Effects of selenium adaptation on intestinal morphology, antioxidant-relate genes expression and intestinal microflora of grass carp (*Ctenopharyngodon idella*)

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Abstract

In the study, the effects of selenium on intestinal tissue morphology, antioxidant-related genes, and intestinal flora of grass carp (*Ctenopharyngodon idella*) were studied. For this purpose, 180 healthy grass carps (20.0±2.0 g) were randomly divided into three groups with three replicates each: the corresponding amount of anhydrous sodium selenite was added to make experimental water solutions of different concentrations, including 0 µg/L Se⁴⁺ (control group), 200 µg/L Se⁴⁺ group and 300 µg/L Se⁴⁺ group. The experiment was carried out for 42 days. The obtained results showed that: at the end of the experiment, the 200 µg/L Se⁴⁺ adaptation can have beneficial effects on the intestinal villi height and goblet cells. The CuZnSOD and CAT genes mRNA levels of grass carp intestine were strongly upregulated in the 200µg/L Se⁴⁺ group. 200µg/L selenium could increase the expression level of the Hsp70 gene in the intestinal tract of grass carp after 42 days of adaptation. At the genus level, the most abundant sequence in the gut of Se-treated grass carp was *Pseudomonas*, while *Aeromon, Flavobacterium*, and *Defluviimonas* were more abundant in the control group. In conclusion, this study demonstrates that 200µg/L Se⁴⁺ selenium adaptation can positively affect gut morphology and antioxidant responses and can alter the gut microbiota structure of grass carp. The results will provide a theoretical basis for further research on the effect of selenium on aquatic animals.

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Introduction

Grass carp is one of the most important aquaculture species. It has the advantages of delicious meat and fast growth and is intensively cultured. However, deteriorating water quality due to intensive farming resulted in excessive breeding densities, making grass carp more susceptible to environmental stress and pathogens (Pei et al., 2019). In the long-term evolution process, aquatic animals can adapt to the changing external environment through the body's physiological, biochemical, and molecular reactions (Shang et al., 2021).

Selenium (Se) is an essential micronutrient that can maintain the body's regular metabolism and play an indispensable role in anti-oxidant, immunoregulation, anti-inflammatory and anti-viral, and Selenium deficiency leads to susceptibility to disease and decreased immunity in animals (Khan et al., 2016). Selenium protects the animal body from oxidative stress mainly by forming thioredoxin reductase and glutathione peroxidase. Therefore, selenium supplementation can effectively promote the physiological health and growth, and reproduction of fish (Yu et al., 2005; Hoffmann and Berry, 2008). However, the current research on selenium primarily focuses on the effects of adding selenium in feed to fish. At present, Se added in feed have been reported in many fish, for example, Nile tilapia (Oreochromis niloticus), rainbow trout (Oncorhynchus mykiss), channel catfish (Ictalurus punctatus), crucian carp (Carassius auratus), grouper (Epinephelus malabaricus) and Snub-Nosed Dart (Trachinotus Blochii) (Hilton et al., 1980; Gatlin and Wilson 1984; Lin and shiau, 2005; Hancz et al., 2012; Zhu et al., 2017; Lee et al., 2016; Sang et al., 2016). Selenium plays an essential physiological role in animals. Both deficiency and excess will cause adverse effects on animals. Lack of selenium in animals will lead to adverse symptoms in the body. Studies have reported that selenium deficiency in Atlantic salmon manifests increased mortality, growth retardation, decreased glutathione transferase activity, and muscle wasting (Poston et al., 1976). However, selenium has specific toxicity, and excessive intake will cause harm to the body (Saffari et al., 2018).

So far, there are few reports on the long-term adaptation of grass carp to selenium solution. In this study, we assessed histological changes in the gut of grass carp. We analyzed the antioxidant-relate gene expression and some types of gut microbiota after adaptation to selenium in water. The results will provide a theoretical basis for further research on the effect of selenium on aquatic animals.

Materials and methods

Experimental design

The grass carp used in this experiment was purchased from a local standardized grass carp farm. The purchased fish were first sterilized in a sodium chloride bath (2%, 15 minutes) and then put into three 500L water tanks for 7 days of acclimation, the water quality was detected every day, and the water body was continuously oxygenated. The fish were fed twice a day, and observed the ingestion of grass carp. Then 180 grass carp (20.0±2.0 g) were randomly divided into 3 treatment groups with 3 tanks (800mm×500mm×600mm) in each group. 3 treatment groups including: control group (0µg/L Se⁴⁺), 200µg/L Se⁴⁺ group and 300µg/L Se⁴⁺ group. The corresponding amount of anhydrous sodium selenite was added to make experimental solutions of different concentrations. Change the test solution every 48 hours to ensure proper Se⁴⁺ concentration. During the trial, the water was continuously aerated and changed by 50% every 48h, and checked the water quality regularly to make sure that the water quality stayed within the following ranges: water temperature (21.5-26.5°C), PH (7.2-7.8), dissolved oxygen (DO) (5.2-6.8 mg/L), NH₃–N (<0.11mg/L) and the photoperiods followed a natural daylight cycle. During the trial period, grass carp were fed twice daily at 8:30 and 14:30 (3% of body weight per day), and the feeding rate was adjusted every 2 weeks.
At the end of the experiment, grass carp fasted 24 hours in advance. Then 6 fish were randomly selected from each replicate and anesthetized with MS-222 (100 mg/L), of which 3 fish were sacrificed, and the intestinal tissue was fixed for histomorphological analysis. The intestines of the other 3 fish were snap-frozen in liquid nitrogen for gene expression analysis and gut microbiome analysis.

**Preparation of tissue slices**

The intestines of grass carp collected from the experiment were fixed with 10% formalin solution, dehydrated, embedded, sectioned, and finally stained with hematoxylin and eosin (H&E).

**RNA extraction and determination of the expressions of antioxidant-relate genes**

Quickly slaughtered grass carp to take the intestine, quick-frozen in liquid nitrogen, and stored in a -80°C refrigerator until analysis, using the Trizol method (Trizol Reagen kit, Takara, Japan) to extract total RNA from the intestine, and measuring the RNA concentration and purity with a micro-UV-visible spectrophotometer. 2 µg of total RNA was reverse transcribed into cDNA using PrimeScript RT-PCR kit (Takara, Japan). RT-qPCR was performed on each treated cDNA sample by RT-qPCR (SYBR Green II) on a Thermocycler Dice TP800 Sequence Detection System (Takara, Japan). β-actin was used as an internal reference to normalize the mRNA expression levels of the genes in each sample. The relative gene expression was calculated by the 2^−ΔΔCt method. Sequences of primers for RT-qPCR are shown in Table 1.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer direction</th>
<th>Primer sequence (5′→3′)</th>
<th>Tm (℃)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP70</td>
<td>Forward</td>
<td>CTGCTGGATGTTGGCTCTCTGTC</td>
<td>60</td>
<td>GU475146.1</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AAGGTCTGGGTCTTGGTGTTGGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAT</td>
<td>Forward</td>
<td>GAAGTCTTGCCCGATGAGG</td>
<td>60</td>
<td>FJ560431</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CGAAATCCCCAACCAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>Forward</td>
<td>CGACTTCAACCGTTACCTTACA</td>
<td>58</td>
<td>GU901214</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ACTTTCTCATTGCTTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward</td>
<td>GTCCGTGACATCAAGGAGAGC</td>
<td>60</td>
<td>DQ211096.1</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGATACCGCAAGACCTCCATACC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**DNA extraction and 16S rRNA gene analysis**

The preserved samples were sent to Shanghai Yuanshen Biotechnology Company for DNA extraction, library construction, and high-throughput sequencing of 16S rRNA amplicon. The process is as follows: use 16S V3-V4 variant region primers to amplify the DNA of the extracted samples, and use the TruSeq DNA PCR-Free Sample Preparation Kit for library construction. The constructed library was quantified by Qubit and Q-PCR. After the library was qualified, the NovaSeq6000 was sequenced on the computer.

**Statistical analysis**

After data were processed by Excel, a one-way analysis of variance (one-way ANOVA) was performed with SPSS 18.0, and multiple comparisons were performed with Duncan's method. Data presented as Mean ± standard deviation (SD), P< 0.05 indicates a significant difference.
**Results**

*Growth performance and survival*

The growth performance and survival of grass carp following waterborne adaptation to selenium for 42 days are in **Table 2**. Results showed that FBW and SGR in the control group and 200ug/l Se⁴⁺ group were significantly higher than those of the 300ug/l Se⁴⁺ group. At the end of the experiment, the survival rates of the control group and the 200ug/l Se⁴⁺ group were 100%.

**Table 2** Effects of selenium adaptation on growth performance and survival of grass carp

<table>
<thead>
<tr>
<th>Items</th>
<th>Control</th>
<th>200ug/l Se⁴⁺</th>
<th>300ug/l Se⁴⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBW (g)</td>
<td>20.12±0.35</td>
<td>20.34±0.42</td>
<td>20.23±0.26</td>
</tr>
<tr>
<td>FBW(g)</td>
<td>47.45±1.21ᵃ</td>
<td>52.62±1.11ᵃ</td>
<td>42.37±1.36ᵇ</td>
</tr>
<tr>
<td>SGR (%)</td>
<td>2.04±0.03ᵃ</td>
<td>2.26±0.04ᵃ</td>
<td>1.76±0.06ᵇ</td>
</tr>
<tr>
<td>SR (%)</td>
<td>100.00</td>
<td>100.00</td>
<td>91.67</td>
</tr>
</tbody>
</table>

Note: Values are mean ± S.E.M, and values in the same row with different letters indicate a significant difference (p < 0.05). IBW, initial body weight; FBW, final body weight; SR, survival rate; SGR, specific growth rate; SGR== (Ln final weight − Ln initial weight) × 100/days;

*Effects of selenium on histopathological changes of intestine*

**Figure 1** shows the intestinal epithelial layer was orderly, lamina propria dense, and nuclei regular in the control group (**Figure 1A**), whereas the grass carp gut showed lamina propria enlargement in microvilli after exposure to 200 µg/l Se⁴⁺ and 300 µg/l Se⁴⁺; The villus space was increased (**Figure 1B, C**). **Figure 2** displays that the VH (intestinal villi height) and GC (goblet cells) of grass carp treated with 200 µg/L Se⁴⁺ were significantly higher than those in the control group and 300 µg/L Se⁴⁺ groups (P<0.05). However, the intestinal MT (muscular layer thickness) of grass carp in the control group was significantly higher than that in the selenium treatment group (P<0.05).

![Figure 1](image)

**Figure 1** Intestinal morphology in grass carp following waterborne adaptation to selenium for 42 days. The panels include, A: control; B: 200µg/L Se⁴⁺; C: 300µg/L Se⁴⁺. GC, goblet cells; LP, lamina propria; L, lumen; E, epithelium. H&E stain (100×).
**Effects of selenium on intestinal morphology, genes expression and intestinal microflora of grass carp**

Figure 2 Effect of Intestinal morphology in grass carp following waterborne adaptation to selenium for 42 days.

**Expressions of antioxidant-relate genes in the intestine of grass carp**

Figure 3 demonstrates the expression levels of CAT, CuZnSOD, and Hsp70 genes in the intestinal tract of grass carp in the 200ug/L Se⁴⁺ group were significantly higher than those in the control group (P<0.05). In contrast, the gene levels of CAT and Hsp70 in the 200ug/L Se⁴⁺ group were significantly higher than those in the 300ug/L Se⁴⁺ group (P<0.05).
Figure 3 Expressions of antioxidant-relate genes in the intestine of grass carp following waterborne adaptation to selenium for 42 days.

The abundance of differences in microbial taxa at the genus level

Figure 4 compares the control group; Aeromon, Flavobacterium, and Defluviimonas were the most dominant genus at the genus level. However, Pseudomonas is abundant in sequences in the selenium-treated samples. The abundances of Aeromon, Flavobacterium, and Defluoromonas were significantly reduced in the selenium-treated group compared with the control group ($P<0.05$).
Effects of selenium on intestinal morphology, genes expression and intestinal microflora of grass carp

Discussion

Selenium is an essential trace element for animals. The information about selenium in aquatic animals studies have focused on the effects of adding a certain amount of selenium to the feed on aquatic animals, including crucian carp (Carassius auratus gibelio), Japanese seabass (Lateolabrax japonicus), cobia (Rachycentron canadum), common carp (Cyprinus carpio) and marron (Cherax cainii-marron) (Zhou et al., 2009; Liang et al., 2006; Yang et al., 2016; Saffari et al., 2018; Nugroho and Fotedar, 2013). However, little information about the effect of selenium in an aqueous solution on the intestinal function of fish has been reported. As an essential part of fish’s digestive system, the development of the intestine is closely related to absorption efficiency. The higher the intestinal fold, the stronger the ability of the intestine to absorb nutrients; the thicker the muscle layer, the stronger the intestinal peristalsis (Lee et al. 2017). In this study, the 200ug/L Se⁴⁺ treatment group had beneficial effects on both the villus height and the number of goblet cells in the intestinal tract of grass carp. A couple of previous studies also suggest that dietary selenium has a promoting effect on the morphological development of fish intestines (Saffari et al., 2018; Shang et al., 2021). Goblet cells are known to lubricate and protect the mucosa from chemical and physical damage by secreting mucus (Begam and Sengupta, 2015). Healthy and complete intestinal folds can
prevent harmful bacteria from colonizing the intestines, reduce the incidence of enteritis and enhance the disease resistance of fish (Huang et al., 2010).

As an essential antioxidant in animals, selenium can not only improve the antioxidant capacity of animals but also remove excess free radicals, protect cell membranes, and resist lipid peroxidation damage, thereby maintaining the health of the body (Yu et al., 2005; Hoffmann and Berry, 2008; Khan et al., 2016). Environmental stressors affect the antioxidant system of fish, causing oxidative stress in the body (Zhang et al., 2019). Essential micronutrients, such as selenium, are strong antioxidant micronutrients that play a key role in inhibiting oxidative stress and promoting cell stability in fish (Martínez-Álvarez et al., 2005). At present, some studies are only limited to adding selenium in feed to improve the activity of antioxidant enzymes in tissues and organs, and there is no report on the research on improving the activity of antioxidant enzymes by adapting to selenium in water (Jovanovic et al., 1997; Lin and shiau, 2005; Zhu et al., 2017; Lee et al., 2016).

To study the relationship between the molecular mechanism of selenium adaptation and the expression of antioxidant-related genes in the intestine of grass carp (Ctenopharyngodon idellus). The results showed that the gene expression levels of CuZnSOD and CAT in the intestine of grass carp were increased in the 200ug/l Se₄⁺ Group, suggesting that proper selenium adaptation may enhance the antioxidant capacity of grass carp. Hsp70 has various functions, such as antioxidant and synergistic immunity, which can maintain cell homeostasis and enhance the body’s ability to resist stress (Feder and Hofmann, 1999; Daugaard et al., 2007). In this study, the Hsp70 expression level of the intestine was increased in the 200ug/L Se₄⁺ group. This study showed that the expression levels of Hsp70 in the intestines of the 200ug/L Se₄⁺ group increased. 200ug/L selenium adaptation can increase the expression level of the Hsp70 gene, thereby enhancing stress resistance.

In this study, many fish gut microbes were discovered through high-throughput sequencing and bioinformatics analysis. The most abundant sequence in the selenium-treated samples is Pseudomonas. The abundance of Aeromon, Flavobacterium, and Defluviimonas decreased significantly in the selenium-treated groups. This study also found that there are many unknown gut microbes in the gut of the experimental grass carp, and the types and functions of these gut microbes need to be further studied. These findings reveal that selenium is closely related to the gut microbiota and may influence the healthy development of the gut.

**Conclusion**

In conclusion, we demonstrate for the first time the adaptive response of grass carp intestine to selenium. The 200ug/L Se₄⁺ solution has a specific promoting effect on the morphological development of grass carp intestines and antioxidant responses. Meanwhile, the 200ug/L Se₄⁺ could alter the intestinal microbiota structure of grass carp. The research results will provide a theoretical basis for further in-depth study of the application of selenium in aquaculture.

**Acknowledgments**

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