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THE EFFECT OF NATURAL IMMUNOMODULATORS APPLIED IN IMMERSION ON NON-SPECIFIC IMMUNE RESPONSES IN RUSSIAN STURGEON (*Acipenser gueldenstaedti* Brandt)

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A B S T R A C T. The effect of immersion in 1,3/1,6 β-Glucan, Chitosan, and FinnStim on non-specific immune responses was studied in Russian sturgeon fry reared in cages in power plant effluents. In the first month of treatment, an increase of total leucocyte numbers due to high increase of lymphocyte level, stimulation of metabolic and phagocytic activity of PMN cells, an increase of activity and index of lysozyme, and a decrease of ceruloplasmin activity, level of γ-globulin fraction, and total protein in plasma were observed. Seasonal variability of these indices was also noted. Application of natural immunomodulators in *Acipenser gueldenstaedti* Brandt fry resulted in a pleiotropic effect, modulating cellular and humoral responses which differed in various treatments.

Key words: RUSSIAN STURGEON, CELLULAR AND HUMORAL IMMUNE RESPONSES, NATURAL IMMUNOMODULATORS

INTRODUCTION

Under intensive rearing conditions lacking complex water purification, the fish are exposed to infections caused not only by the pathogens, but also by opportunist microorganisms. In such situations, disease prevention becomes an important issue. A possibility of stimulating non-specific immune responses against infectious diseases was observed in many teleost fish (Siwicki, Studnicka 1986, Siwicki 1987, 1989, 1990, 1991, Anderson, Siwicki 1989, Siwicki et al. 1989, 1990, 1993 a, b, c, Siwicki, Cossarini-Dunier 1990). Such data for sturgeons are generally lacking. Among the immunoprophylactic methods, application of natural immunomodulators of plant or animal origin seems quite intersting (Azuma, Jolles 1987, Kono et al. 1987, Kajita et al. 1990, Raa et al. 1990). The present study deals with the effect of three natural substances on certain non-specific cellular and humoral immune mechanisms in Russian sturgeon (*A. gueldenstaedti* Brandt) fry.

MATERIAL AND METHODS

The study was performed on a pre-reared Russian sturgeon fry kept in cages placed in a channel with power plant effluents, under fish farm conditions. The following immunomodulators were applied:

- 1, 3/1, 6 β-Glucan (polymer of glucose bound with beta-bonds) obtained from yeast *Saccharomyces cerevisiae* supplied by USDI – Tunison Laboratory of Fish Nutrition , Cortland, NY, USA.
- Chitosan – deacetylated chitin (polymer of N-acetyl-D-glucosamine, bound with b-bonds) obtained from shrimp shells, produced by the Marine Fisheries Institute in Gdynia, Poland.
- FinnStim – bioformula prepared from betaine. Betaine itself is obtained from beetroot by-products (Kasumian, Kazhlaev 1992). The formula was made by Finnish Sugar Bioproducts Co. Ltd, Helsinki, Finland.

The experiment started after several weeks of adaptation, when the average body mass of fish (aged 0+) was 218 g. The immunomodulators were applied in immersion using the following concentrations: 1, 3/1, 6 β-Glucan – 100 mg/l, Chitosan – 100 mg/l, and FinnStim – 50 mg/l. Immersion duration was 30 minutes. Blood was sampled from 10 randomly harvested fish of each group, 1 and 4 months after treatment. At that time, average body mass was 374 (age 0^+), and 760 (age 1^+) respectively. The fish were anesthetized before sampling in a Propiscin solution. Plasma was isolated by blood centrifugation (Krzemińska-Ławkowicz, Maj 1993) and stored at –20°C. Total number of the leucocytes was determined using a hemocytometer and a staining fluid. Leucograms were eveluated in blood smears stained according to R. May – L. Grunwald and Giemsa (Goledec 1955, Ivanova 1983, Glagoleva 1981, Krzemińska-Ławkowicz, Maj 1993).

Metabolic activity of the neutrophils was estimated using NBT test (reduction of nitrotetrazolium blue) – a spectrophotometric method described by Studnicka et al. (1985). 0.2% NBT solution (Sigma) was used. Percentage of polymorphonuclear NBT-positive cells (PMN) was evaluated using the cytochemic method (Szczylik et al. 1979). Samples were fixed with alcohol, and stained with safranine according to Van Oss et al. (1973). NBT index was calculated according to Siwicki et al. (1985). Phagocytic activity of the cells was assessed using the method described by Avtalion, Shahrabani (1975) and O'Neill (1985), and expressed as phagocytic index (IF). Suspension of *Staphylococcus aureus* 209 P was used.

Lysosyme activity (LZM) in fish blood plasma was evaluated using the turbidimetric method described by Studnicka et al. (1986). For the analysis, suspension of *Micrococcus lysodeikticus* (Sigma) in phosphate buffer solution was applied. Extinction was measured with Eskalal spectrophotometer (Smith Kline Instruments, USA). Lysosyme of chicken egg was used as a standard (Sigma). Lysosyme index was calculated according to Siwicki and Studnicka (1987). Plasma ceruloplasmin activity (Cp), total protein level, and γ-globulin fraction level were eveluated using the micromethods described by Siwicki and Anderson (1993b). The results were subjected to Duncan's test.

RESULTS

Immersion of sturgeon fry in natural immunomodulators 1, 3/1, 6 β-Glucan, Chitosan, and FinnStim resulted in a statistically significant increase of the total leucocyte number from 30 to about 50 cells/mm^3 (Fig. 1), and of the lymphocte number from 20 to about 40 cells/mm^3, which persisted in all experimental groups until the end of the study (Fig. 2). The following changes in the neutrophil, eosinophil and monocyte numbers were also observed:

- Chitosan application resulted in a considerable increase (about 50%) of the neutrophil number 4 months post treatment (Fig. 3), and an increase of the eosinophil number 1 month after treatment from about 3 to over 5 thousand/mm^3 (Fig. 4);
- 1, 3/1, 6 β-Glucan treatment caused a significant increase (over 50%) of the monocyte number within 1 month post the application (Fig. 5).
- FinnStim produced a slight increase of the neutophil number (Fig. 3), and a significant drop of the monocyte number in the blood (Fig. 5).

Four months after treatment, a statistically significant (over two fold) increase of the number of eosinophils was observed in all experimental groups compared to the control (Fig. 4).

Percentage of NBT-positive PMN cells increased after 1 month by about a half (Fig. 6), and NBT reduction ability increased (particularly 1 month after treatment) almost twice. In cases of Chitosan and FinnStim treatments, NBT reduction remained elevated until the end of the experiment (Fig. 7). NBT index increased significanly within 1 month after the immersion, but within 4 months it returned to the control

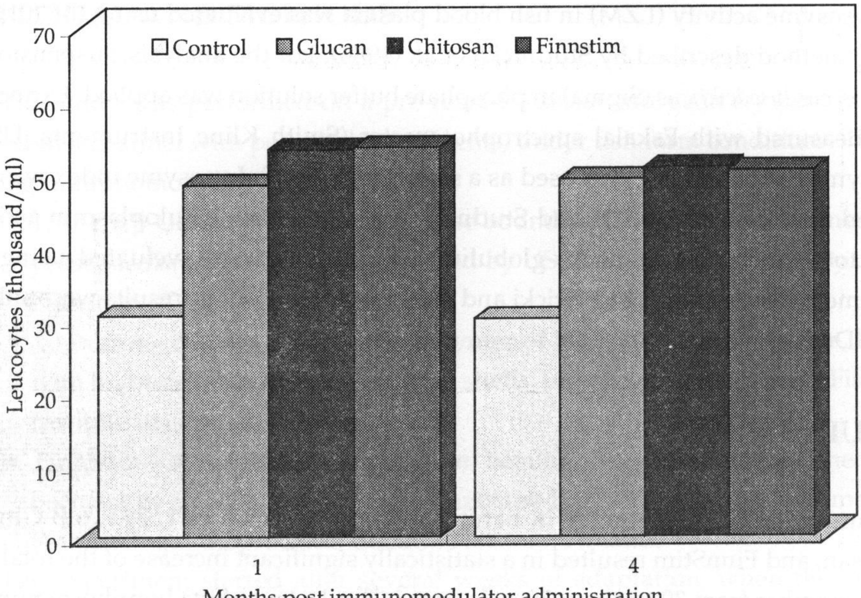

Fig. 1. The effect of natural immunomodulators on total leucocyte count in Russian sturgeon *Acipenser gueldenstaedti* Brandt

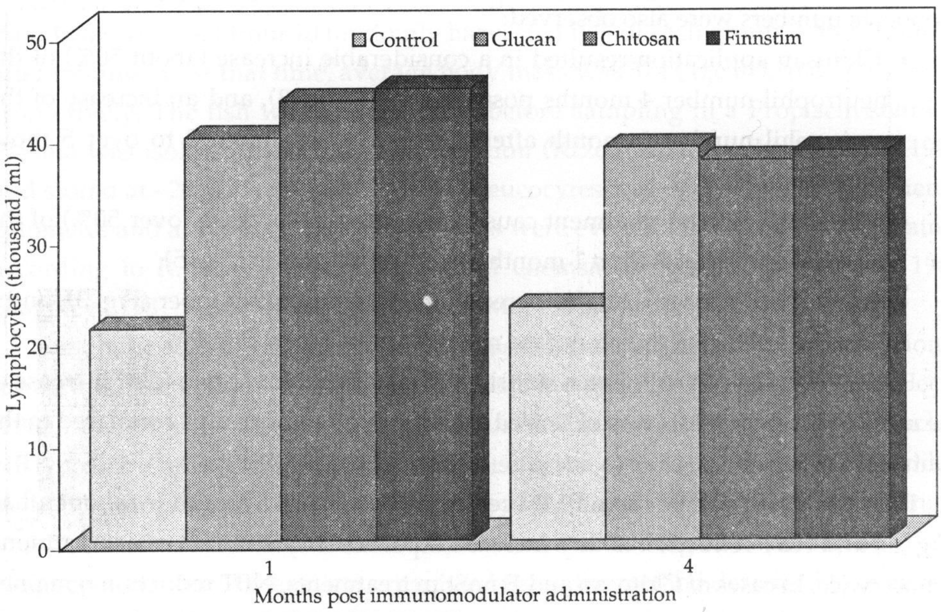

Fig. 2. The effect of natural immunomodulators on total lymphocyte count in Russian sturgeon *Acipenser gueldenstaedti* Brandt

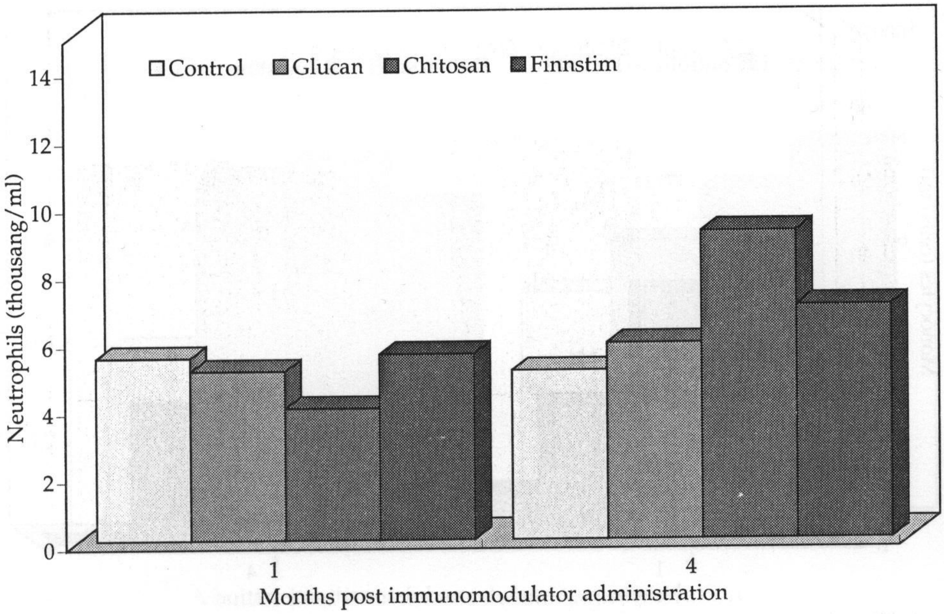

Fig. 3. The effect of natural immunomodulators on total neutrophil count in Russian sturgeon *Acipenser gueldenstaedti* Brandt

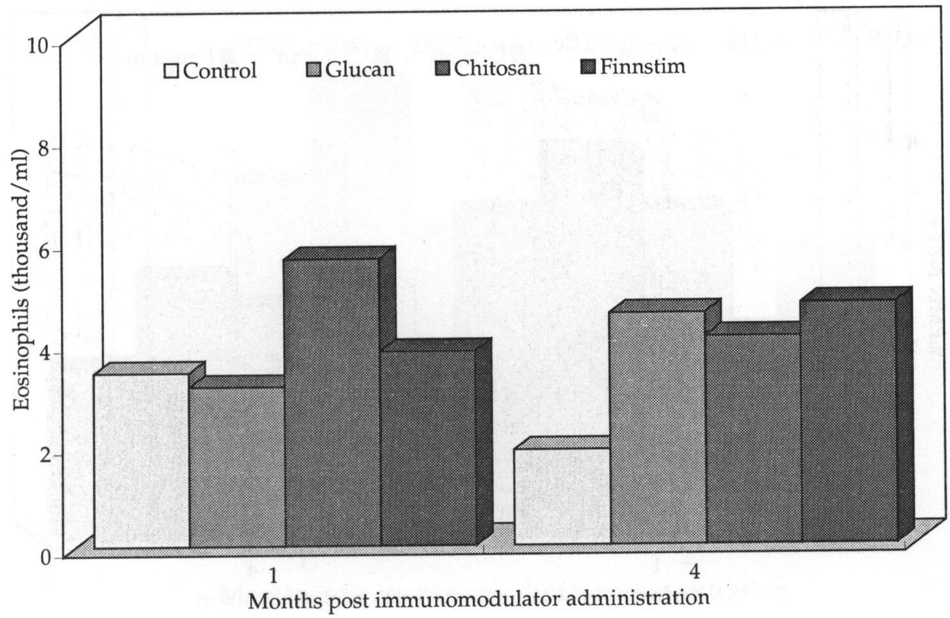

Fig. 4. The effect of natural immunomodulators on total eosinophil count in Russian sturgeon *Acipenser gueldenstaedti* Brandt

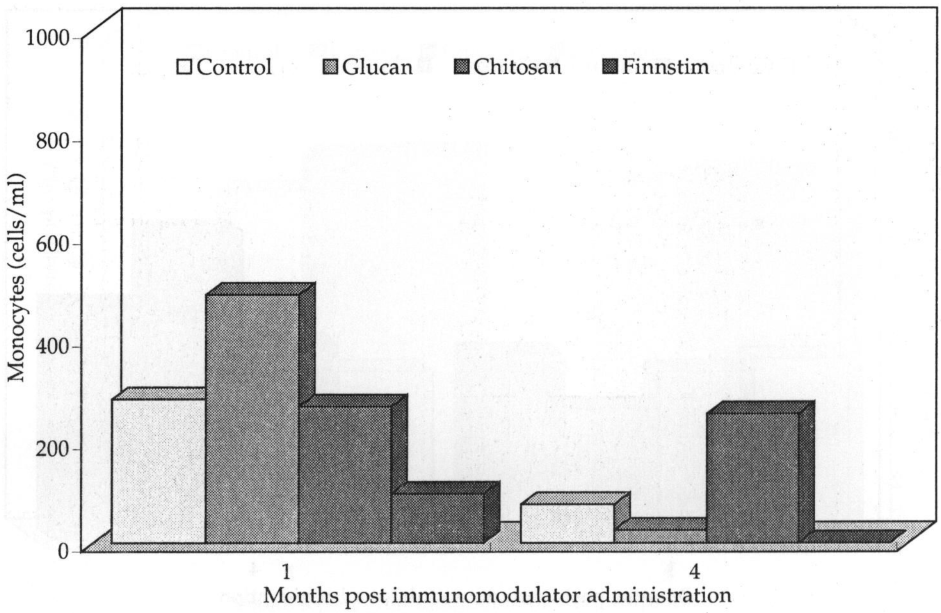

Fig. 5. The effect of natural immunomodulators on total monocyte count in Russian sturgeon *Acipenser gueldenstaedti* Brandt

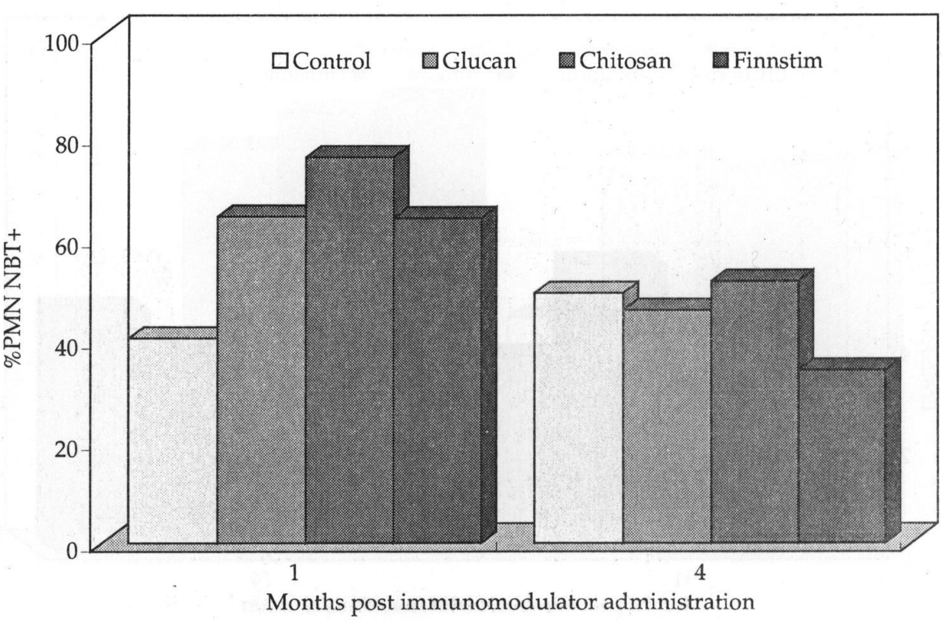

Fig. 6. The effect of natural immunomodulators on percentage of NBT-positive PMN cells in Russian sturgeon *Acipenser gueldenstaedti* Brandt

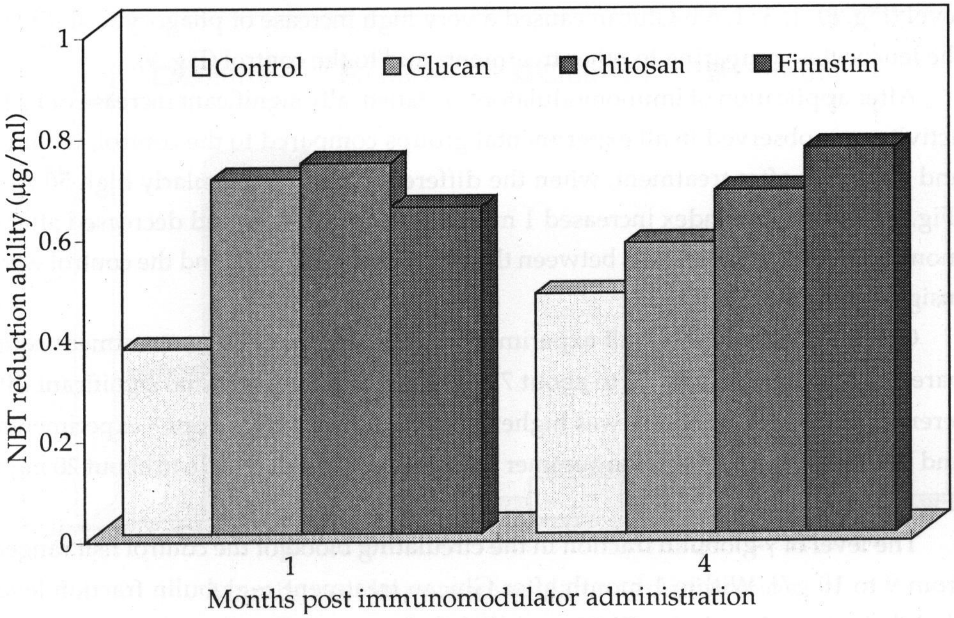

Fig. 7. The effect of natural immunomodulators on NBT reduction ability in Russian sturgeon *Acipenser gueldenstaedti* Brandt

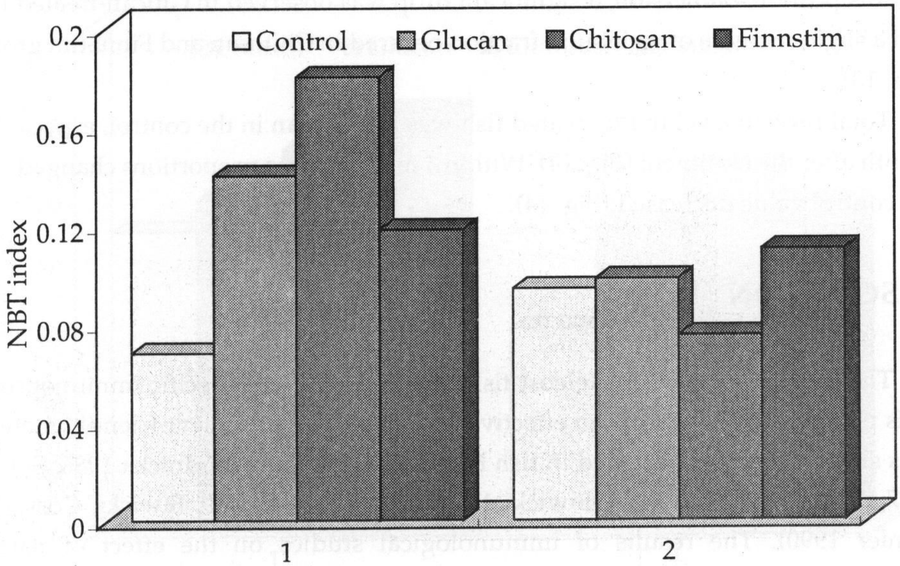

Fig. 8. The effect of natural immunomodulators on phagocytic index in Russian sturgeon *Acipenser gueldenstaedti* Brandt

level (Fig. 8). 1, 3/1, 6 b-Glucan caused a very high increase of phagocytic ability of the leucocytes comparing to other treatments and to the control (Fig. 9).

After application of imunomodulators, a statistically significant increase of LZM activity was observed in all experimental groups compared to the control, within 1 and 4 months after treatment, when the differences were particularly high 50-70% (Fig. 10). Lysozyme index increased 1 month post-immersion, and decreased after 4 months, when the differences between the experimental groups and the control were insignificant (Fig. 11).

Cp activity dropped in all experimental groups 1 month after treatment compared to the control: from 12 to about 7 mg%, but after 4 months no significant differences occured. Cp activity was higher in the autumn in all groups – experimental and control ones - compared to summer values, and ranged from 16 to about 20 mg% (Fig. 12).

The level of γ-globulin fraction in the circulating blood of the control fish ranged from 9 to 10 g/l. Within 1 month after Glucan treatment, γ-globulin fraction level slightly increased, and after Chitosan – slightly increased. FinnStim treatment resulted in a significant drop of gamma globulins in sturgeon blood, down to 2 g/l. Four months post the immersion, a significant drop was observed in Glucan-treated fish, and a slight increase of γ-globulin fraction occured in Chitosan and FinnStim groups (Fig. 13).

Total protein level in the treated fish was lower than in the control, especially 1 month after the treatment (Fig. 14). Within 4 months these proportions changed, and the control value decreased (Fig. 14).

DISCUSSION

The results obtained for teleost fish revealed that non-specific immunopropylaxis using natural agents is an effective, cheap, and environment-friendly method, thus should be widely applied in fish health-care (Siwicki, Studnicka 1986, Siwicki 1987, 1990, 1991, Anderson, Siwicki 1989, Siwicki et al. 1989, Siwicki, Cossarini-Dunier 1990). The results of immunological studies on the effect of natural immunomodulators in contaminated water indicate their usefulness under wide variety of environmental conditions (Siwicki et al. 1990b, Dunier et al. 1995, Studnicka et al. 1997).

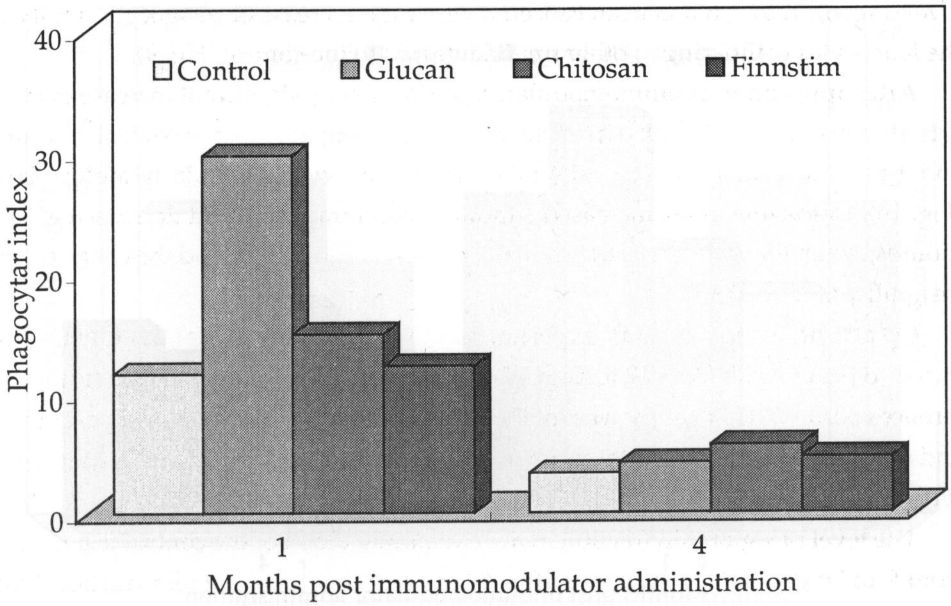

Fig. 9. The effect of natural immunomodulators on NBT index in Russian sturgeon *Acipenser gueldenstaedti* Brandt

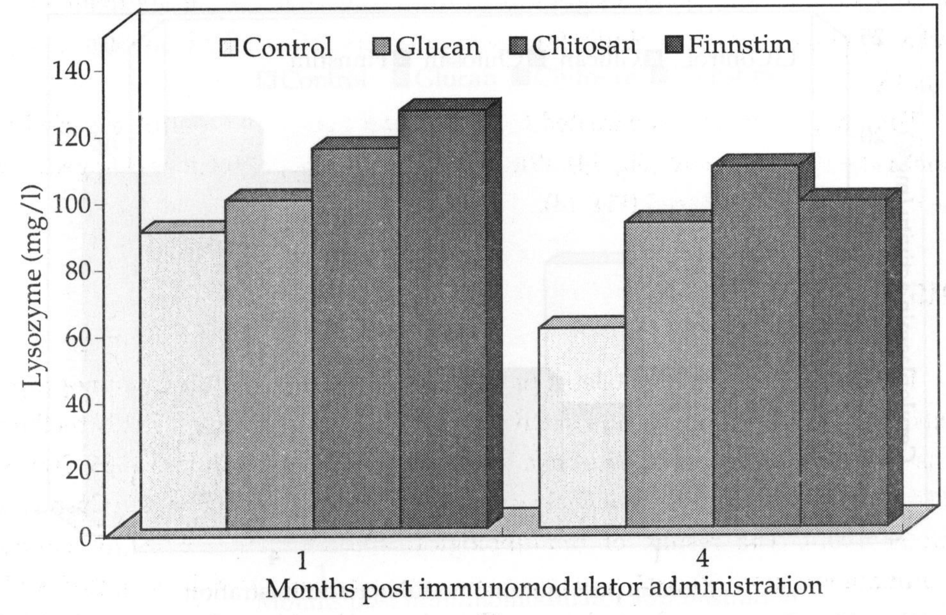

Fig. 10. The effect of natural immunomodulators on lysozyme activity in Russian sturgeon *Acipenser gueldenstaedti* Brandt

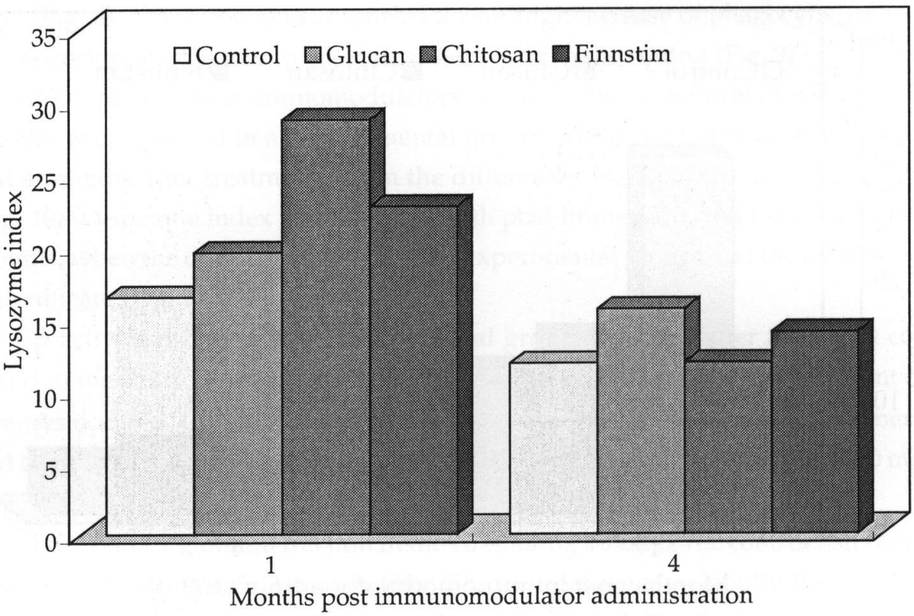

Fig. 11. The effect of natural immunomodulators on lysozyme index in Russian sturgeon *Acipenser gueldenstaedti* Brandt.

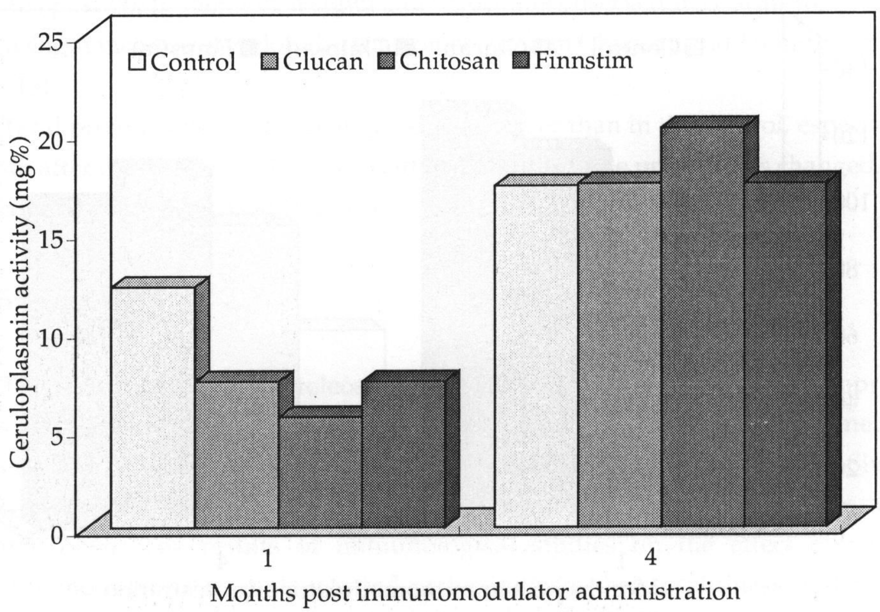

Fig. 12. The effect of natural immunomodulators on ceruloplasmin activity in Russian sturgeon *Acipenser gueldenstaedti* Brandt

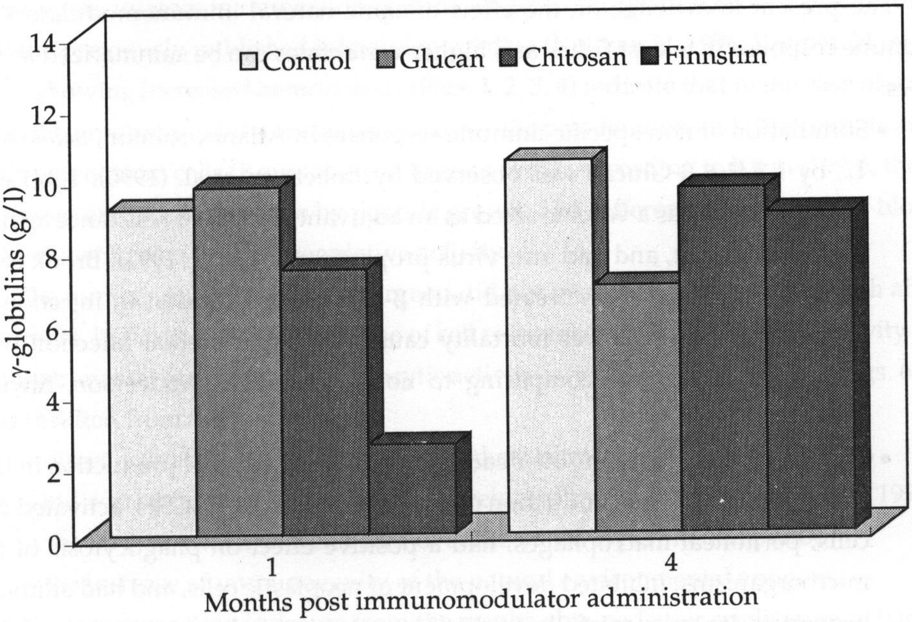

Fig. 13. The effect of natural immunomodulators on ã-globulin fraction level in plasma of Russian sturgeon *Acipenser gueldenstaedti* Brandt

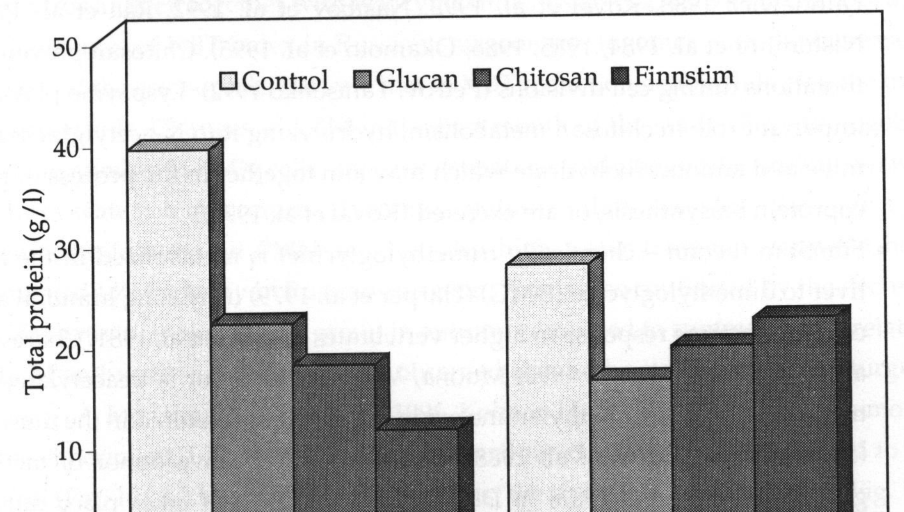

Fig. 14 . The effect of natural immunomodulators on total plasma protein level in Russian sturgeon *Acipenser gueldenstaedti* Brandt

The present knowledge on the effect of some natural immunomodulators on immune response in teleost fishes and higher vertebrates can be summarized as follows:

- Stimulation of non-specific immune responses in Atlantic salmon, *Salmo salar* L., by 1,3/1,6 β-Glucan was observed by Robertsen et al. (1990). 1,3/1,6 b-Glucan applied as a vaccine acted as an adjuvant, increased resistance against diseases in trout, and had anti-virus properties (Raa et al. 1992). Brook trout (*Salvelinus fontinalis*) fry treated with β-Glucan or Chitosan in injection or immersion showed lower mortality caused by experimental infection with *Aeromonas salmonicida* comparing to non-treated fish (Andeerson, Siwicki 1994).
- Chitosan (particularly in 70% deacetylated) stimulated IFN production in laboratory animals, and production of many cytokines (IL-1, CSF), activated NK cells, peritoneal macrophages, had a positive effect on phagocytosis of the microorganisms, inhibited development of neoplastic cells, and had adjuvant properties. It was succesfully applied in treatment of ulcers, wounds, and dermatological diseases in warmblooded animals (Suzuku et al. 1982, 1984, 1985, Dutkiewicz 1988, Koval et al. 1992, Nasibov et al. 1992, Raa et al. 1992, Nishimura et al. 1984, 1985, 1986, Okamoto et al. 1995). Chitosan prevented mutations during cell divisions (Petrov, Tarasenko 1992). Lysozyme plays an important role in chitosan metabolism, hydrolyzing it to N-acetylglucosammine and aminocarbohydrate which may join together in the process of glycoprotein biosynthesis, or are excreted (Koval et al. 1992).
- FinnStim (betain – chemically trimethyloglycine) is metabolized in the fish liver to dimethyloglycine (DMG) (Harper et al. 1979) increasing humoral and cellular immune response in higher vertebrates (Graber et al. 1981, Charest et al. 1988, Reap, Lawson 1990, Muona, Virtanen 1993), or is deacetylated to trimethylamine or trimethylamine oxide. Most betaine is stored in the muscles (*Salmonidae*) (Charest et al. 1988). Betaine is an effective donor of methyl groups (Stekol et al. 1953) in DNA methylation which takes place during immunological recognition, and antibody production (Sano et al. 1988, Bestor 1990).

In the present study, a modulation of non-specific humoral and cellular responses after treatment of Russian sturgeon, *Acipenser gueldenstaedti* Brandt, with natural immunomodulators in immersion was investigated for the first time. These results, and the previously published hematological data (Kolman H. 1996, Kolman H. et al. 1997) showing increased hemopoiesis (Figs. 1, 2, 3, 4) indicate that in the case of sturgeon the immunomodulators increase mainly the proliferation and differentiation of multipotent stem cells (Koumans-van Diepen 1993). According to Klein (1993), these processes are induced in vivo by peptide growth and differentiation factors of blood cells, showing also immunoregulative activity.

The number of PMN cells in sturgeon did not increase over the first month after treatment, indicating that the increase of cell resistance indices - metabolic activity (in all experimental groups), and phagocytic activity (after Glucan treatment) (Figs. 6, 7, 8, 9) resulted from the following:

- direct activation of the leucocytes by the treatments (Jeney et al. 1994),
- indirect stimulation by the cytokines released by the lymphocytes (Klein 1993), the level of which was elevated over the entire experimental period,
- the increase of immunogenity of the natural environmental antigens.

The last suggestion is drawn from the results obtained after application of bacterial antigen LPS together with 1,3 β-D-Glucan (Guz, Sopińska 1997), as well as isolated virus antigen together with lysozyme dimer (KLP-602) (Siwicki et al. 1997a).

Dynamics of NBT index in Russian sturgeon fry, together with the increase of number of the eosinophils, indicate that in NBT reduction neutrophils play the most important role. Changes of LZM in the first month of the study (Fig. 10), together with the number of PMN cells, and their metabolic and phagocytic activity, viewed against water temperature dynamic, indicate that, besides the effect of immunomodulators on PMN activity, also high water temperature might have stimulated LZM activity in Russian sturgeon. This is also confirmed by the decrease of LZM activity in the control group in autumn compared to summer. Close relation of LZM and water temperature was observed also in the studies on other sturgeon species – *A. baeri* Brandt (Kolman H. 1996, Kolman H. et al. 1997). In the forth month of the experiment, LZM activity significanly differed in all groups compared to the control (Fig. 10). LZM activity viewed against the dynamics of LZM index (Fig. 11), suggests that higher lysozyme activity might have been related to the increase of number of PMN cells in that period (Fig. 2, 3), and their activity increased in the experimental groups by high number of lymphocytes (Fig. 2) (Koumans-van Diepen 1993).

Treatment of Russian sturgeon with immunomodulators inhibited Cp activity only in the first month after the immersion. Later on, activity of the enzyme increased in all experimental groups (Fig. 12) and in the control. The increase took place at the time when LZM activity dropped. Similar reaction was observed in the intoxicated carps (Siwicki et al. 1990, Studnicka et al. 1997). This suggests a negative feedback between these humoral factors in fish, activated in extreme situations, when the risk of homeostasis breakdown appears. In the studies on Siberian sturgeon, however, a similar change in Cp activity was observed during the first year of life (Kolman H. 1996). It is possible that Cp activity, at least in the first year of sturgeon life, depends mainly on fish body mass (Kolman H. 1996).

Slight changes of γ-globulin fraction level after Glucan and Chitosan application, and considerable decrease after FinnStim treatment suggest that no natural stimulus for differentiation of B-lymphocytes occured, and/or that immunomodulators inactivated plasmatic cells (Sikora 1996). Further studies on the phenotypes of differentiation antigens (CD) in the lymphocyte subpopulations (Koumans-van Diepen 1993), and some cytokines (Siwicki et al. 1997b) in sturgeon might bring an explanation of this effect. The effects of immunomodulators on Russian sturgeon are similar to those obtained for other animals:

The study performed *in vitro* on teleost tissues incubated with viruses isolated from carp and wels revealed that immunostimulation caused changes in IFN and TNF synthesis (Siwicki et al. 1997a);

The study on young mammals showed that application of lysozyme dimer caused no changes in γ-globulin fraction level compared to the control, despite considerable stimulation of the metabolic and phagocytic activity of PMN and MN cells, and the increase of cytokin Il-1, Il-2 and INF_{α} secretion (Siwicki et al. 1997c).

Over the last years, molecular mechanisms affecting cell proliferation and differentiation have been discussed (Koumans-van Diepen 1993, Michieli et al. 1994, Sikora 1996). The data indicate that these processes depended on the expression of various gene groups. Based on the suggestions of the authors mentioned above, it may be assumed that the decrease of γ-globulin fraction level in the experimental groups of Russian sturgeon after treatment with the immunomodulators was an indirect result of an inhibition of B-lymphocyte differentiation after a period of intense proliferation of the cells of the lymphoid line (Fig. 2). Taking this into consideration, as well as the dynamics of the parameters under study in the first and forth month post-treatment compared to the controls, it may be assumed that natural immunomodulators induced a multi-stage immunological responces in Russian sturgeon. Their duration in case of various modulators is not known as yet; however, it seems longer than in the teleost fish (Siwicki 1990).

Decrease of total protein level in Russian sturgeon blood plasma in the first month after treatment resulted in an opposite directon of the changes in the concentrations of various immunoproteins, and possibly also other proteins which were not analyzed in the present study. In autumn, the level of total plasma protein might have been considerably affected by Cp activity increase (Fig. 12), and the increase of relative share of γ-globulin fraction was related to the overall decrease of total protein level, which could be caused by the drop of LZM activity (Fig. 10) and other protein fractions (Eiszporn-Orecka 1970, Kuz'min 1996).

Comparison of non-specific immunne responses in Russian sturgeon pre-reared in cages with the same indices in sturgeons reared in water recirculation system reveals considerable differences (Kolman H. 1996, Kolman H. et al. 1997), indicating high lability of these parameters in sturgeons due to environmental factors, and their adaptative significance.

Application of natural substances in Russian sturgeon modulated indices of cellular and humoral non-specific immune response, stimulating cellular mechanisms, LZM activity, and LZM index. Dynamics of the parameters under study suggests that modulated mechanisms are complex, multi-stage and separate. The results of the present study indicate that immunoprophylaxis in sturgeons (*Acipenseridae*) is possible without the risk for the environmet or the consumers. Further studies should involve optimation of the doses and application methods for particular immunomodulators.

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STRESZCZENIE

WPŁYW NATURALNYCH IMMUNOMODULATORÓW PODANYCH W IMMERSJI NA NIESWOISTĄ ODPOWIEDŹ ODPORNOŚCIOWĄ U JESIOTRA ROSYJSKIEGO (*Acipenser gueldenstaedti* Brandt)

Badano wpływ 1,3/1,6-β-Glukanu, Chitozanu i FinnStim'u, podanych w immersji, na nieswoistą odpowiedź odpornościową u narybku jesiotra rosyjskiego podchowywanego w sadzach na wodzie zrzutowej z elektrociepłowni. W pierwszym miesiącu po podaniu biopreparatów stwierdzono znaczny wzrost ogólnej liczby leukocytów na skutek szczególnie wysokiego wzrostu liczby limfocytów, stymulację aktywności metabolicznej i fagocytarnej komórek PMN, wzrost aktywności lizozymu oraz indeksu lizozymu; obniżenie aktywności ceruloplazminy, poziomu frakcji γ-globulinowej i zawartości białka całkowitego w osoczu krwi. Wykazano również występowanie zmian sezonowych badanych wskaźników. Zastosowanie badanych naturalnych immunomodulatorów u narybku *A. gueldenstaedti* Brandt miało plejotropowy efekt, modulujący odpowiedź komórkową i humoralną, która była zróżnicowana w zależności od rodzaju podanego preparatu oraz typu odpowiedzi.

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