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Short communication

Effects of dietary nucleotides on growth, non-specific immune response and disease resistance of sea cucumber *Apostichopus japonicas*

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ABSTRACT

A 9-week feeding trial was conducted to investigate the effects of dietary nucleotides (NT) on growth, immune response and disease resistance of sea cucumber *Apostichopus japonicas* (initial weight: 5.87 ± 0.03 g). Four graded levels of dietary NT were designed as 0, 150, 375 and 700 mg/kg, respectively. After the feeding trial, sea cucumbers were challenged with *Vibrio splendidus* for the determination of disease resistance. The results showed that the specific growth rates were significantly higher in sea cucumber fed the diet with 375 mg/kg NT than those fed the basal diet without NT supplementation ($P < 0.05$). The highest total coelomocytes counts in coelomic fluid were found in the treatment with 150 mg/kg of dietary NT ($P < 0.05$). Compared to those fed with the basal diet, sea cucumber fed diets with nucleotides (≥375 mg/kg) had significantly higher phagocytic activities in coelomic fluid ($P < 0.05$). Respiratory burst activities in coelomic fluid significantly increased with increasing dietary NT supplementations up to 700 mg/kg ($P < 0.05$). No significant differences in the activities of superoxide dismutase, total nitric oxide synthase and acid phosphatase in coelomic fluid were found among all the treatments ($P > 0.05$). After being challenged with *V. splendidus*, the cumulative mortalities of sea cucumber fed diets with 150 and 375 mg/kg NT were significantly lower than that in the treatment without dietary nucleotide supplementation ($P < 0.05$). Under the experimental conditions, the present results confirmed that a diet supplemented with 375 mg/kg NT is able to enhance both non-specific immune response and growth of sea cucumber in vivo. In conclusion, it was showed that dietary NT does increase the growth performance, non-specific immunity and disease resistance of sea cucumber. The optimum dietary NT supplementation level for sea cucumber was found to be 375 mg/kg. The application of dietary NT may present a novel strategy for health management in sea cucumber's aquaculture.

1. Introduction

Sea cucumber *Apostichopus japonicas* (Selenka) is a custom food which is higher in protein and lower in fat than most foods and contains the amino acids and trace elements essential for keeping humans healthy. It is also used as tonic and a traditional medicine in China [1,2]. As an economically important farmed species, its production broke through 193,705 metric tons in China in 2013 [3]. However, the rapid expansion and high intensity of sea cucumber farming resulted in serious diseases, such as skin ulceration and peristome tumescence. These diseases caused serious economic losses and limited the sustainable development of this industry. Therefore, it is very urgent to find ways to control the diseases [4]. Because of the restriction of antibiotics and chemotherapeutics in controlling those diseases, more attention is being drawn to immune-stimulants, which enhance immunity and disease resistance of sea cucumber [5–7].

Dietary nucleotides (NT) have received a lot of attention in relation to promote growth and to exhibit immunomodulatory effects in fish [8]. They play key roles in many biological processes serving as nucleic acid precursors, physiological mediators, components of coenzymes and sources of cellular energy, and were described as “semiessential” or “conditionally” essential nutrients [9,10]. There are two biosynthetic pathways for nucleotide metabolism: The *de novo* synthesis from amino acids and the salvage

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pathways from the breakdown of nucleotides. Dietary nucleotides contribute to the salvage pathway, providing preformed nucleosides and nitrogenous bases [11]. Under the conditions of rapid growth or physiological stresses which increase the susceptibility to disease, the *de novo* synthesis of nucleotides may become limiting and an additional source of exogenous nucleotides through the diet may exert a positive effect on growth and health [9,12–14].

In fish, Burrells et al. [12,13] reported that dietary NT of 2 g/kg increased the resistance of Atlantic salmon (*Salmo salar* L.) against infections with bacterial, viral and rickettsial diseases as well as ectoparasitic infestation. It also increased the capacity for osmoregulation as well as the growth rate. Similar results have been reported in fish, such as hybrid striped bass (*Morone chrysop*×*Morone saxatilis*) fed a diet with 5 g/kg NT [15], grouper (*Epinephelus malabaricus*) fed diets with 1.5 g/kg of AMP or 1.0–1.5 g/kg mixed-NT [16], rainbow trout (*Oncorhynchus mykiss*) fingerlings fed a diet with 1.5–2 g/kg of NT [17], red drum (*Sciaenops ocellatus*) fed a diet with 10 g/kg NT [18] and olive flounder (*Paralichthys olivaceus*) fed diets with 0.46–1.84 g/kg of inosine monophosphate (0.1–0.4% IMP product) [19]. Studies were also reported in crustacean, indicating that final mean weight was significantly higher in white shrimp *Litopenaeus vannamei* fed a diet supplemented with 5 g/kg NT than when feeding a basal diet [20], and the optimum supplemented level in the diet was 2 g/kg NT [21].

Sea cucumber is one of the holothurian species belonging to Echinodermata. No published study was found in sea cucumber fed diets with NT. The purpose of this study was to determine the effects of dietary NT on growth, immune response and disease resistance of sea cucumber.

2. Materials and methods

2.1. Experimental diets

The formulation and proximate analysis of the basal diet are shown in Table 1. The brown alga *Sargassum thunbergii* powder was used as the main sources of the diet. The diets were formulated to contain four graded levels of NT (0, 150, 375 and 700 mg/kg, respectively). Nucleotides were supplemented in the form of 'ROVIMAX NX' (40% nucleotides, DSM Nutritional Products, Kaiseraugst, Switzerland), which contained cytidine-5V-monophosphate (CMP), disodium uridine-5V-monophosphate (UMP), adenosine-5V-monophosphate (AMP), disodium inosine-5V-monophosphate (IMP), disodium guanidine-5V-monophosphate (GMP) and RNA. Ingredients were ground into fine powder through a 149 μm mesh sieve. The powder was thoroughly mixed with fish oil and about 30% cold water was added until a stiff dough was produced. The dough was extruded through a granulator at 1.0 mm diameter, and then spread out and dried in an oven at 40 °C. After drying, the diets were broken up and sieved into the appropriate pellet length through a mesh sieve between 180 μm and 425 μm, and then stored at −20 °C.

Table 1
Formulation and proximate composition of the basal diet (% dry matter).

Ingredients	%
Sargassum thunbergii powder	68.5
fish meal	5
Soybean meal	7
Shrimp shell meal	5
Wheat meal	10
Fish oil	0.5
Lecithin	1
Vitamin premix[a]	1
Mineral premix[a]	1
$Ca(H_2PO_4)_2$	1
Proximate composition	
Crude protein (%)	20.93
Crude lipid (%)	8.43

[a] Kindly provided by Qingdao Master Biotechnology Co. Ltd, Qingdao, China.

2.2. Sea cucumbers and feeding trial

Sea cucumbers were obtained from a commercial hatchery in Jimo, Qingdao, China. Prior to the initiation of the feeding trial, all animals were fed the basal diet without NT supplementation for 2 weeks to acclimatize them to the experimental diets and the rearing conditions. After that, 600 healthy sea cucumbers with similar sizes (initial mean body weight 5.87 ± 0.03 g) were randomly assigned to twenty four 60 L aquaria (25 sea cucumbers per aquarium). Each diet was fed to 6 aquaria. Animals were fed twice daily (07:00 and 14:00, respectively) for 9 weeks. During the feeding trial, the water temperature remained at 17–19 °C, pH 7.9 to 8.3 and the salinity at 28–30‰. Low pressure electrical blowers provided aeration via air stones and maintained dissolved oxygen levels at or near air-saturation.

2.3. Growth and survival

At termination of the feeding trial, the sea cucumbers were not fed for 24 h. All sea cucumbers were counted and weighed for the estimation of the specific growth rate (SGR) and survival rate. Growth and survival were expressed as follows:

$$\mathrm{SGR}\left(\%\,\mathrm{day}^{-1}\right) = 100 \times (\ln \text{final body weight} - \ln \text{initial body weight})/\text{feeding days}$$

$$\text{Survival rate}(\%) = 100 \times (\text{final amount of fish})/(\text{initial amount of fish})$$

2.4. Immune assays

2.4.1. Sample collection

Four sea cucumbers per aquarium were randomly sampled for the collection of the coelomic fluid, which was then thoroughly mixed with an equal volume of anticoagulant (0.02 M EGTA, 0.48 M NaCl, 0.019 M KCl and 0.068 M Tris–HCl, pH = 7.6). The diluted coelomic fluid of four sea cucumbers from one aquarium was pooled for the analysis of total coelomocytes counts (TCC), phagocytosis activity, respiratory burst activity, superoxide dismutase (SOD) activity, the total nitric oxide synthase (T-NOS) activity and acid phosphatase (ACP) activity.

2.4.2. The total coelomocytes counts (TCC)

A 50 μl of coelomic fluid was diluted by 150 μl of 2.5% glutaraldehyde solution immediately. The TCC was measured with a hemocytometer (Qiujing Inc., Shanghai, China) under an optical microscope at 400 × magnification.

2.4.3. Phagocytic activity

Neutral red method [22,23] with slight modifications was used to evaluate the coelomocytes phagocytosis. A total of 100 μl of coelomic fluid was placed in a flat-bottomed 96 well microtiter plate and incubated for 30 min for adhesion, and then the supernatant was carefully removed. After that, 100 μl of 0.001 M neutral red was added to the coelomocyte monolayer and swallowed for 30 min. The supernatant was removed by rinsing three times with

0.85% saline. A 100 μl of cell lysis buffer (acetic acid: ethanol = 1:1) was added and dissolved for 20 min. The results were recorded with a universal microplate spectrophotometer using a test wavelength of 540 nm. The absorbance of 10^6 cells represented the capability of coelomocytes phagocytosing neutral red.

2.4.4. Respiratory burst activity

The method of Song and Hsieh [24] with slight modifications was adopted to evaluate the production of intracellular anion by coelomocytes using the nitroblue tetrazolium (NBT, Sigma). In brief, 50 μl 0.2% poly-L-lysine (Sigma) solution was added to wells of 96-well plate for increasing coelomocytes adhesion. A 100 μl of coelomic fluid was added to wells and then centrifuged at 3,000 g for10 min at 4 °C. The supernatant was removed and 100 μl of 1 μg/ml phorbol myristate acetate (PMA, Sigma) was added for incubation for 30 min at 30 °C. The cells in each well were then stained with 100 μl 0.3% NBT at 37 °C for 30 min. The supernatant was discarded after centrifuging for 10 min (560 g, 4 °C) from each well, and then the cells were killed by adding 20 μl 100% methanol and incubating for 10 min. Then 200 μl 70% methanol was used to wash wells carefully for three times. After air-drying, 120 μl 2 mol/L KOH and 140 μl dimethyl sulfoxide was added to each well. The results were recorded with a universal microplate spectrophotometer using a test wavelength of 630 nm. The absorbance of 10^6 cells represented the capability of coelomocytes respiratory burst activity.

2.4.5. Superoxide dismutase activity (SOD)

The SOD activity was assayed using the commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) by its ability to inhibit superoxide anion generated by xanthine and xanthine oxidase reaction system. The optical density was measured at 550 nm with a universal microplate spectrophotometer. One unit of SOD was defined as the amount required for inhibiting the rate of xanthine reduction by 50% in 1 ml reaction system. Specific activity was expressed as SOD unit per 10^7 cells.

2.4.6. The total nitric oxide synthase activity (T-NOS)

The T-NOS was measured by its catalytic ability to convert L-Arginine into NO using a T-NOS detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The NO was oxidized to nitrite (NO^{2-}) and nitrate (NO^{3-}), which was also converted to NO^{2-} finally by nitrate reductase. The colour produced by NO^{2-} was measured at 530 nm with a universal microplate spectrophotometer. One unit of T-NOS activity was defined as the amount of T-NOS producing 1 nmol NO/min. Specific activity was expressed as T-NOS unit per 10^7 cells.

2.4.7. The acid phosphatase activity (ACP)

The ACP activity was measured with a chemical detection kit (Nanjing Jiancheng, Bioengineering Institute, Nanjing, China). Disodium phenyl phosphate was converted to phenol and phosphoric acid by ACP. Then quinone was produced. The optical density was measured at 520 nm with a universal microplate spectrophotometer. One unit of T-NOS activity was defined as the amount required to degrade 1 mg phenol at 37 °C within 30 min. Specific activity was expressed as ACP unit per 10^7 cells.

2.5. Challenge test

After the feeding trial, 15 sea cucumbers per aquarium were used for the challenge test. The bacteria pathogen *Vibrio splendidus* was obtained from the Yellow Sea Fisheries Research Institute, Chinese Academy Fishery Sciences (Qingdao, China). The *V. splendidus* was grown in fluid nutrient medium at 28 °C for 24 h. The bacterial culture was re-suspended in PBS to form different density bacteria liquids. The LD50 was determined prior to challenge and the result showed that the LD50 for 7 days was 2×10^7 CFU/ml. For the challenge test, sea cucumbers from each aquarium were injected with 0.1 ml 0.85% PBS containing 2×10^7 CFU bacteria. The cumulative mortality was monitored for 14 days.

2.6. Statistical analysis

All data were analyzed by the one-way analysis of variance (ANOVA) using the SPSS 17.0 for Windows. The results were presented as means ± SE (standard error of the means). When overall differences were found, differences between means were determined and compared by Tukey's honest significant difference post hoc test. All differences were considered significant at $P < 0.05$.

3. Results

3.1. Growth and survival

The growth data of sea cucumbers are shown in Table 2. Final weight and SGR were beneficially influenced by dietary NT supplementation ($P < 0.05$). Sea cucumbers fed the diet with 375 mg/kg NT had a significantly higher final weight and SGR than those of the other treatments.

There were no significant differences in survival rate among the various treatments, which ranged from 91.33% to 98.67% among treatments (Table 2).

3.2. Immune parameters

3.2.1. Total coelomocytes (TCC)

The data on the immune parameters are shown in Table 3. Dietary NT supplementation significantly influenced the TCC of coelomic fluid ($P < 0.05$). Sea cucumber fed the diet supplemented with 700 mg/kg NT had significantly lower TCC than those of the treatment with 150 mg/kg dietary NT ($P < 0.05$). There was no significant difference among sea cucumbers feed the basal diet, 150 and 375 mg/kg NT diet.

3.2.2. Phagocytic activity

The phagocytic activities significantly increased with increasing dietary NT supplementations ($P < 0.05$) (Table 3). When the dietary NT supplementation was 700 mg/kg, the phagocytic activity reached the highest level of 0.98 OD540/10^6 cells, which was significantly higher than those in the treatments with the basal diet without NT supplementation and 150 mg/kg NT supplementation, respectively ($P < 0.05$).

3.2.3. Respiratory burst

Like the phagocytic activities, respiratory burst activities also significantly increased with increasing dietary NT supplementations ($P < 0.05$) (Table 3). When the dietary NT supplementation was 700 mg/kg, the respiratory burst activity reached the highest level of 0.39 OD630/10^6 cells, which was significantly higher than that in the treatment with basal diet without NT supplementation ($P < 0.05$).

3.2.4. Activities of SOD, T-NOS and ACP

Activities of SOD, T-NOS and ACP were not significantly influenced by dietary NT supplementation (Table 3). They ranged from 5.20 to 5.81 U/10^7 cells, 0.15–0.20 U/10^7 cells and 1.65–2.44 U/10^7 cells, respectively.

Table 2
Growth and survival of sea cucumber fed the experimental diets for 9 weeks (means ± S.E., n = 3).

Dietary nucleotides (mg/kg)	Initial weight (g)	Final weight (g)	SGR[a] (%/d)	Survival rate (%)
0	5.88 ± 0.08	13.87 ± 0.64[a]	1.22 ± 0.07[a]	95.33 ± 0.02
150	5.87 ± 0.01	16.67 ± 0.83[ab]	1.48 ± 0.07[ab]	98.67 ± 0.08
375	5.87 ± 0.01	17.53 ± 0.92[b]	1.56 ± 0.08[b]	98.00 ± 0.01
700	5.88 ± 0.01	16.85 ± 0.89[ab]	1.49 ± 0.07[ab]	91.33 ± 0.03

Data in the same column with different letters are significantly different (P < 0.05; Tukey's test).
[a] SGR: specific growth rate.

Table 3
The effect of the experimental diets with different levels of nucleotide on non-specific immunity of sea cucumber after 9 weeks (means ± S.E.).

Dietary nucleotides (mg/kg)	TCC[a] (× 10^7 cells/ml)	Phagocytic activity (OD540/10^6 cells)	Respiratory burst (OD630/10^6 cells)	SOD[b] (U/10^7 cells)	T-NOS[c] (U/10^7 cells)	ACP[d] (U/10^7 cells)
0	0.88 ± 0.06[ab]	0.53 ± 0.05[a]	0.18 ± 0.03[a]	5.38 ± 0.48	0.20 ± 0.03	1.95 ± 0.37
150	0.98 ± 0.04[b]	0.66 ± 0.04[ab]	0.25 ± 0.05[ab]	5.45 ± 0.22	0.17 ± 0.02	2.44 ± 0.27
375	0.82 ± 0.05[ab]	0.92 ± 0.05[bc]	0.34 ± 0.06[ab]	5.20 ± 0.18	0.15 ± 0.03	1.65 ± 0.25
700	0.70 ± 0.09[a]	0.98 ± 0.17[c]	0.39 ± 0.06[b]	5.81 ± 0.52	0.19 ± 0.04	2.16 ± 0.44

Data in the same column with different letters are significantly different (P < 0.05; Tukey's test).
[a] The total coelomocytes counts.
[b] Superoxide dismutase activity.
[c] The total nitric oxide synthase activity.
[d] The acid phosphatase activity.

3.3. Challenge test

Cumulative mortality of sea cucumbers after having been challenged with *V. splendidus* was significantly affected by dietary NT (P < 0.05) (Fig. 1). The sea cucumbers fed with the basal diet showed statistically the highest cumulative mortality (63.33%), which was significantly higher than those fed diets with 150 mg/kg and 375 mg/kg NT, respectively.

4. Discussion

The present study showed that supplementation of dietary NT with 375 mg/kg significantly improved the final weight and SGR of sea cucumber after 9 weeks of feeding. This finding is in agreement with the previous studies in fish, like grouper (*E. malabaricus*) [16] and rainbow trout (*O. mykiss*) fingerlings [17]. A similar result was also reported in shrimp *L. vannamei* fed the NT-supplemented diet, which had a significantly higher SGR than those fed the control diet without NT supplementation [21]. Though, most cell types can synthesise nucleotides from purines and pyrimidines provided endogenously, but the *de novo* synthesis of nucleotides is an energy-expensive process. An additional source of exogenous nucleotides in the diet may therefore be preferentially utilised by the organism to optimise the functions of rapidly expanding tissues, particularly when growth is fast [12].

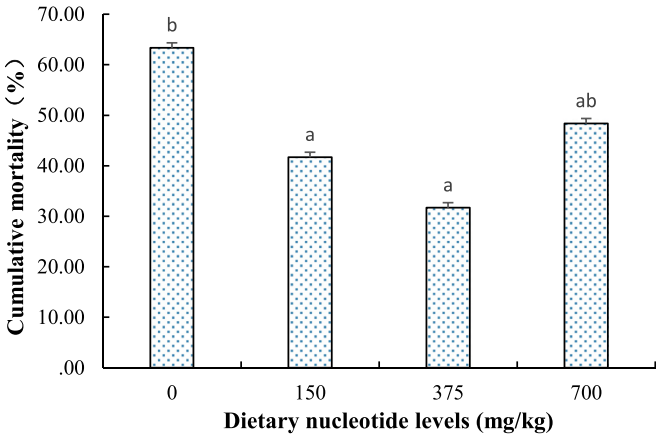

Fig. 1. Effects of dietary nucleotide on the cumulative mortality of sea cucumber after *Vibrio splendidus* challenge. Data were expressed as mean ± S.E. Data with different letters are significantly different (P < 0.05).

However, in the present study, performance of sea cucumber fed the diet with 700 mg/kg of NT was not further increased. Although not being significant, the final weight and SGR decreased in that treatment. Reduced growth performance by high NT levels might be explained by the toxicity of serum uric acid derived from purine base [25]. Adamek et al. [26] and Song et al. [19] reported that the optimal dietary NT levels increased the growth of rainbow trout, wels (*Silurus glais*) and olive flounder, while the higher levels of dietary NT caused growth depression. It was suggested that a suitable dose of NT in diet is important to improve the growth of fish, shrimp and sea cucumber.

Results from the previous studies on human and terrestrial vertebrates support the theory that dietary nucleotides are important to the function of several components of the immune system [27]. Exogenous nucleotides can influence both humoral and cellular components of the innate immune system in fish [8,11]. The immune enhancement by dietary NT in fish and shrimp includes increasing complement and lysozyme activity [28], respiratory burst activity [16] and the total haemocyte count [21].

Sea cucumber belong to marine invertebrates, which lack adaptive immune responses [29]. The coelomocytes play an important role in the non-specific immune processes, which have the ability to distinguish between different foreign antigens (e.g., pathologically modified tissues, microorganisms, parasites and grafts) and to express variable effector mechanisms which are elicited specifically and repeatedly after a variety of non-self-challenges [30,31]. In the present study, the highest value of TCC was found in the diet with 150 mg/kg NT. The total haemocyte count in shrimp was also increased by dietary nucleotide supplementation [20,21]. However, in sea cucumber fed a diet with 700 mg/kg NT, the TCC was significantly lower than that in the treatment with 375 mg/kg of dietary NT. It was confirmed that an over dose of dietary NT decreased the TCC.

The phagocytosis is one of the primary mechanisms of defense against invasion of pathogenic organisms [32,33]. It exerted by phagocytic cells, an important function of which is degradation of material after phagocytosis [34]. Respiratory bursts are produced by phagocytes in order to attack invasive pathogens during phagocytosis. Both phagocytosis and respiratory bursts have been extensively used to evaluate the immunity of sea cucumber [5,7,35]. The current study found that the phagocytic activity was significantly increased with the increasing dietary NT supplementation. Like phagocytosis, the respiratory bursts gradually increased with dietary supplementation of NT and exerted the highest activity when adding 700 mg/kg NT in the diet. Similar results were reported in carp (*Cyprinus carpio* L.) [36], grouper [16] and white pacific shrimp [21]. Contrary to that in hybrid striped bass [15] and salmonids [12], no enhancement of the respiratory burst activity was found in the head kidney macrophage. It was suggested that the effects of dietary NT on the immunity of aquatic animals could be species dependent.

Skin ulceration syndrome is one of the serious diseases causing mass mortality in sea cucumber. It is displayed as anorexia, shaking head, mouth timidity, general atrophy, and skin ulceration in sea cucumber [37]. Data from the present study showed that sea cucumber fed the diet with 150 mg/kg and 375 mg/kg of NT had significantly lower cumulative mortality than those fed diet without NT supplementation. The lowest cumulative mortality was 31.67%, which was almost half of that (63.33%) in the treatment without dietary NT supplementation. Similar results were reported in fish and shrimp. Li et al. [15] found that 40% mortality was observed in fish fed the basal diet after *Streptococcus iniae* exposure, while only 13.3% mortality occurred in hybrid striped bass fed the nucleotide-supplemented diet. Tahmasebi-Kohyani et al. [17] reported that the cumulative mortality of rainbow trout fed NT-supplemented diets was about 39% after challenge with *S. Iniae*, while in those fed no dietary NT mortality was about 85%. After a challenge test, cumulative mortalities (4–15%) of olive flounder fed inosine monophosphate (IM) supplemented diets were significantly lower than that (87%) of fish fed the control diet without IM supplementation [19]. Also in the shrimp, survival rate of test animals upon challenge with white spot syndrome virus infection was significantly higher in the NT group [21]. These results indicate that dietary supplementation of NT could enhance the resistance of animals against infections with various pathogens.

5. Conclusion

In conclusion, the present results showed that dietary NT could increase growth performance, non-specific immunity and disease resistance of sea cucumber. The optimum dietary NT supplementation level for sea cucumber was found to be 375 mg/kg. The application of dietary NT may present a novel strategy for health management in sea cucumber's aquaculture.

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