [8], and pond culture has become the most important method of Chinese mitten crab breeding industrialization [9]. Water quality is the primary factor influencing the growth of crabs, which are more sensitive to un-ionized ammonia (NH_3) [10,11]. Increased NH_3–N content in water has been reported to reduce hemolymph cell number and hemocyanin levels, suppress the hemocyte phagocytosis rate, decrease the activities of lysozyme and phenol oxidase, and destroy the immune defense system, including nonspecific immune cells and immune response factors in the hemolymph [12–14]. Increased NH_3–N in water also was reported to reduce immunity against pathogens and the ability of crabs to remove free radicals [15–17]. These studies suggested that the free radical-mediated pathway was involved in the adverse effects of NH_3–N

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exposure in crabs.

Cerium (Ce) is a rare earth element that the National Research Council of the National Academies (NRC) in USA does not classify as an essential nutrient; however, in East Asia it is widely used as a growth promoter for aquaculture species and livestock [18]. Ce has a wide range of biological activities, including bactericidal, anti-inflammatory, growth promotion, resistance to oxidative stress, and immune regulation [19–23]. Nano cerium oxide (CeO_2) particles are small (6–25 nm), and their absorption efficiency is much higher than that of traditional cerium nitrate and CeO_2 [24]. These features allow nano CeO_2 to achieve an observable supplement effect at a lower usage dose and reduce environmental residues of this rare earth element [25]. As an immune enhancer, the immune function of nano CeO_2 mainly comes from its antioxidant capability [26]. At the nano scale, Ce^{4+} is reduced to Ce^{3+} to stabilize surface oxygen defects. Therefore, Ce^{3+} and Ce^{4+} in nano CeO_2 particles can be reversibly transformed, which makes nano CeO_2 an antioxidant that can be recycled [27]. It has been reported that CeO_2 is a free radical scavenger with a long half-life, which allows it to catalyze the decomposition of excessive free radicals, reduce the potential damage caused by free radicals, and protect the integrity of proteins and DNA [28,29]. Nano CeO_2 also provides a protective effect against certain harmful environmental factors by enhancing the activity of natural killer cells and the expression of immune factors, thereby improving the immune ability of the body and protecting hematopoietic tissue from damage *in vivo* [30,31].

Pathogens are important factors that can induce disease, affect growth, and even cause death of crabs. The bacterium *Aeromonas hydrophila* is widespread in fresh water and can induce disease in many aquatic animals, resulting in growth retardation and in outbreaks of ascites infection, edema, enteritis, and liver necrobacillosis in *Eriocheir sinensis* [32]. Hematocytes are the major cellular components of the crustacean immune system, and they clear pathogens by adhering to them, triggering phagocytosis, and producing highly toxic reactive oxygen species (ROS) [33,34]. However, ROS are highly reactive molecules that can damage DNA, proteins, and cell membranes, resulting in pathological damage to tissues [35,36]. Nano CeO_2 particles are known to catalyze the decomposition of ROS such as superoxide radical and hydrogen peroxide because of their superoxide dismutase (SOD)-like and catalase (CAT)-like activities [37].

The goals of this study were to determine whether the adverse health effects of NH_3–N on *Eriocheir sinensis* can be prevented by dietary supplementation with nano CeO_2 and whether nano CeO_2 can promote *Eriocheir sinensis* growth and resistance to the pathogen *A. hydrophila*.

2. Materials and methods

2.1. Synthesis and characterization of nano CeO_2 particles

Nanosized CeO_2 particles were synthesized by the conventional hydrothermal method. Half a gram of polyvinylpyrrolidone (molecular weight 30,000 Da) and 1 mmol of cerium nitrate hexahydrate ($Ce(NO_3)_3{\cdot}6H_2O$) (Shanghai Aladdin, Shanghai, China) were dissolved in 50 mL of ultrapure water. Next, 1 mmol of ammonia water was added to the mixture, which was stirred for 30 min at room temperature. The product was transferred to a 50 mL Teflon lined autoclave and heated at 120 °C for 12 h. The precipitate was collected by centrifugation, then washed several times with ethanol and ultrapure water. Finally, the CeO_2 powder was obtained by drying the precipitates in an oven at 60 °C for 2 h. Structural analysis of CeO_2 nanocrystals was performed using a JEM-2100F transmission electron microscopy (TEM, JEOL, Tokyo, Japan) with an Energy-dispersive X-ray (EDS) accessory and an X-ray diffractometer (XRD) (Bruker AXS, Billerica, MA, USA) with Cu Kα radiation (λ = 1.5406 Å).

Table 1
Composition of the complete formula feed.

Ingredient	Content (%)	Ingredient	Content (%)
Fish meal	17	Blood meal	3
Cotton seed meal	17	Shrimp shell meal	3
Rape seed meal	16	Soybean oil + rape seed oil	2
Wheat meal	10.5	$Ca(H_2PO_4)_2$	1.5
Soybean meal	10	Zeolite powder	2
Corn	9	Crab additive	1
Rice bran	5	Vitamin premix	1
Adhesive	1	Mineral premix	1

Notes: The crab mineral premix provided the following per 100 g: NaH_2PO_4 3.5 g, KH_2PO_4 6.0 g, $CaCO_3$ 3.5 g, KCl 0.6 g, $MgSO_4{\cdot}7H_2O$ 3.2 g, $AlCl_3{\cdot}6H_2O$ 0.55 g, $ZnSO_4{\cdot}7H_2O$ 0.157 g, ferric citrate 0.019 g, $MnSO_4{\cdot}4–6H_2O$ 0.043 g, KI 0.016 g, $CuCl_2$ 0.014 g, $CoCl_2{\cdot}6H_2O$ 0.055 g, calcium lactate 5.15 g.

2.2. Preparation of test diet

The experimental diet containing nano CeO_2 was prepared following the procedure previously described [38]. Table 1 shows the composition of the crab commercial complete formula feed (Suzhou Xinyu Feed, Suzhou, China), including the mineral premix. Nano CeO_2 at 0, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, or 12.8 mg/kg was added and mixed with the complete formula feed and then pelletized using a mincer. Granular test diets (2 mm in diameter, 3 mm in length) for crabs were dried, packaged, and stored at −20 °C. The content of cerium in the test diets containing different supplement levels of nano CeO_2 was measured, and the results are shown in Table 2.

2.3. Animals and experimental setup

In experiment I, healthy juvenile Chinese mitten crabs (11.56 ± 0.54 g) were obtained from Modern Fishery Development Co. LTD in Linhu, Suzhou, China. The crabs were immediately transferred to plastic tanks (300 L) in the Aquatic Laboratory of Soochow University. The animals were kept at a 12 h/12 h light-dark cycle and held in tanks equipped with pre-aerated running water obtained from municipal waterworks at a temperature of 25 ± 1 °C. The quality parameters of water were maintained daily at pH, 7.86 ± 0.05; salinity, 0.3%; oxygen concentration, 6.3 ± 0.2 mg/L; total ammonia 0.35 ± 0.02 mg/L; and basal nitrite < 0.05 mg/L. The crabs were fed with basic pellets twice daily at 3% of body weight. Fecal matter was removed from each tank daily, and the water exchange rate was 20% per day. Four hundred crabs were randomly divided into eight groups of 50 each. They were fed for 60 d with diets supplemented with nano CeO_2 (0, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, or 12.8 mg/kg). Their weights were recorded on alternate days. The weight gain rate (WGR) and feed coefficient (FC) were determined as follows, and the optimum addition level of nano CeO_2 was obtained.

$$WGR = 100\% \times (Wt - W0) / W0$$

$$FC = F/(Wt - W0)$$

Table 2
Content of cerium in the test diet for different supplement levels of nano CeO_2.

Added levels of nano CeO_2 (mg/kg)	Ce content in diet (mg/kg)
0	0.007 ± 0.001
0.2	0.158 ± 0.006
0.4	0.334 ± 0.012
0.8	0.661 ± 0.014
1.6	0.962 ± 0.019
3.2	2.578 ± 0.027
6.4	4.200 ± 0.067
12.8	9.614 ± 0.115

Table 3
Experimental design groups in Experiment II.

Groups		Nano CeO_2	NH_3–N exposure	*A. hydrophila*
Control (0 mg/kg)	Blank	–	–	–
	NH_3–N	–	+	–
	NH_3–N + *A. hydrophila*	–	+	+
Nano CeO_2 (0.8 mg/kg)	Blank	+	–	–
	NH_3–N	+	+	–
	NH_3–N + *A. hydrophila*	+	+	+

Where W0 is the initial average weight (g); Wt is the final average weight (g); and F is the consumed feed weight (g).

Experiment II was conducted to determine whether dietary nano CeO_2 could relieve the stress induced by NH_3–N and enhance crab resistance to bacterial infection by regulating immunity. In this experiment, 720 crabs (10.79 ± 0.62 g) were randomly divided into control and nano CeO_2 experimental groups. The control group received 0 mg/kg nano CeO_2 in the diet, whereas the experimental group received 0.8 mg/kg nano CeO_2: this dosage was based on the best WGR and FC levels observed in experiment I. At the end of 90 d, each group was divided into three sub-groups: blank, NH_3–N stress, and double stress of NH_3–N and *A. hydrophila* infection (NH_3–N + *A. hydrophila*) (Table 3). Crabs in the latter group were injected with *A. hydrophila* (Strain CL99920) bacterial suspension (4×10^6 CFU/kg diluted with saline) through the third pereiopoda basipodite before exposure or not to NH_3–N (25 mg/L). After 96 h, the number of dead crabs was recorded to determine the efficiency of immune protection provided by nano CeO_2. Each live crab was sacrificed and hemolymph was collected and stored in a heparin sodium anticoagulant tube to determine hemocyte counts, antioxidant index (SOD, CAT, and malondialdehyde (MDA)), and immune enzyme activities (lysozyme (LZM), acid phosphatase (ACP), and alkaline phosphatase (AKP)). Half of the hepatopancreas was used for the histopathology assay, and the other half was stored at −80 °C to measure the expression level of cathepsin L using real time polymerase chain reaction (PCR) and Western blot analyses. The immune protection efficiency was calculated for each treatment using the following formula: (control mortality rate - nano CeO_2 mortality rate) × 100/control mortality rate. Each experiment was repeated three times, and each repeat contained 40 crabs kept in two tanks.

2.4. Hepatopancreas histopathology

For the histopathology analysis, the hepatopancreas of each crab was fixed in neutral buffered formalin and processed for paraffin embedding. Paraffin blocks were sectioned at 5 μm thickness using a microtome (Leica RM2245, Wetzlar, Germany). Each sample was stained with hematoxylin and eosin (H&E) and observed with a Leica microscope DM2000 (Wetzlar, Germany). Histological components in hepatic tubules, including the basement membrane, lumen, R cells, B cells, and other cells, were observed and compared between the blank group and the NH_3–N/NH_3–N + *A. hydrophila* stress groups without nano CeO_2 and between crabs supplemented with 0 mg/kg and 0.8 mg/kg nano CeO_2 under the same stress conditions.

2.5. Hemolymph analysis

The hemolymph from each crab was divided into two aliquots [38]. One was used to count the number of hemocytes, and the other was centrifuged at relative centrifugal force of 3,752 g at 4 °C for 15 min. The supernatant was stored at −80 °C and later used to measure the antioxidant index and immune enzyme activities.

2.5.1. Hemocyte level

A hemocytometer and DMIL microscope (Leica, Wetzlar, Germany) were used to record the total number of hemocytes (THC), granulocytes (LGC), semi-granulocyte (SGC), and hyalinocytes (HC) per mL.

2.5.2. Antioxidant indexes and immune enzyme activities in the hemolymph supernatant

Commercially available kits purchased from Jiancheng Bioengineering Institute (Nanjing, China) were used to measure the antioxidant index following the manufacturer's protocols. MDA (nmol/mL, thiobarbituric acid method), SOD (U/mL, hydroxylamine method), CAT (U/mL, ammonium molybdate method), LZM (U/mL, colorimetric method) and ACP and AKP (U/L, potassium ferricyanide colorimetric method) were all measured in the hemolymph supernatant using a UV/visible-610s spectrophotometer (Shanghai Precision Instrument Co., Ltd., Shanghai, China).

2.6. Expression of the cathepsin L gene

The hepatopancreas from each crab was collected after 96 h of exposure to NH_3–N stress with or without *A. hydrophila* infection for real time PCR analysis. Total RNA was isolated using the Mini Kit (Qiagen, Duesseldorf, German), and the quality and quantity were determined using a BioPhotometer (Analytik Jena AG, Jena, Germany). Quantitative real-time PCR was conducted on the QuantStudio™ 7 Flex Real-Time PCR system (Life Technologies, Carlsbad, CA, USA) using 2.5 × SYBR Green Abstart One Step RT-PCR Mix (Sangon Biotech Co., Ltd, Shanghai, China) according to the manufacturer's instructions. Primers were designed and synthesized as follows: cathepsin L (GenBank: GU593246.1), 5′-TACCGCAGCACCAAGGAGGAC-3' (forward) and 5′-GCAGA CAGGAAGCCGTTCATAGC-3' (reverse); β actin (GenBank: NM_007393.5), 5′-GCGAGGCTACACCTTCACGA-3' (forward) and 5′-CAGCGGAACCTCTCATTGCC-3' (reverse) (Sangon Biotech). β-actin, a house-keeping gene, was used as the internal control. Relative changes in gene expression levels were determined using the $2^{-\Delta\Delta CT}$ method. Every sample were performed in triplicate.

2.7. Expression of the cathepsin L protein

Extraction and quantification of total protein from the hepatopancreas of each crab were conducted using a Protein Extraction Kit and BCA Protein Assay Kit (Merck, Darmstadt, Germany). For each sample, 40 μg of protein were loaded onto a sodium dodecyl sulfate polyacrylamide gel, transferred onto Immobilon™-P PVDF membranes (Sigma-Aldrich, Shanghai, China), and blocked as described in a previous study [38]. The membranes were incubated overnight at 4 °C using primary rabbit polyclonal *E. sinensis* cathepsin L antibody (1:500) (WuXi AppTec, Suzhou, China) and rabbit monoclonal anti-actin antibody (1:1,000) (Merck). After the second incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000) (Thermo Scientific, Rockford, IL, USA) at room temperature, the immunoblot signals were visualized using the Pierce SuperSignal West Pico chemiluminescent kit (Thermo Scientific, Waltham, MA, USA).

2.8. Statistical analysis

All data were analyzed using SPSS software (SPSS version 17), and the results are presented as mean ± standard deviation. They were subjected to one-way analysis of variance. Duncan's multiple range test was used to compare multiple stress treatments (NH_3–N stress with or without bacteria infection). *T*-tests were used to compare data from the control group (without nano CeO_2) and nano CeO_2 group. $P < 0.05$ was considered to be statistically significant.

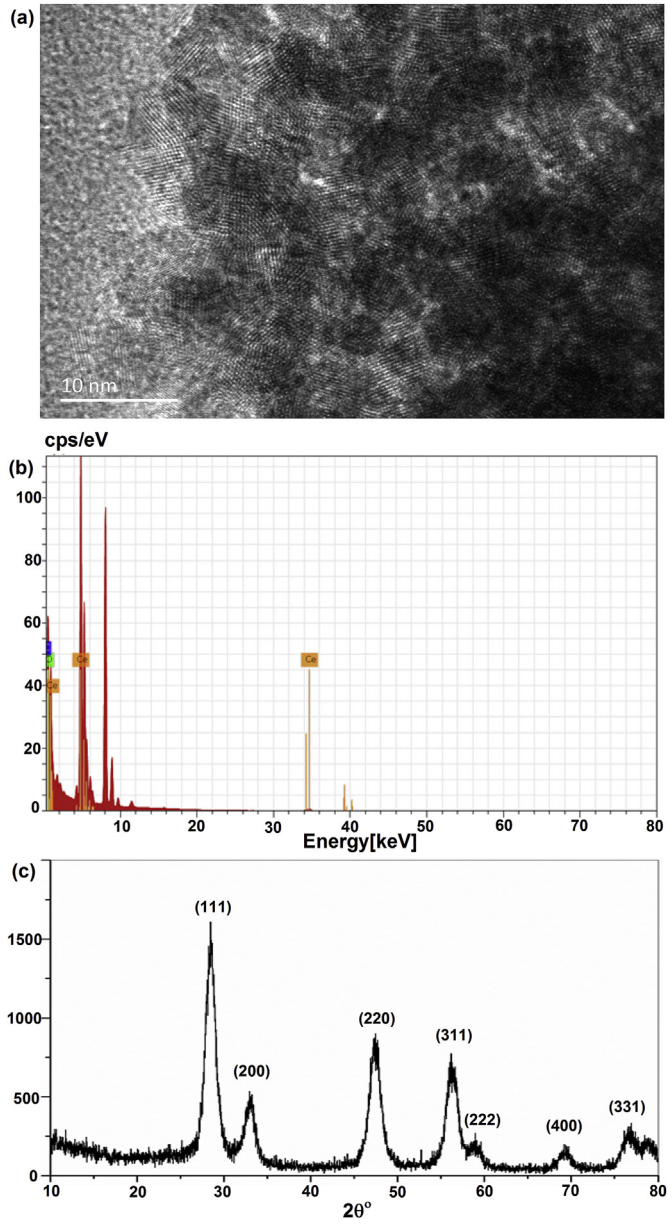

Fig. 1. Characterization of nano CeO_2: (a) HRTEM image of nano CeO_2 particles; (b) EDS spectrum of nano CeO_2 particles; (c) XRD pattern of nano CeO_2 particles.

3. Results

3.1. Nano CeO_2 particles were successfully synthetized

Fig. 1a shows the shape and size of the nano CeO_2 particles observed under TEM. The average size of the particles was 5.06 ± 0.48 nm. The EDS spectrum confirmed the presence of the representative peaks of Ce and O (Fig. 1b). The corresponding XRD pattern (Fig. 1c) displays the crystal structure and the phase of nano CeO_2. The low diffraction peaks of 28.6°, 33.04°, 47.5°, 56.06°, 59.02°, 69.44°, and 76.65° corresponded to the (111), (200), (220), (311), (222), (400), and (331) planes of CeO_2 (JCPDS 34–0394), and the broadened diffraction peaks illustrate the relatively low crystallinity and lessened size of the crystallites of the synthetized nano CeO_2.

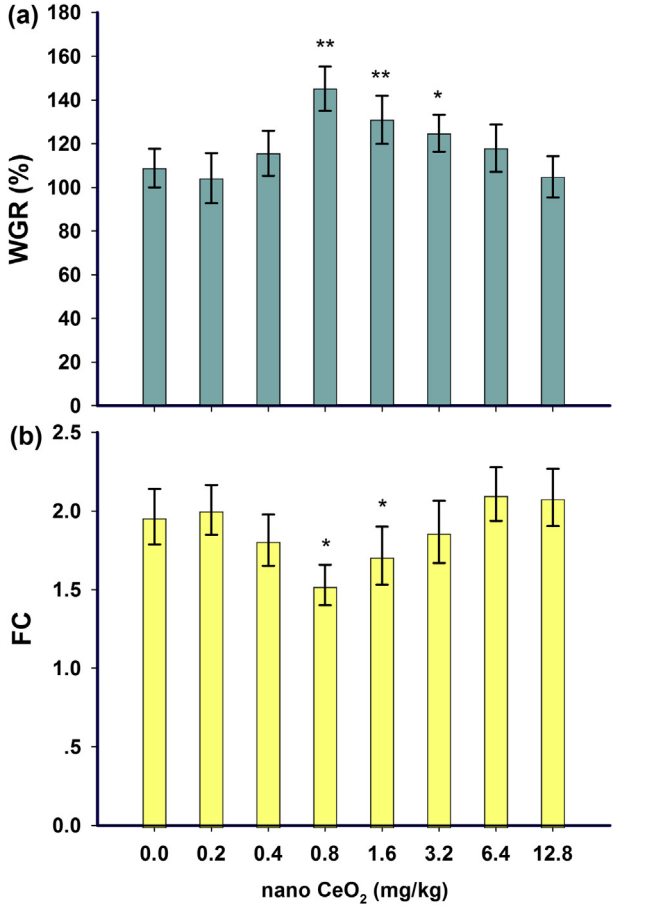

Fig. 2. Changed levels of weight gain rate (WGR) (a) and feed coefficient (FC) (b) in crabs fed with a diet supplemented with various doses of nano CeO_2. Significance of difference: groups with different doses of nano CeO_2 compared with the 0 mg/kg group, WGR, $^{*}P < 0.05$, $^{**}P < 0.01$; FC, $^{*}P < 0.05$.

3.2. Effects of dietary nano CeO_2 on growth of juvenile Chinese mitten crabs

Fig. 2 shows the results of the growth performance experiment in which crabs were fed diets supplemented with eight different levels of nano CeO_2. Compared to the control (0 mg/kg), the WGR was significantly higher in the 0.8, 1.6, and 3.2 mg/kg nano CeO_2 supplemented groups ($P < 0.05$), and FC was significantly lower in the 0.8 and 1.6 mg/kg nano CeO_2 groups ($P < 0.05$). The most significant changes (up- or downregulation) in WGR and FC were observed in the 0.8 mg/kg nano CeO_2 supplemented group, as indicated by the increase in WGR from 108.90% (0 mg/kg) to 145.26% and the decrease in FC from 1.96 (0 mg/kg) to 1.52, respectively. Therefore, 0.8 mg/kg nano CeO_2 was considered to be the optimum supplementation dose in the diet of the crabs.

3.3. Mortality

The results of mortality in Fig. 3 showed that there is no death in blank groups with or without nano CeO_2. Compared to the blank group, NH_3–N stress alone or the double stress of NH_3–N and *A. hydrophila* infection for 96 h significantly increased mortality rates from 0% to 30.67% ($P < 0.01$) or 77.33% ($P < 0.01$), respectively. However, 0.8 mg/kg nano CeO_2 supplementation significantly reduced mortality from 30.67% to 24.00% ($P < 0.05$) and from 77.33% to 42.67% ($P < 0.01$) for the single stress or double stress treatments, respectively. Thus, 0.8 mg/kg nano CeO_2 supplementation improved immune protection efficiency by 44.82% when crabs were infected with *A. hydrophila* under NH_3–N stress for 96 h.

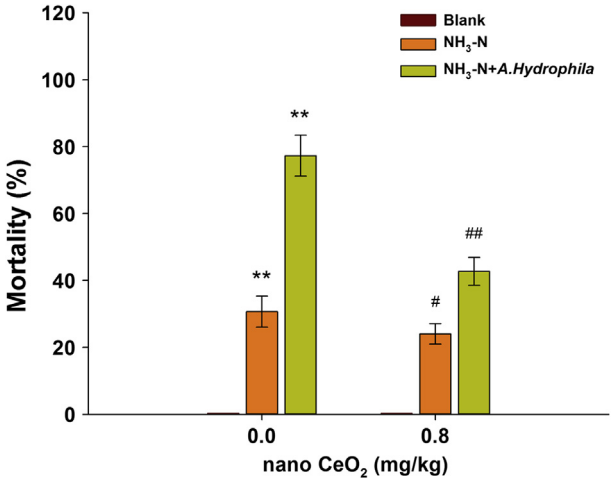

Fig. 3. Increased mortality rates of crabs exposed to single NH_3–N or the double threat of NH_3–N and *A. hydrophila* for 96 h and the remission effect of feeding them with the 0.8 mg/kg nano CeO_2 supplemented diet. Significance of difference: NH_3–N stress alone or the NH_3–N + *A. hydrophila* double stress group compared with the mortality (0%) in the blank group without nano CeO_2 (0 mg/kg): $^{**}P < 0.01$; the 0.8 mg/kg nano CeO_2 group compared with the 0 mg/kg nano CeO_2 group under the same stress condition, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

3.4. Hepatopancreas histopathology

The hepatopancreas from blank group crabs without nano CeO_2 supplementation had normal hepatic tubules that consisted of basement membrane, lumen, R cells, B cells, and other cells (Fig. 4). After 96 h of NH_3–N exposure alone, the hepatopancreas structure of crabs without nano CeO_2 supplementation became loose, and the number of B cells increased and individual cells were enlarged; in contrast the hepatopancreas histological structure of crabs treated with 0.8 mg/kg nano CeO_2 was not significantly changed. The double stress of NH_3–N + *A. hydrophila* markedly damaged the hepatopancreas tissue structure, making it observably loose, causing the tubules to appear atrophic, and inducing hepatopancreas cell detachment from the basement membrane. For crabs fed with 0.8 mg/kg nano CeO_2, the double stress also induced structural looseness of the hepatopancreas, but almost no atrophy was detected. Thus, nano CeO_2 supplementation partly relieved the damage induced by NH_3–N single stress and NH_3–N + *A. hydrophila* double stress.

3.5. Hemolymph analysis

3.5.1. Immunase activity in hemolymph plasma

Fig. 5 shows the changes in LZM, ACP, and AKP activities in the plasma of the crabs. Compared to the blank group crabs without nano CeO_2, NH_3–N stress alone or NH_3–N + *A. hydrophila* double stress induced significant increases in the activities of all three immune enzymes in the hemolymph plasma of crabs without nano CeO_2 supplementation ($P < 0.05$). Nano CeO_2 supplementation further enhanced LZM activity in the hemolymph plasma of crabs under both types of stress ($P < 0.01$). Further enhancement of activities of ACP ($P < 0.01$) and AKP ($P < 0.05$) by nano CeO_2 diet supplementation were seen only in crabs under the double stress of NH_3–N + *A. hydrophila*.

3.5.2. Hemocyte level (THC, LGC, SGC, and HC counts)

As showed in Fig. 6, THC, LGC, and SGC counts in the hemolymph of crabs under NH_3–N stress for 96 h were significantly lower than those of crabs in the blank group crabs without nano CeO_2 supplementation ($P < 0.05$) while there was no significant change in HC cells ($P > 0.05$). The double stress of NH_3–N + *A. hydrophila* caused the same significant decrease in THC, LGC, and HC counts ($P < 0.01$) in the hemolymph, although SGC count increased ($P < 0.01$). The nano CeO_2 supplemented diet (0.8 mg/kg) increased the counts of THC, SGC, and HC in the crab hemolymph in blank group ($P < 0.05$), and this enhancement effect happened in the counts of THC, LGC and HC in the two stress groups ($P < 0.05$).

3.5.3. Antioxidant capacity of hemolymph plasma

Compared to the blank group, exposure to NH_3–N stress alone or to NH_3–N + *A. hydrophila* double stress for 96 h decreased the SOD and CAT activities and increased the MDA content in the hemolymph plasma of crabs in the group without nano CeO_2 supplementation ($P < 0.05$) (Fig. 7a–c). In contrast, the nano CeO_2 supplemented diet (0.8 mg/kg) increased the SOD activity and decreased the MDA content in the crab hemolymph ($P < 0.01$) in the blank group, and it improved the SOD and CAT activities and decreased the MDA content in the two types stress groups ($P < 0.05$) (Fig. 7 a–c). These data suggested that nano CeO_2 supplementation improved the antioxidant capacity indexes in crabs under NH_3–N stress alone or combined with *A. hydrophila* infection.

3.6. Cathepsin L expression

Without nano CeO_2 supplementation, expression of the cathepsin L

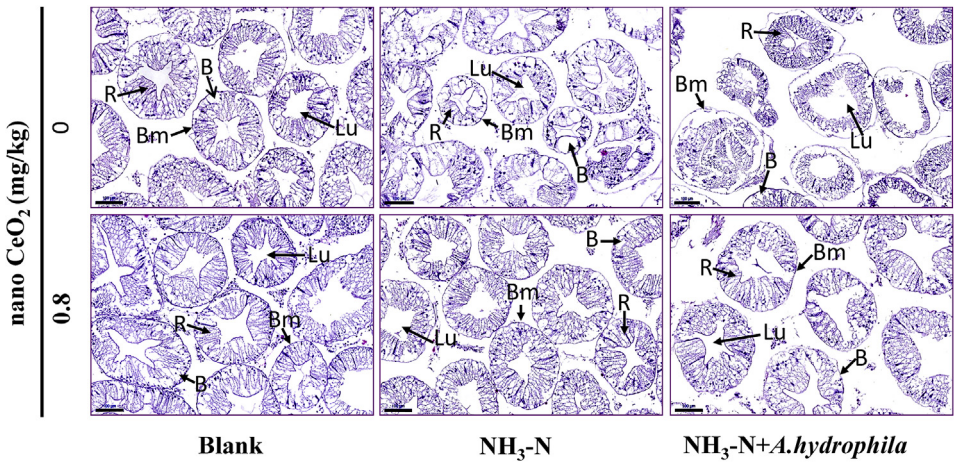

Fig. 4. Histopathology change in the hepatopancreas of crabs with or without the nano CeO_2 supplemented diet exposed to NH_3–N or NH_3–N + *A. hydrophila* for 96 h. Basement membrane (Bm); Lumen (Lu); B cells (B); R cells (R). Paraffin sections were sectioned at 5 μm and stained with H&E. Bar length = 100 μm.

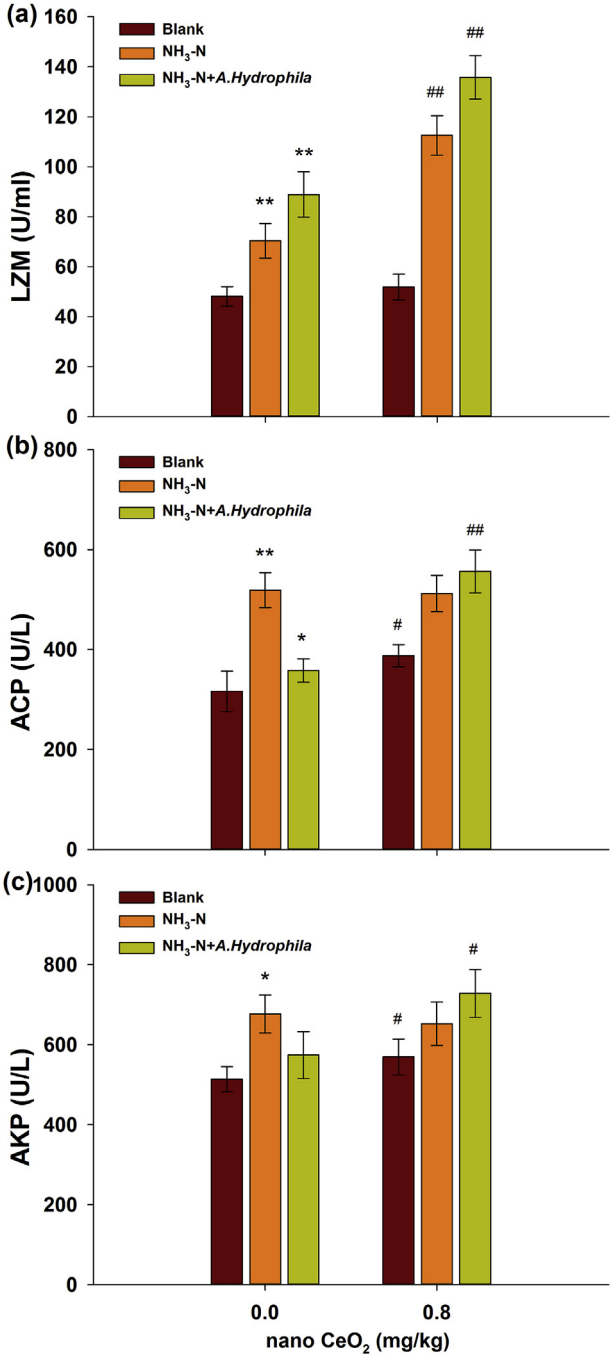

Fig. 5. Changed activities of immune enzymes [lysozyme (LZM), acid phosphatase (ACP), and alkaline phosphatase (AKP)] in crabs with or without the 0.8 mg/kg nano CeO_2 supplemented diet exposed to NH_3–N or NH_3–N + *A. hydrophila* for 96 h. Significance of difference: NH_3–N stress alone or NH_3–N + *A. hydrophila* double stress group compared with the blank group without nano CeO_2 (0 mg/kg), $^{*}P < 0.05$; $^{**}P < 0.01$; the 0.8 mg/kg nano CeO_2 group compared to the 0 mg/kg nano CeO_2 group under the same stress condition, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

gene in the hepatopancreas was significantly downregulated in crabs under NH_3–N stress for 96 h (0.36-fold) or NH_3–N + *A. hydrophila* double stress (0.29-fold) compared to that of the blank group ($P < 0.01$). Nano CeO_2 supplementation increased cathepsin L gene expression in the hepatopancreas of crabs (1.66-fold) in the blank group, under NH_3–N alone stress (from 0.36-fold to 0.63-fold, $P < 0.05$), and under NH_3–N + *A. hydrophila* double stress (from 0.29-fold to 3.08-fold. $P < 0.01$) (Fig. 8a). Similar effects on cathepsin L protein expression were observed for NH_3–N stress or NH_3–N + *A. hydrophila* double stress and intervention with nano CeO_2 (Fig. 8b). Gene and protein expression of cathepsin L were downregulated when crabs were exposed to both stress treatments, whereas nano CeO_2 supplementation (0.8 mg/kg) improved both cathepsin L gene and protein expression, especially when crabs were infected by *A. hydrophila* under NH_3–N stress.

4. Discussion

Nano CeO_2 is an oxide of a rare earth element, and due to its antioxidative properties it has potential uses in biomedical applications, such as protecting cells against radiation damage, oxidative stress, and inflammation [20,37]. The small size of nano CeO_2 particles allows them to enter the cells *in vitro* via clathrin-mediated endocytosis, where they can be distributed to multiple subcellular compartments such as the cytoplasm, mitochondria, endoplasmic reticulum, and nucleus [39]. In hens, dietary supplementation with CeO_2 (50 nm) was reported to increase egg laying and up-regulate mRNA expression of digestive enzyme activities such as pancreatic amylase and trypsin [40]. In the current study, Chinese mitten crabs were fed a diet supplemented with different levels of nano CeO_2 (0, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 mg/kg) for 60 d. The optimum supplementation level was 0.8 mg/kg nano CeO_2 based on WGR and FC, thus this dosage could provide an economic benefit to the aquaculture industry.

In the next experiment, crabs were fed a diet supplemented with either 0 mg/kg or 0.8 mg/kg nano CeO_2 for 90 d; crabs in each group then were randomly divided into three subgroups (blank, NH_3–N, NH_3–N + *A. hydrophila*), and they were treated with or without single NH_3–N stress or the double stress of NH_3–N and *A. hydrophila* for 96 h. NH_3–N stress increased the mortality of crabs, whereas the diet supplemented with nano CeO_2 decreased the mortality of crabs under NH_3–N stress. Higher levels of NH_3–N have been shown to have harmful effects on the immune system [41]; for example, phagocytic activity and clearance efficiency of pathogenic bacteria decreased in crustaceans living in water containing high NH_3–N concentration [42]. In the current study, the mortality rate of crabs infected with *A. hydrophila* under NH_3–N stress was reduced when they were given a diet supplemented with nano CeO_2 (0.8 mg/kg) for 90 d. These results suggested that dietary nano CeO_2 lowered the mortality risk of crabs that was induced by elevated NH_3–N levels and increased immune protection from bacterial infection under NH_3–N stress. In other words, a diet containing nano CeO_2 0.8 mg/kg alleviated immune damage under NH_3–N stress.

Crustaceans have a primitive immune system that depends mainly on phagocytosis, wrapping, and agglutination [43]. A cellular response mediated by hemocytes, which constitutively induce LZM and antimicrobial peptides to dissolve the invading microorganisms, is an important method of immune resistance [33,44]. Hemocytes play an important role in the immune response of crustaceans [45]. A common evaluation index of the immune status of crustaceans is the change in THC, which reflects cellular immunity and the secretion of humoral immune factors [46]. It was previously reported that NH_3–N stress or *A. hydrophila* infection both significantly reduced the number of hemocytes in *E. sinensis* [47,48]. In the present study, 96 h of NH_3–N stress decreased the THC in the hemolymph of the crabs, and the double stress of NH_3–N exposure and *A. hydrophila* infection induced an even greater reduction in THC. LGC in the hemolymph showed similar changes under the same stress conditions. However, HC and SGC varied in different ways. Overall, dietary supplementation with nano CeO_2 (0.8 mg/kg) significantly improved the THC, LGC, and HC in the hemolymph of crabs under NH_3–N stress alone or in combination with *A. hydrophila* infection for 96 h.

Hemocytes produce highly toxic ROS and trigger phagocytosis [49]. In this study, 96 h of NH_3–N stress increased the levels of MDA in the hemolymph of crabs, and the double stress of *A. hydrophila* infection

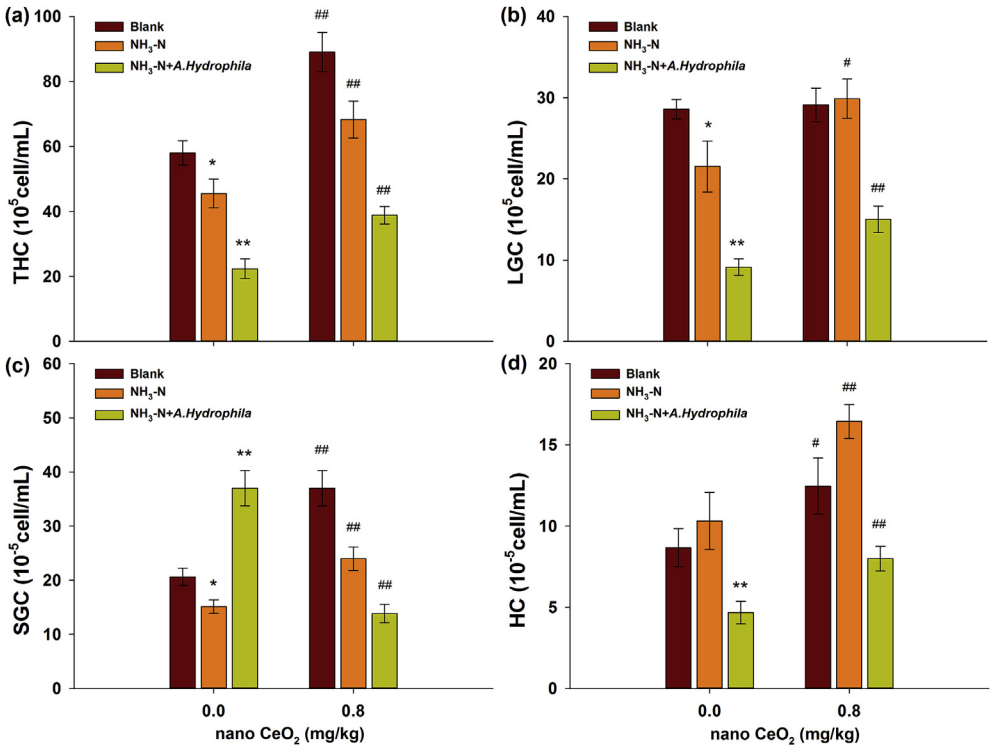

Fig. 6. Changed numbers of hemocytes [total number of hemocytes (THC), granulocytes (LGC), semi-granulocytes (SGC), and hyalinocytes (HC)] in crabs with or without the 0.8 mg/kg nano CeO_2 supplemented diet exposed to NH_3–N or NH_3–N + *A. hydrophila* for 96 h. Significance of difference: NH_3–N stress alone or NH_3–N + *A. hydrophila* double stress group compared with the blank group without nano CeO_2 (0 mg/kg), $^{*}P < 0.05$; $^{**}P < 0.01$; the 0.8 mg/kg nano CeO_2 group compared to the 0 mg/kg nano CeO_2 group under the same stress condition, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

and NH_3–N exposure induced an even sharper increase in MDA level. However, the activities of CAT and SOD decreased significantly. Thus, these results suggested that the decreased immunity induced by NH_3–N stress may have been due to oxidative injury. Nano CeO_2 has been shown to possess antioxidant properties and to scavenge superoxide radicals [50]. Furthermore, nano CeO_2 was shown to induce SOD and CAT activities in a cell-free system [51,52]. In our previous studies, nano CeO_2 prevented damage induced by sleep deprivation by improving the antioxidant capacity of tissue in mice [53]. In the current study, dietary supplementation with nano CeO_2 (0.8 mg/kg) significantly increased activities of SOD and CAT and decreased the level of MDA in the hemolymph of crabs under NH_3–N stress alone or the double stress of NH_3–N and pathogen infection. These observations suggested that nano CeO_2 reduced the oxidative stress induced by NH_3–N or the double stress of NH_3–N and pathogen infection.

The body fluids of crustaceans do not contain immunoglobulins for an antibody-mediated immune response, but they do contain enzymes that participate in immune reactions, such as LZM, AKP, and ACP [54]. LZM is mainly produced by neutrophils and mononuclear cells, although a small amount is produced by macrophages [55] and is important for phagocyte sterilization. NH_3–N stress for 96 h was shown to promote the activities of LZM, AKP, and ACP in the hemolymph serum of the crab *Scylla paramamosain*, indicating that short-term treatment with NH_3–N caused a stress response in crabs and enhanced their disease resistance [6]. The results of the current study are consistent with the above reports. The combined action of the two stress factors (NH_3–N and pathogenic bacteria) induced similar changes, whereas dietary nano CeO_2 increased the activities of LZM, AKP, and ACP.

Cathepsin L, a lysosomal cysteine protease, is an important member of the cathepsin family, which plays critical roles in a variety of biological processes, such as immune responses [56], molting [57], and antigen processing [58]. Cathepsin L exhibits strong endopeptidase activity in the degradation of intracellular and extracellular protein and may play an important role in the defense against pathogens in the crayfish *Procambarus clarkii* [59]. It also acts as a biomarker of exposure of crustaceans to harmful environmental factors in water [60]. Cathepsin L is known to be associated with the evolution of the innate immune system and serves as a chemical barrier against microbial invasion [61]. In previous studies, the enzyme activity and mRNA and protein expression of cathepsin L were reported to be significantly upregulated in the hepatopancreas of Pacific abalone and freshwater mussels after 8 h of *Vibrio parahaemolyticus* or *A. hydrophila* infection [62,63]. In the present study, gene and protein expression of cathepsin L decreased significantly after 96 h of exposure to NH_3–N alone or to NH_3–N + *A. hydrophila* double stress. These results were not consistent with the above reports, but the difference may be due to the different exposure durations. Supplementation with dietary nano CeO_2 for 90 d increased the gene and protein expression of cathepsin L in the hepatopancreas of crabs exposed to NH_3–N stress or the combined stress of NH_3–N and *A. hydrophila* infection.

To evaluate the safety of using nano CeO_2 in aquatic products, we measured the Ce concentration of the edible hepatopancreas in crabs fed a diet supplemented with 0.8 mg/kg nano CeO_2 for 90 d. Compared to the crabs without nano CeO_2 supplementation, the levels of Ce in the hepatopancreas of crabs supplemented with 0.8 mg/kg nano CeO_2 increased from 0.4949 to 0.6805 mg/kg. However, the overall Ce content in the crab hepatopancreas was below 0.700 mg/kg, which was lower than values that have been reported in rice (average value, 0.75 mg/kg) and wheat (average value, 1.5 mg/kg) [64]. It should be noted that NRC has not established maximum tolerable levels of Ce, but it stated that dietary concentrations of 100 mg/kg should be considered safe [18].

In conclusion, supplementation with 0.8 mg/kg dietary nano CeO_2 (nanoparticles, 5 nm) significantly increased the growth of juvenile crabs and the efficiency of feed utilization. The supplementation also increased survival of crabs exposed to NH_3–N and/or *A. hydrophila* by up-regulating immunity as showed in Fig. 9. This is the first study that shows the beneficial effects of adding nano CeO_2 to the diet of *E. sinensis*, which include increased immune parameters and protection against NH_3–N exposure or pathogen infection under ammonium-N stress.

Declaration of interest

The authors report no conflicts of interest. The authors alone are

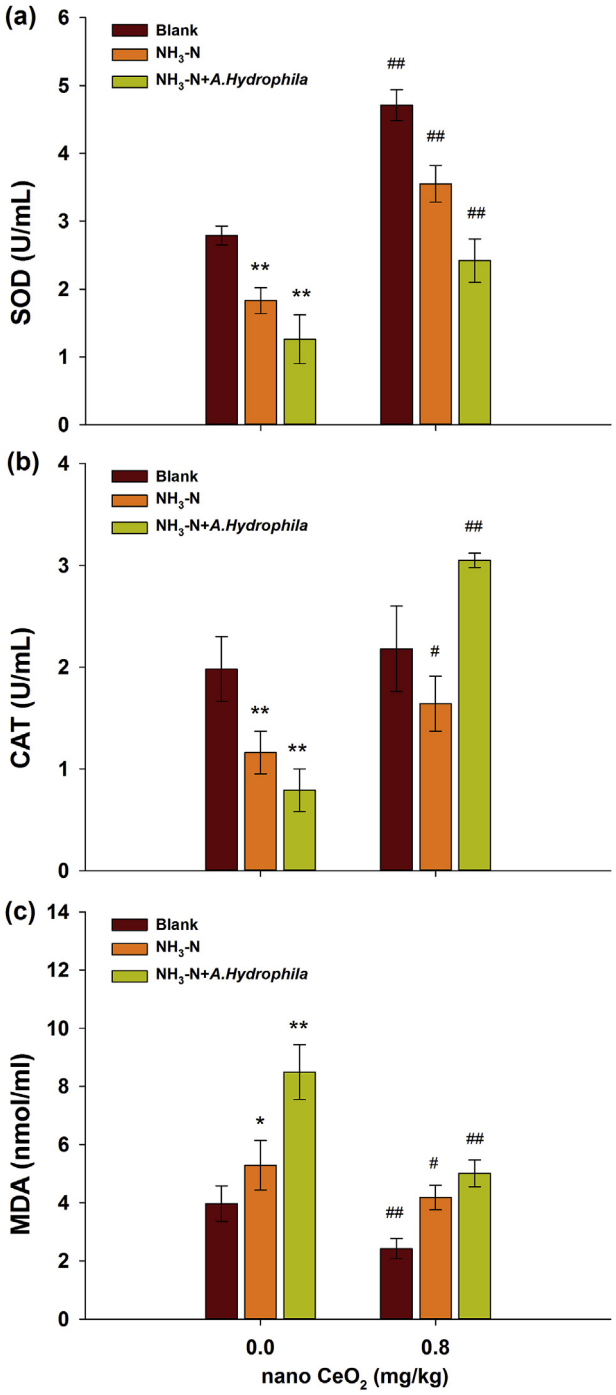

Fig. 7. Changed antioxidant capacity [superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA)] in crabs with or without the 0.8 mg/kg nano CeO_2 supplemented diet exposed to NH_3–N or NH_3–N + *A. hydrophila* for 96 h. Significance of difference: NH_3–N stress alone or NH_3–N + *A. hydrophila* double stress group compared with the blank group without nano CeO_2 (0 mg/kg), $^{*}P < 0.05$, $^{**}P < 0.01$; 0.8 mg/kg nano CeO_2 group compared to the 0 mg/kg nano CeO_2 group under the same stress condition, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

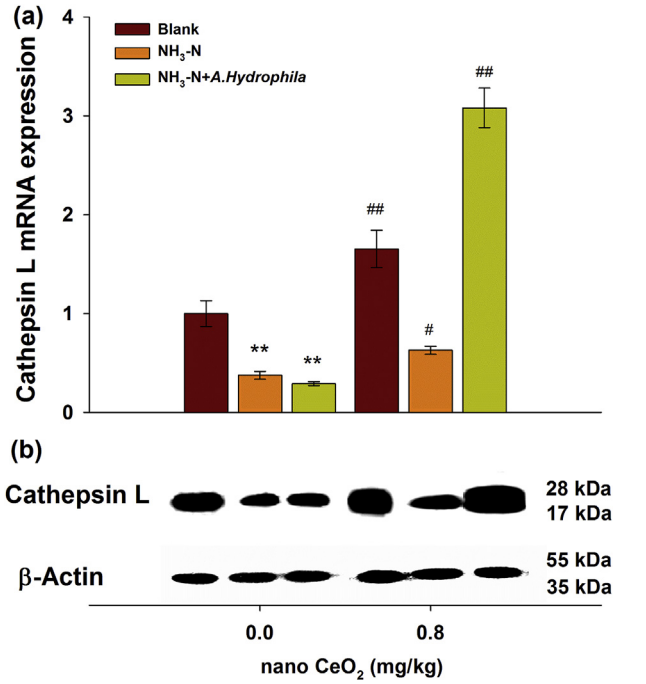

Fig. 8. Changed gene and protein expression of cathepsin L in crabs with or without the 0.8 mg/kg nano CeO_2 supplemented diet exposed to NH_3–N or NH_3–N + *A. hydrophila* for 96 h. Significance of difference: NH_3–N stress alone or NH_3–N + *A. hydrophila* double stress group compared with the blank group without nano CeO_2 (0 mg/kg), $^{**}P < 0.01$; the 0.8 mg/kg nano CeO_2 group compared to the 0 mg/kg nano CeO_2 group under the same stress condition, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

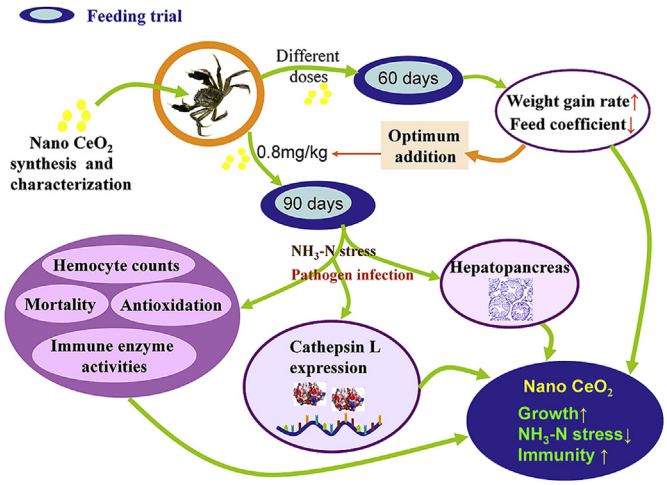

Fig. 9. Proposed hypothesis of nano CeO_2 as a growth regulator and immune enhancer against ammonium nitrogen stress.

responsible for the content and writing of the paper.

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