Effect of HMB (β-Hydroxy-β-Methylbutyrate) on *in vitro* Proliferative Responses of Sheatfish (*Silurus glanis*) and Catfish (*Ictalurus melas*) Lymphocytes Stimulated by Mitogens

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Abstract

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The aim of this work was to study the *in vitro* effect of β-hydroxy-β-methylbutyrate (HMB) on the proliferative response of sheatfish (*Silurus glanis*) and catfish (*Ictalurus melas*) lymphocytes stimulated by mitogens at temperatures of 20 °C and 30 °C. The pronephros lymphocytes were isolated from 20 sheatfish and 20 catfish, respectively. Mitogens, ConA at concentration of 64 μg/ml or LPS at concentration of 160 μg/ml were used for stimulation of lymphocytes. The plates were incubated for 72 h at 20 °C and 30 °C without CO_2. The MTT method was used for the study of proliferative responses of lymphocytes stimulated by mitogens. The plates were read on a microreader, using the test wavelength of 620 nm. Net optical density (OD) values were obtained by subtracting the mean OD of negative control cells (not stimulated by mitogens) from that of stimulated cells. The results showed that the HMB at concentrations 10, 50 and 100 μg HMB/ml of medium significantly ($p < 0.05$) increased T-lymphocyte proliferation stimulated by ConA and B-lymphocyte proliferation stimulated by LPS, respectively, at 20 °C and 30 °C, compared to control. The highest proliferative response of T-cells at 30 °C was observed at concentrations 50 and 100 μg HMB/ml in both species. The results showed that HMB stimulated the lymphocyte activity and suggested a possible application of HMB for modulation of cell-mediated immunity in sheatfish and catfish.

Sheatfish, catfish, HMB, different temperature, in vitro lymphocyte activity

Calcium β-hydroxy-β-methylbutyrate (calcium HMB) is a calcium salt of β-hydroxy-β-methylbutyrate (HMB). The latter is a leucine metabolite produced in the body via oxidation of the ketoacid of leucine (ketoisocaproate, KIC). Since leucine has an important protein metabolism regulatory role and because of the obligatory conversion of leucine to KIC, several researchers (Buckspan et al. 1986; Nissen and Abumrad 1997) have postulated that KIC was the active ingredient responsible for most of the beneficial effects of leucine on protein metabolism. In addition to its effects on protein metabolism, some studies have established a major role for added leucine and leucine metabolites (HMB, β-hydroxy methyl glutarate – HMG, β-hydroxy butyrate – BHB) in modulating the immunocompetence cells, especially of lymphocyte activity (Ichihara 1975; Nonnecke et al. 1991). The results showed that the only direct leucine metabolite to affect lymphocyte blastogenesis was HMB (Kuhlman 1989). In our preliminary *in vitro* study we presented that HMB at different concentrations activated the lymphocyte activity in fish (Siwicki et al. 2000).

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In this study, we continue to examine the *in vitro* effects of HMB on the proliferative response of sheatfish (*Silurus glanis*) and catfish (*Ictalurus melas*) lymphocytes stimulated by mitogens at temperatures of 20 °C and 30 °C.

Materials and Methods

Experimental design

The pronephros were isolated from 20 healthy sheatfish (*Silurus glanis*) with a mean body mass 50 g and 20 healthy catfish (*Ictalurus melas*) with a mean body mass 50 g purchased from French (TAG) and Polish (IFI) catfish and sheatfish culture. Before dissection, the fish were anaesthetized in Propiscin (IFI, Poland) and bled from the caudal vein to reduce the blood volume in the pronephros. Single cell suspensions were obtained by placing the pronephros in RPMI-1640 medium (Sigma) and teasing it through a steel mesh. They were isolated on Histopaque-1077 (Sigma) gradients.

The calcium salt of β-hydroxy-β-methylbutyrate (HMB) from Metabolic Technologies Inc. Ames, IA, USA, at concentrations 0, 1, 10, 50 and 100 µg/ml of RPMI medium was used in this study.

Assay procedures

The proliferative response of the pronephros lymphocytes was determined by the MTT [3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] colorimetric assay method according to Mosmann (1983), as modified for fish by Siwicki et al. (1996). Isolated lymphocytes were distributed in 96-well plates (Nunclon, Denmark), 100 l/well at a concentration of 1×10^6 cells/ml in RPMI-1640 containing 2 mM L-glutamine, 0.02 mM 2-mercaptoethanol, penicillin/streptomycin (100 U/100 µg per ml), 1% Hepes buffer and 10% foetal calf serum (FCS) with or without HMB at different concentrations. Mitogens concanavalin A (ConA, Sigma) at concentration of 64 µg/ml or lipopolisaccharide (LPS, Sigma) at concentration of 160 µg/ml was then added (100 µl/well) to each well. The plates were incubated for 72 h at 20 °C and 30 °C without °CO_2. After incubation, 50 µl of MTT solution (dissolved in PBS at a concentration of 5 mg MTT/ml and sterilised by filtration) were added to all the wells and the plates were incubated at 22 °C for 4 h. After centrifugation of the plates, the media were removed and 100 µg/ml of isopropanol (Sigma) was added to all wells and mixed for 2 min. The micro-plates were read on a microreader, using the test wavelength of 620 nm. All samples were tested in triplicate and the mean value used as the results. Net optical density (OD) values were obtained by subtracting the mean OD of negative control cells (not stimulated by mitogens) from that of stimulated cells.

Statistical analyses were performed using the Student *t*-test. Differences between the treatment means were considered statistically significant at $p < 0.05$.

Results and Discussion

The current study used an *in vitro* model to examine the effects of different concentrations of HMB on proliferative response of sheatfish and catfish lymphocytes stimulated by mitogens at 20 °C and 30 °C. The influence of different concentrations of HMB on the proliferative response of sheatfish pronephric lymphocytes stimulated by ConA at the

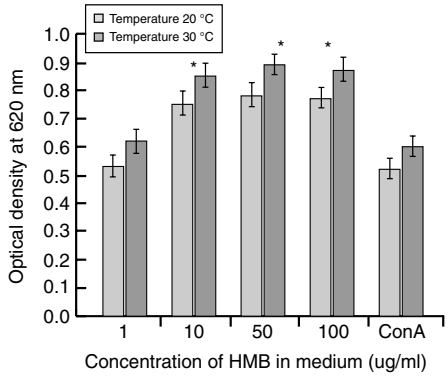

Fig. 1. *In vitro* influence of various concentrations of HMB on the proliferative response of sheatfish pronephric lymphocytes stimulated by ConA at the temperatures of 20 °C and 30 °C (mean ± SD; n = 20; * $p < 0.05$)

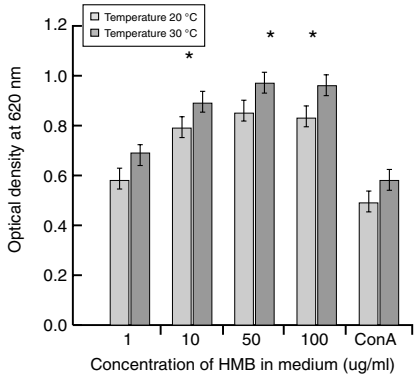

Fig. 2. *In vitro* influence of various concentrations of HMB on the proliferative response of catfish pronephric lymphocytes stimulated by ConA at the temperatures of 20 °C and 30 °C (mean ± SD; n = 20; * $p < 0.05$)

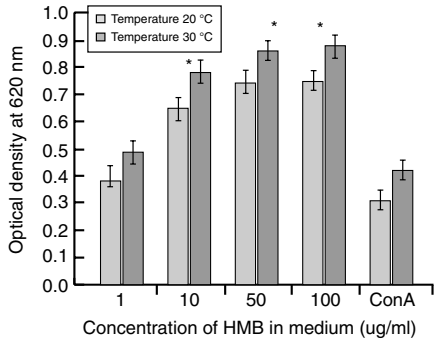

Fig. 3. *In vitro* influence of various concentrations of HMB on the proliferative response of sheatfish pronephric lymphocytes stimulated by LPS at the temperatures of 20 °C and 30 °C (mean ± SD; n = 20; * $p < 0.05$)

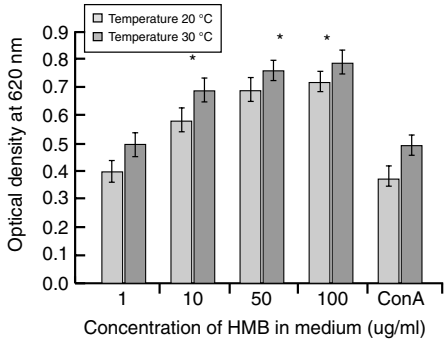

Fig. 4. *In vitro* influence of various concentrations of HMB on the proliferative response of catfish pronephric lymphocytes stimulated by LPS at the temperatures of 20 °C and 30 °C (mean ± SD; n = 20; * $p < 0.05$)

temperatures of 20 °C and 30 °C is shown in Fig. 1. The results showed that the HMB at concentrations 10, 50 and 100 μg HMB/ml of medium significantly ($p < 0.05$) increased T-lymphocyte proliferation stimulated by ConA, respectively, at 20 °C and 30 °C, compared to control (only mitogen). But no significant differences were found in cultures stimulated with 10, 50 and 100 μg/ml of HMB. The highest proliferative response of sheatfish T-cells at 30 °C was observed at concentrations 50 and 100 μg HMB/ml.

Similarly, the proliferative response of pronephric T-lymphocytes stimulated by ConA in catfish was also increased by HMB at concentration between 10 to 100 μg HMB/ml of medium (Fig. 2), and also the highest proliferative response of T-cells at 30 °C was observed at concentrations of 50 and 100 μg HMB/ml.

The effects of different concentrations of HMB on the proliferative response of sheatfish pronephric lymphocytes stimulated by LPS at 20 °C and 30 °C is shown in Fig. 3, and the effects of different concentrations of HMB on the proliferative response of catfish pronephric lymphocytes stimulated by LPS at 20 °C and 30 °C is shown in Fig. 4. The results showed that the HMB at concentration between 10 to 100 μg HMB/ml of medium significantly ($p < 0.05$) increased B-lymphocyte proliferation in sheatfish and catfish stimulated by LPS, respectively, at 20 °C and 30 °C, compared to control (only mitogen). But no significant differences were found in cultures stimulated with 10, 50 and 100 μg/ml of HMB. The highest proliferative response of sheatfish and catfish B-cells at 30 °C were observed at the concentrations of 50 and 100 μg HMB/ml.

The results of these *in vitro* studies have shown that HMB increased the lymphocyte T and B activity both in sheatfish and catfish measured by proliferative response to the ConA or LPS mitogen. The highest proliferative response was observed at a temperature of 30 °C. Similar to our previous *in vitro* studies, the stimulatory influence of HMB on macrophage and lymphocyte activity in rainbow trout and carp were observed (Siwicki et al. 2000). Recent *in vitro* studies in chickens strongly suggested that HMB increased macrophage activity (Peterson et al. 1999). In general, the animal research clearly shows that in moderate to severe stress, HMB counteracts many of the negative effects of stress and can improve the health of animals. Lastly, this extensive database in animals shows that over a wide range of doses, HMB is safe and does not have demonstrable toxic effects in any species tested (Gatnau et al. 1995; Nisses et al. 1994; Van Koevering et al. 1993).

In conclusion, HMB has been shown to be an immunopotentiating compound of proliferative response of T and B lymphocytes in both catfish species at different temperatures and suggested that HMB could be used to provide the nonspecific cell-mediated

immunity responses suppressed by temperature in catfish and sheatfish intensive culture. Several previous *in vitro* and *in vivo* studies with HMB in rainbow trout strongly suggest that HMB affects the macrophage and lymphocyte function, enhancing the fish ability to fight disease effectively and resist infection (Siwicki et al. 2000; Siwicki et al. 2003).

Účinek HMB (β-hydroxy-β-methylbutyrát) na *in vitro* proliferaci lymfocytů sumce velkého (*Silurus glanis*) a sumečka černého (*Ictalurus melas*) stimulovaných mitogeny

Cílem práce bylo studovat *in vitro* účinek β-hydroxy-β-methylbutyrátu (HMB) na proliferaci lymfocytů sumce velkého a sumečka černého stimulovaných mitogeny za teplot 20 °C a 30 °C. Lymfocyty byly izolovány z pronefros 20 sumců velkých a 20 sumečků černých. Pro stimulaci lymfocytů byly použity mitogeny ConA o koncetraci 64 $\mu g \cdot ml^{-1}$ nebo LPS o koncetraci 160 $\mu g \cdot ml^{-1}$. Plotny byly inkubovány 72 hod při teplotě 20 °C a 30 °C bez přístupu CO_2. Pro zjištění stupně proliferace byla použita metoda MTT. Destičky byly vyhodnoceny při vlnové délce 620 nm. Přesné hodnoty absorbance (optical density-OD) stimulovaných buněk byly získány odečtením průměru OD buněk negativní kontroly (nestimulovaných mitogeny) od OD stimulovaných buněk. Výsledky ukázaly, že HMB, ve srovnání s kontrolou, v koncentracích 10,50 a 100 μg $HMG \cdot ml^{-1}$ v médiu signifikantně ($P < 0.05$) zvýšilo proliferaci T-lymfocytů stimulovanou ConA a proliferaci B-lymfocytů stimulovanou LPS, při teplotách 20 °C a 30 °C. Nejvyšší proliferativní odpověď T-buněk při 30 °C byla u obou druhů pozorována při koncentraci 50-100 μg $HMG \cdot ml^{-1}$. Výsledky ukázaly, že HMG stimuloval aktivitu lymfocytů a naznačují možnou aplikaci HMG pro modulaci buněčné imunity u sumce velkého a sumečka černého.

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