Effect of dietary administration of the β-hydroxy-β-methylbutyrate on the innate immunity and protection against motile *Aeromonas* septicaemia in fish

ANDRZEJ K. SIWICKI, EDWARD GŁĄBSKI, KRZYSZTOF KAZUŃ, BARBARA KAZUŃ, AGNIESZKA LEPA, MAGDALENA MAJEWICZ-ZBIKOWSKA

Department Pathology and Immunology, Inland Fisheries Institute in Olsztyn, Poland

Abstract

In the present study examined the influence of leucine metabolite β-hydroxy-β-methylbutyrate (HMB) on the nonspecific cellular and humoral defence mechanisms and protection against motile Aeromonas septicaemia in common carp (Cyprinus carpio). The carp were fed with 50 mg or 100 mg HMB/kg body weight per day (HMB-fed group) for 4 weeks. The control group of carp was fed pellets without HMB (HMB-free group). After feeding HMB, 20 healthy carp of approximately 150 g were anaesthetised and blood was drawn from the caudal vein into heparinized syringes. Also the pronephros and spleen of each fish was removed aseptically and single cells suspensions were obtained for immunological study. A disease challenge test using Aeromonas hydrophila were conducted after 4 weeks of feeding. Briefly, 100 fish from each group were each given a single intraperitoneal injection of a 48 h growth of A. hydrophila. Mortalities were tabulated and the presence of pathogens was confirmed by isolation from the kidney. The results of this experimental study showed that HMB at a dose of 50 and 100 mg/kg body weight statistically stimulated the non-specific cellular and humoral defence mechanisms and protection against motile Aeromonas septicaemia (MAS) in carp.

Key words: *fish, metabolite β-hydroxy-β-methylbutyrate (HMB), innate immunity, MAS.*

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Introduction

Nutritional support is important for optimum health by providing the building blocks of nonspecific cellular and humoral defence mechanisms and thus protection against infectious diseases. Certain nutrients can be supplemented in the food to stimulate or modulate directly host defence mechanisms. At present, substances for treating fish diseases including antibiotics, other drugs and chemicals such as these used for sterilising fish-holding ponds can influence fish immune system. While each therapeutic agent is at least partly effective in the treatment of a particular disease, problems arise with accumulation of these substances in the environment as well as the emergence of resistant pathogenic strains when using antibiotics [1, 2].

The substance β-hydroxy-β-methylbutyrate (HMB) is a catabolite of the amino acid leucine. It is produced in the body via oxidation of the ketoacid of leucine (ketoisocaproate – KIC). Since leucine has an important role in protein metabolism regulatory and because of the obligatory conversion of leucine to KIC, several researches [3, 4] have postulated that KIC was the active ingredient responsible for most of the beneficial effects of leucine on protein metabolism. In addition to its influence on protein metabolism, some studies have confirmed a major role of leucine and leucine metabolites (HMB, β-hydroxy-methyl glutarate – HMG, β-hydroxy butyrate – BHB) in modulating the immunocompetence cells, especially of lymphocyte activity [5, 6]. Other study showed that only direct leucine metabolite to affect lymphocyte blastogenesis was HMB [7]. The hypothesis that HMB could be an effective sup-

Correspondence: Andrzej K. Siwicki, Department of Microbiology and Clinical Immunology, University of Warmia and Mazury in Olsztyn, Oczapowskiego 13, 10-719 Olsztyn, Poland, phone number: +48 89 523 32 17, e-mail: aksiw@infish.com.pl

plement in maximising muscle growth and preventing muscle loss during stressful situation was first tested in animals. Domestic animals such as dogs, chickens, cattle and swine are all susceptible (as are humans) to the negative metabolic effects to stress. This stress leads to weight loss and a marked increase in susceptibility to disease, both of which can impact the economics of animal production. In general, the animal research clearly shows that in moderate to severe stress, HMB counteracts many of the negative effects of stress and can increase the growth and health of animals [8, 9]. Moreover, this extensive database in animal's shows that over a wide range of doses, HMB is safe and does not have demonstrable toxic effects in any species tested [3, 4, 8]. Also in different species of fish HMB activated cell-mediated immunity and protection against diseases.

Motile *Aeromonas* septicaemia (MAS) is among the most common bacteria in freshwater habitats throughout the world and these bacteria frequently cause disease among cultured and feral aquatic animals [10]. Motile *Aeromonas* septicaemia cause diverse pathologic conditions that include acute, chronic, and latent infections. Severity of disease is influenced by a number of interrelated factors, including bacterial virulence, the kind and degree of stress exerted on a population of animals. Motile Aeromonas septicaemia often concurs with viral infections such as Spring Viraemia of Carp (SVC) or other bacterial diseases. In addition, motile *Aeromonas* cause disease in frogs, turtles and snakes and also cause disease in warm-blooded vertebrates. Moreover, in human motile *Aeromonas* are associated with various conditions including gastro-enteritis, wound infections and septicaemia. Also *Aeromonas hydrophila* may cause septic arthritis, diarrhoea, corneal ulcers, skin and wound infections, meningitis, and fulminating septic cemias [11]. Clinical isolates of *A. hydrophila* have been obtained from seafood, fish, raw milk, red meats and poultry and all isolates had biotypes identical to those of enterotoxin-positive strains [12]. The ability of these bacteria to grow competitively at 5°C may be indicative of their potential as a public health hazard. Resistance of *Aeromonas* sp. against the most commonly used antimicrobials in aquaculture has developed greatly in the recent years. Those antimicrobials have been so abused by fish farmers that they have been accumulated in the edible muscles and different internal organs of the treated fish. So other environmentally safe alternatives including natural or synthetic immunostimulants are recommended.

The goal of the study is to examine the influence of feeding the leucine metabolite HMB on the innate immunity and on resistance against MAS in carp (*Cyprinus carpio*) grown in an intensive system of culture.

Material and methods

The fish were reared at the Experimental Station of Inland Fisheries Institute in Olsztyn, Poland. The juvenile carp were reared circular fibreglass tanks with a water volume of 200 liters each. The tanks were part of a recilculation system equipped with biological and mechanical filters. The water temperature was maintained at a constant level of about 22°C. The fish of approximately 150 g were fed with a commercial carp feed (Bestfeed, Poland) using automatic band feeders. The carp were fed with 50 mg or 100 mg HMB/kg body weight per day (HMB-fed group) for 4 weeks. The control group of carp was fed pellets without HMB (HMB-free group). The leucine metabolite HMB was obtained as a monohydrate calcium salt with a purity > 98% (Metabolic Technologies, Ames, USA). The feed was distributed *ad libitum*, which was confirmed by observations of feed waste. The fish were observed daily for unusual behaviour, morphological changes and any mortality. Four weeks after feeding HMB, 100 healthy carp from each experimental and control groups were anaesthetised in Propiscin (IFI, Poland) and blood was drawn from the caudal vein into heparinized syringes. Also the pronephros of each fish was removed aseptically and single cells suspension were obtained for isolating individual cells using either a Gradisol (Polfa) or Percoll (Pharmacia) gradient.

The metabolic activity of pronephros phagocytes by their respiratory burst activity (RBA) stimulated by Phorbol myristate acetate (PMA, Sigma) was measured by the technique presented by Siwicki *et al.* [1]. Potential killing activity (PKA) of the pronephric phagocytes was measured by the method presented by Siwicki and Anderson [13]. The lymphocytes proliferation (LP) was determined by the MTT colorimetric assay methods modified by Siwicki *et al.* [14] for the fish species. The mitogens concanavaline A (ConA, Sigma) or lipopolisaccharide (LPS, Sigma) were used for the stimulation of lymphocytes. The lysozyme activity in the plasma was measured in a turbidimetric assay presented by Siwicki and Anderson [13] and ceruloplasmine activity in the plasma was determined according to Siwicki and Studnicka [15] which was modified for micro-methods. Total protein level in serum was measured by the colorimetric Lowry micro-methods (Sigma, Diagnostic Kits) and total immunoglobulin (Ig) levels in the serum were measured by spectrophotometric methods [13].

A disease challenge test using *A. hydrophila* were conducted after 4 weeks of feeding. Briefly, 100 fish from each group were each given a single intraperitoneal injection of a 48 h growth of *A. hydrophila* (0.2 ml). Mortalities were tabulated and the presence of pathogens was confirmed by isolation from the kidney.

The results were verified statistically by a one-way ANOVA analysis of variance (GraphPad Prims software package) and the significance of differences between the groups was verified with a Tukey's test.

Results and discussion

The current study used a fish model to examine the influence of different concentrations of HMB on innate

immunity and protection against motile *Aeromonas* septicaemia. The effect of different doses of HMB on cell-mediated and humoral-mediated immunity are presented in Table 1. The results of this experimental study showed that HMB at a dose of 50 and 100 mg/kg body weight statistically ($P < 0.05$) stimulated the nonspecific cellular and humoral defence mechanisms in common carp, compared to the control HMB-free group. The phagocytic ability (RBA) and potential killing activity (PKA) of pronephros phagocytes were statistically significant higher ($P < 0.05$) from HMB-fed carp, compared to fish from control group. But in this experimental study, we not observed statistically significant differences between the group fed HMB at dose 50 mg/kg body weight and 100 mg/kg body weight. The similar pattern was observed in proliferative response of pronephros lymphocytes stimulated by mitogens ConA or LPS. The lymphocytes proliferation was statistically significant ($P < 0.05$) higher in the group fed with HMB, compared to the control group of fish. Either we do not observed statistically significant differences between the group fed HMB at dose 50 and 100 mg/kg body weight. The results of the study showed that HMB activated nonspecific humoral-mediated immunity as well. The lysozyme activity in plasma and total Ig levels in serum were significantly ($P < 0.05$) higher that in the control group of carp. Moreover HMB at examined doses significantly increased total protein level is serum. On the other hand we not observed significant differences in the activity of ceruloplasmine which is very important acute phase-protein. The results suggested that HMB at examined doses does not affect hepatocytes' ability to produce acute phase mediators.

The challenge tests showed that HMB applicated orally decrease the mortality due to experimental infection with *A. hydrophila.* Cumulative mortality rates was of 30% in the group fed with HMB at dose 100 mg/kg body weight and 35% in the group fed with 50 mg/kg, compared to the control HMB-free group of fish with cumulative mortality of 70%.

Several natural and synthetic drugs and biological modifiers have been tested in fishes *in vitro* and *in vivo* and many of these products were applicated for stimulation of innate immunity and protection against infectious diseases [16, 17]. In this study we observed immunomodulatory action of HMB on the nonspecific cellular and humoral immunity and it's protective role against MAS on the fish model. Moreover oral administration of HMB in several animal species demonstrated immunomodulatory effects on the macrophage and lymphocyte activity [18]. The similar pattern was observed on the carp model, where HMB applicated orally increased the phagocyte and lymphocyte activities. Additionally the humoral-mediated immunity statistically significant increased and demonstrated very important interaction with cell-mediated immunity.

These results showed that HMB is very effective in reducing of mortality induced by acute form of MAS disease. This is a very important for the development of therapy of this disease in aquaculture. Chloramphenicol and oxytetracycline are effective in treating fish when was administered orally for 10 days. This treatment sometime produces dramatic results when it is administered for even 2 or 3 days, and is particularly effective when fish become infected after they have been handled, crowded, or held under stress for short periods of time. However, chloramphenicol is prohibited in food fishes and discouraged in other fishes because it is a drug of last choice in certain human diseases e.g. typhoid fever. Uncontrolled use of chloramphenicol can result in drug resistance and thus reduce the value of this antibiotic in human medicine. Also oxytetracycline is an antibiotic that induces very strong immunosuppression and inhibited the protein synthesis in fish [1, 2].

The oral application of HMB demonstrated to have a practical and economical impact in intensive aquatic animal culture. The use of natural immunomodulators in order

Table 1. The innate cellular and humoral defence mechanisms levels in control-fed and HMB-fed carp at doses 50 and 100 mg/kg body weight ($n = 20$; mean ±SD)

Immunological parameters	Control group	HMB-fed groups	
		50 mg/kg	100 mg/kg
RBA of phagocytes (OD 620 nm)	0.45 ±0.04	0.59 ±0.05*	0.60 ±0.04*
PKA of phagocytes (OD 620 nm)	0.41 ±0.04	0.62 ±0.04*	0.65 ±0.05*
LP stimulated by ConA (OD 620 nm)	0.49 ±0.05	0.57 ±0.05*	0.59 ±0.06*
LP stimulated by LPS (OD 620 nm)	0.34 ±0.04	0.51 ±0.05*	0.54 ±0.06*
Lysozyme activity (mg/l)	45.5 ±5.8	57.5 ±3.9*	58.4 ±4.0*
Ceruloplasmine activity (IU)	22.8 ±3.5	21.5 ±2.5	20.8 ±4.5
Total protein in serum (g/l)	54.5 ±4.0	62.5 ±2.5*	64.5 ±3.5*
Total Ig in serum (g/l)	18.0 ±1.4	21.5 ±1.5*	21.8 ±1.6*

**statistically significant P < 0.05*

to modulate or restore proper functioning of the immune system is a new, intensively developing branch of the experimental and clinical immunology. The creation of such possibilities may be vital practice, especially in the environment of a great farming and public health.

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