Fish & Shellfish Immunology 67 (2017) 119–128

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Efficacy of *Spirulina platensis* diet supplements on disease resistance and immune-related gene expression in *Cyprinus carpio* L. exposed to herbicide atrazine

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ARTICLE INFO

Article history:
Received 4 January 2017
Received in revised form
24 May 2017
Accepted 31 May 2017
Available online 1 June 2017

Keywords:
Atrazine
Complement C3
Cyprinus carpio
Immunoglobulin M
Interleukin-1β
Lysozyme
Spirulina platensis

ABSTRACT

The present study evaluated the immunotoxicological effects of the herbicide atrazine (ATZ) at sub-lethal concentrations and the potential ameliorative influence of *Spirulina platensis* (SP) over a sub-chronic exposure period on *Cyprinus carpio* L., also known as common carp. Common carp was sampled after a 40-days exposure to ATZ (428 μg/L) and SP (1%), individually or in combination to assess the non-specific immune response, changes in mRNA expression of immune-related genes [lysozyme (LYZ), immunoglobulin M (IgM), and complement component 3 (C3)] in the spleen, and inflammatory cytokines (interleukins IL−1ß and IL−10) in the head kidney using real-time PCR. Additionally, disease resistance to *Aeromonas sobria* was evaluated. The results revealed that ATZ exposure caused a significant decline in most of the hematological variables, lymphocyte viability, and lysozyme and bactericidal activity. Moreover, ATZ increased the susceptibility to disease, reflected by a significantly lower post-challenge survival rate of the carp. ATZ may induce dysregulated expression of immune-related genes leading to downregulation of mRNA levels of IgM and LYZ in the spleen. However, expression of C3 remained unaffected. Of the cytokine-related genes examined, *IL−1B* was up-regulated in the head kidney. In contrast, the expression of *IL−10* gene was down-regulated in the ATZ-exposed group. The SP supplementation resulted in a significant improvement in most indices; however, these values did not match with that of the controls. These results may conclude that ATZ affects both innate and adaptive immune responses through the negative transcriptional effect on genes involved in immunity and also due to the inflammation of the immune organs. In addition, dietary supplements with SP could be useful for modulation of the immunity in response to ATZ exposure, thereby presenting a promising feed additive for carps in aquaculture.

1. Introduction

The contamination of various water sources from chemicals of anthropogenic origin may render the water unsuitable for household use, irrigation, and fish cultivation and may adversely affect the aquatic life forms. Unfortunately, the unpredictable use of herbicides to control weeds and improve agricultural production and yields introduce them to aquatic environments located in the vicinity of agricultural areas, triggering a variety of impacts on non-target organisms, including fish and their surrounding environments, thereby hampering fish productivity [1]. Evidently, water contaminated with agrochemical pesticides associated with stressful raising conditions might increase the occurrence of infections produced by both pathogenic and opportunistic bacteria [2,3].

Atrazine (ATZ) is one of the most widely used agrichemicals. It is mainly applied as a pre-emergent herbicide to control broadleaf and grassy weeds in a variety of crops such as maize, cereals, lucerne, sugarcane, and submerged vegetation in slow running and stagnant waters [4]. ATZ is considered to be a potential contaminant in aquatic habitats, where it may gain entry through runoff from treated fields or spillage or accidental release during

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http://dx.doi.org/10.1016/j.fsi.2017.05.065
1050-4648/

production, packaging, warehousing, and waste disposition, as well as frequent torrential rains. Therefore, residues are found in various environments, particularly in surface and groundwater [5]. Because of its relative persistence in soil, moderate solubility in water, and a half-life extended from a few days to months, surface and groundwater contamination is on the rise in many countries [6]. As indicated by the reviews and studies conducted by the United States Environmental Protection Agency (US EPA) and different researchers, the extremely high concentrations of ATZ were observed in the water of some agricultural and urban areas. For instance, ATZ level of up to 330 μg/L was reported in rivers and surface waters in the USA [7]. Doses as low as 0.1 μg/L of ATZ were found to cause dramatic effects on amphibians and fish [8].

A number of early studies have been conducted to evaluate the impact of ATZ on various aquatic animals. Most of them have focused on the acute toxicity of ATZ on fish. It was reported that ATZ adversely affects immune function in juvenile rainbow trout [9] and developing *Xenopus laevis* tadpoles [10]. Furthermore, ATZ is considered to be a potent endocrine disruptor in various taxa [11,12] and known to affect the reproductive yield in fathead minnow [8]. It acts as an enzyme inhibitor in the Neotropical fish *Prochilodus lineatus* leading to impairment of hepatic metabolism and genotoxic damage to different cell types [13]. Moreover, ATZ mediated alteration in acetylcholinesterase activity and mRNA levels in the brain, muscles [14], liver, kidneys, and gills [15] of common carp induces oxidative stress [16] and disturbs the swimming performance of larval zebrafish [17]. Therefore, many European and African countries have restricted its use [18].

Spirulina platensis (SP) is one of the most commonly utilized dietary supplements in humans and many animal species, including fish [19]. Recently, there has been a growing interest in its application in fish for its antioxidant activity, growth-promoting role and immunomodulatory effect [20]. SP is a filamentous microalga belonging to the class of cyanobacteria with a characteristic photosynthetic ability [21]. It is one of the most commonly used microalgae and is a rich source of protein, vitamins, minerals, essential fatty acids, essential amino acids, and pigments [19]. SP is also a source of bioactive compounds such as ß-carotene, phycocyanin, and allophycocyanin with antioxidant and anti-inflammatory activities, sulfated polysaccharides with anti-viral properties, and sterols, which are mainly responsible for antimicrobial activity [22].

Studies conducted in the past using SP as a supplement and as a partial substitution in the diets of fish and crustaceans have reported it to enhance the specific and non-specific immune responses in common carp, *Cyprinus carpio* [23], tilapia, *Oreochromis niloticus* [24], white sturgeon, *Acipenser transmontanus* [25], Mekong giant catfish, *Pangasianodon gigas* [26], and African sharp tooth catfish, *Clarias gariepinus* [27]. The use of SP has been observed to improve the innate immunity and increase the resistance against infection caused by *Vibrio alginolyticus* in the shrimp, *Litopenaeus vannamei* [28]. Furthermore, SP is accounted for encountering organ toxicities induced by heavy metals, including lead, and cadmium [29]. SP administration minimizes the pesticide-induced toxic effects of deltamethrin on *Oreochromis niloticus* by its free radical scavenging and potent antioxidant activity [19]. Moreover, SP polysaccharide was reported to decrease mutagenesis in animal studies and activate hematopoietic system [30].

A comprehensive study of the physiological mechanisms of ATZ mediated sub-lethal toxicity and simultaneous pathogenic infections under experimental conditions along with counteractive measures has rarely been attempted. Although, the effect of ATZ pesticides on the immune system has been investigated in other aquatic organisms; few studies have considered the effect on common carp [31,32]. Moreover, the use of modulating agents to overcome such effects has been a topic of concern for fish farmers and the industry.

The current study targeted to collect more information on the threats imposed by the contamination with ATZ-based pesticides on the immune system of common carp *(Cyprinus Carpio* L.*)*. Also, the study examined the role of SP in ameliorating the responses elicited by exposure of fish to ATZ. This was performed by measuring the immunological endpoints such as differential leukocyte count in peripheral blood, lysozyme activity, protein profile, expression of immune-related genes, and the susceptibility to opportunistic infections such as that caused by *Aeromonas sobria*.

2. Materials and methods

2.1. Tested compounds

ATZ [2-chloro-4-ethylamino-6-isopropyl-amino-s-triazine] (98% purity) was purchased from Sigma-Aldrich Co., St. Louis, MO, USA. It was dissolved in dimethyl sulfoxide (DMSO; SERVA Electrophoresis GmbH, D−69115 Heidelberg, Germany) to obtain the experimental concentration used in the study. *Spirulina platensis* (Sp) is bright, blue-green powder with a characteristic odor, it was stored in fresh, dry, pest-free and hygienic store rooms to prevent deterioration of *Spirulina* pigments. It was purchased from EL-Hellowa for Biological Products.

2.2. Fish

A sample of apparently healthy *Cyprinus carpio* (N = 120, average weight 118 ± 4.2 g) was obtained from Abbassa Fish Hatchery, Sharkia Province, Egypt. Each 10 fish were acclimated in glass aquaria (80 × 40 × 30 cm) filled with 60 L of dechlorinated tap water for 15 days and fed on a basal diet before the initiation of the experiment. All the aquaria were kept under the same conditions of water temperature (25 ± 1.02 °C), pH (6.9 ± 0.1), dissolved oxygen (7.4 ± 0.34 mg/L), and ammonia (0.035 ± 0.01 mg/L) with a controlled photoperiod (10 h light: 14 h dark) in the laboratory. Fish were fed twice daily (9:00 a.m. and 3:00 p.m.) at a rate of 3% of their biomass.

2.3. Experimental design and diet

The experiment lasted for 40 days during which, *Cyprinus carpio* were randomly divided into four groups, each group having three replicates (10 fish/replicate; n = 30 fish/group). The first group served as the control group that was fed on a basal diet without any treatment, DMSO was added to the water at volume (0.02 ml/L; 20 ppm). The second group (SP) was fed on a basal diet supplemented with 1% SP [19]. The third group (ATZ) was fed on a basal diet and exposed to ATZ at a dose of $^{1}/_{5}$ 96 h LC_{50} value (428 μg/L), this concentration was selected to be sub-lethal to carp during a 40 days exposure (the calculated 96-h LC_{50} of ATZ is 2.142 mg/L [14]). The fourth group (ATZ/SP) was fed on a basal diet supplemented with 1% SP and exposed to ATZ at the same dose as the third group. The water was completely replaced every 48 h by transferring the fish to freshly prepared pesticide solutions. Throughout the exposure period, the clinical signs, post-mortem lesions, and mortalities were recorded. The experimental protocol was approved by the Ethics of Animal Use in Research Committee (EAURC) of Cairo University, and experimental procedures were conducted in accordance with the NIH general guidelines for the Care and Use of Laboratory Animals in scientific investigations.

The proximate analysis of basal diet indicated 38.9% crude protein, 10.5% crude lipid, and 3.68% fiber according to the