Effects of dietary isoflavone-genistein on hematological and immunological parameters in pre - brood stock beluga, *Huso huso*

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Abstract

This study was carried out with the aim of detecting the dietary effects of isoflavone-genistein on hematological and immunological parameters in beluga, *Huso huso* in a 12-week feeding period. Five isonitrogenous (45% crude protein) and isoenergetic (19.5 MJ kg $^{-1}$) diets were formulated to contain four graded levels of isoflavone-genistein, namely 0, 0.2, 0.4, 0.8 and 1.6 g kg^{-1} diet. Fish (initial average weight: 26.1 ± 1.8 kg) were stocked in ponds in groups of 3 and fed the experimental diets in triplicate. At the end of experiment, physiological indicators, including hematological and immunological parameters, such as red blood cell (RBC), white blood cell count (WBC), hematocrit (Ht), hemoglobin (Hb), lymphocyte, neutrophil, eosinophil, monocyte, haematological indices, lysozyme, total immunoglobulin (IgM) and complementary activities were determined. Results suggested that mean corpuscular hemoglobin concentration (MCHC) and values of neutrophil had significant differences between treatments. The activities of serum lysozyme, IgM, C_3 and C_4 were significantly influenced by the dietary genistein concentrations. Results indicated that genistein had significant effects on some hematological and immunological parameters in beluga.

Keywords*:* Genistein, Non-specific immunity, Hematological parameters, *Huso huso*

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Introduction

Soybean meal is being applied as a replacement for fishmeal as the principal alternative protein source in fish diet, which is regarded as an economical and nutritious alternative due to its relatively low cost, high crude protein content and reasonably balanced amino acid profile (Hernandez *et al*., 2007; Lilleeng *et al.*, 2007). According to NRC (2011), content of anti-nutritional factors is one of major obstacles toward the increased use of soybean products in diets for fish. Phytoestrogens are a broad group of plant-derived compounds that are structurally and functionally similar to estrogen and have a range of estrogenic activities in animals (Tolman, 1996). Soybean phytoestrogens include several isoflavones (genistein, daidzein, and glycitein) with estrogenic activity. The soybean isoflavone genistein is typically found at a rate of approximately 0.2, 1, and 1.3 mg kg^{-1} soybean (Franke, 1994). Dry soybean on average contains 1.107 milligrams of genistein per kilogram. It has been shown that the dietary estrogenic effects of phytoestrogenic compounds can have a wide range of consequences on various physiological processes in animals. Sturgeons are valuable species, which are currently highly endangered (Moreau *et al*., 1999). The rearing of these species has seen considerable progress in the past years. Beluga, *Huso huso* is one of the most important sturgeon species with high growth rates and appears to be very suitable for aquaculture. Despite the economic importance of beluga, there is little information on the effects of anti-nutrient factors of soybean meal on the physiological status of this species. Several studies have been published only on the effects of isoflavone-genistein on growth performance and reproductive function of some species including turbot, *Scophthalmus maximus* (Burel *et al*., 2000; Day and Plascencia-GonzAlez, 2000); Atlantic salmon, *Salmo salar* (Krogdahl *et al*., 2003; Opstvedt *et al*., 2003), Japanese flounder, *Paralichthys olivaceus* (Saitoh *et al*., 2003; Deng *et al*., 2006), Japanese Medaka, *Oryzias latipes* (Kiparissis *et al*., 2003) and common carp, *Cyprinus carpio* (Turker and Bozcaamutlu, 2009). However, limited knowledge is available at present on the effects of dietary soy isoflavones on hematological and immunological parameters in fish species. So, the aim of the current study was to determine physiological effects of dietary isoflavone-genistein on hematological and immunological parameters in beluga, *H. huso*.

Materials and methods

The basal diet (Table 1) contained fish meal, fish oil and wheat flour (with no inherent content of isoflavone-genistein) to meet the protein (45% crude protein) and energy (19.5MJ kg^{-1}) requirements for beluga. Four experimental diets were formulated to contain 0, 0.2, 0.4, 0.8 and 1.6 g kg^{-1} of genistein (called diets 1 to 4),

respectively by supplementing with isoflavone products (purity, 98%), supplied by the Xinxin Chemistry Technology Co., Ltd., China (Yousefi Jourdehi *et al*., 2014). Experimental ingredients were ground into a fine powder through 320-µm mesh. Isoflavone-genistein was first dissolved in deionized water, mixed with the ingredients thoroughly, and then mixed with fish oil and water to produce stiff dough. The dough was made into 13-mm diameter strands with an experimental feed mill and dried in a warm air cabinet (40ºC, 24 h). The air-dried pellets were stored at 4ºC until used. Pre - brood stock beluga, *H. huso*, was provided from the International Sturgeon Research Institute, in Guilan, Iran. Prior to doing the experiment, fish were transported to the experiment site and stocked in a flow through system (concrete rectangular ponds, 7000-L capacity) to acclimatize to new conditions for 2 weeks. During this period fish were fed twice daily with the diet without isoflavone-genistein for adaptation to experimental diets. At the start of the experiment, the fish were starved for 24 h before weighing. Fish (initial weight 21.6 ± 1.18 kg, mean ± S.E.M.) were stocked in 15 concrete ponds with 3 fish per pond. Each diet was randomly assigned to triplicate concrete ponds. Fish were hand-fed to apparent satiation twice a day for 12 weeks. During the experimental period, the temperature ranged from 18.0 to 23ºC, and pH from 7.7–7.9. Blood samples were taken gently from the caudal vein using 5-mL plastic syringes, for hematological and hemolytical tests. Two different of blood samples were used for different analyses. The first sample was transferred to an eppendorf tube coated with heparin as anticoagulant and was used to determine hematological indices including hematocrit (Ht), number of red blood cell (RBC) and white blood cell (WBC). Red blood cell (RBC) and white blood cell (WBC) counts were determined with a Neubauer using Rees diluting solution. To determine differential count of leukocytes, that is, the measure of lymphocyte, neutrophil, eosinophil and monocyte, the obtained smears were first air dried, fixed in 96% ethanol for 30 min, stained with Giemsa staining for 30 min and were examined for leukocyte differential count under a light microscope (Klontz, 1994). Hemoglobin (Hb) level was determined with Drabkin's reagent reading absorbance at 540 nm (Jain, 1993). According to the procedure of Rehulka (2000), haematocrit (Ht) was measured in microhaematocrit heparinised capillaries, using a microhematocrit centrifuge (13000 rpm for 3 min). The haematological indices including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated according to Haney *et al*. (1992). Other blood samples were collected in centrifuge tubes without anticoagulant for serum. Blood was centrifuged at 3000 rpm for 15 min in cooling

centrifuge for separation of plasma which was stored at -80 ºC until used for biochemical analysis. Lysozyme level was determined by turbidometric assay according to the method of Sankaran and Gurnani (1972) with slight modifications. Aliquots (1.75 mL^{-1}) of *Micrococcus lysodeikticus* suspension (Sigma) (0.375 mg mL^{-1}, 0.05 M PBS, pH 6.2) were mixed with 250 ml^{-1} of each sample and the optical density was measured after 15 and 180 sec by spectrophotometer (Biophotometer Eppendorf) at 670 nm. PBS was used as the blank and results were expressed in amounts of lysozyme (mg) per 1 mg of sample calibrated using a standard curve determined with hens egg white lysozyme (Sigma) in sterile sodium phosphate buffer. Plasma complementary activities were assayed according to Yano (1992). IgM levels were measured according to Fuda *et al*. (1991). Antisera for fish were prepared by immunizing rabbits as previously described by Fuda *et al.* (1991). The procedure for labeling antibody fragments with enzyme was performed. The values were found normal using Kolmogorov - Smirnov test and then data were subjected to one-way analysis of variance (ANOVA) to determine if significant differences occurred in fish fed with different concentrations of genisitein (0.2, 0.4, 0.8 and 1.6 g kg^{-1}) in diets. If a significant difference was identified, differences among means were compared by Tukey's multiple range tests. Results were considered significantly different at the level of $p<0.05$. Statistical analysis was performed using the SPSS 16.0 for Windows.

Results

Data on haematological parameters are shown in Table 2. No significant differences were observed on most haematological parameters in fish fed different levels of genistein. As soy isoflavone levels increased from 0 to 0.8 g kg^{-1}, RBC and WBC counts decreased significantly ($p<0.05$); however, there were no significant difference among other dietary treatments. Values of MCHC showed significant difference among dietary treatments. The highest MCHC was observed in fish fed 1.6g kg^{-1} isoflavone genistein. The highest neutrophil value was observed in fish fed the control diet and the lowest was observed in fish fed 0.4 g kg^{-1}. Significant differences were observed over the 12 weeks of the experimental period in lysozyme and IgM concentrations (Figs. 1 and 2). The lysozyme and IgM concentrations were significantly higher in the control treatment than those in fish fed other diets. A similar trend was observed for C_3 and C_4 concentrations among fish fed different dietary levels of soy isoflavone (Figs. 3 and 4). No statistical difference was observed over the 12 weeks of the experimental period in CH50 concentration (Fig. 5) in beluga fed with different levels of soy isoflavone. The highest and lowest CH50 concentration was found in fish fed 0.4 g kg^{-1} and 1.6 g kg^{-1} of isoflavone genistein, respectively.

Table 1: Ingredients and chemical composition of the basal diet fed to juvenile beluga.

Ingredients (g kg^{-1})	Weight
Fish meal	680
Fish oil	40
Wheat flour	245
Vitamin mixture [a]	20
Mineral mixture [b]	15
Proximate composition	
Crude protein (%)	45
Lipid (%)	14
Crude energy (MJ kg^{-1})	19.5

[a] Vitamin mixture was manually provided according to feed requirements of the fish and ingredients were obtained from Science Laboratories (Ghazvin, Iran); which each 1000 g vitamin mixture provides vitamin A, 1,600,000 I.U; vitamin D3, 400 000 I.U; thiamin, 6 g; riboflavin, 8 g; niacin, 12 g; pantothenic acid, 40 g; pyridoxine, 4 g; folic acid, 2 g; cyanocobalamin, 8 mg; vitamin C, 60 g; vitamin K3, 2 g; biotin, 240 mg and inositol, 20 g.

[b] Aquatic mineral premix, for cold and warm water fish, is manufactured by Science Laboratories (Ghazvin, Iran); which each 1000 g contains mineral trace elements such as ferrous, 6000 mg; zinc, 10 000 mg; selenium, 20 mg; cobalt, 100 mg; copper, 600 mg; magnesium, 5000 mg; iodine, 600 mg. In addition, choline chloride (6000 mg), which is vital for fish and cannot be combined with other vitamins, is also included in mineral premix.

Table2: Hematological parameters of beluga fed experimental diets containing graded levels of soy isoflavone genistein for 12 weeks.

Treatments	Control	Diet 1	Diet 2	Diet 3	Diet 4
RBC ($\times 10^6$) (n/mm^3)	35.1 ± 1.92	30.3 ± 8.33	28.6 ± 8.81	27.1 ± 3.33	25 ± 8.66
WBC ($\times 10^3$)(n/mm^3)	52.2 ± 3.2	50.1 ± 4	49.1 ± 1.6	50.7 ± 6.2	47.3 ± 10.9
Hematocrit (%)	23 ± 1	22.3 ± 3.4	22.6 ± 1.4	24.3 ± 0.66	22.2 ± 1.2
Hemoglobin (g/100mL)	4.45 ± 0.10	4.7 ± 0.50	4.6 ± 0.94	4.5 ± 0.37	4.1 ± 0.16
MCV (fl)	215 ± 15.9	219 ± 15.73	236 ± 10.78	216 ± 13.82	217 ± 22
MCH (pg)	43.62 ± 2.81	45.53 ± 3.14	46.70 ± 5.33	43.94 ± 8.45	44.14 ± 3.20
MCHC (%)	24.62 ± 2.81[c]	35.53 ± 3.14 [a]	33.05 ± 2.06 [ab]	25.93 ± 3.30[c]	33.36 ± 4.35 [ab]

Values within the same row, not sharing common superscript letters are significantly different (p<0.05).

Table 3: Differential count of leukocyte of juvenile beluga fed experimental diets containing graded levels of soy isoflavones for 12 weeks.

Treatments	Control	Diet 1	Diet 2	Diet 3	Diet 4
Lymphocyte (%)	84 ± 6.38	85 ± 1.85	85 ± 1.33	83 ± 3.46	84 ± 2.90
Monocyte (%)	1.5 ± 0.33	1.7 ± 0.88	1.8 ± 0.57	1.5 ± 0.57	1.6 ± 0.33
Neutrophil (%)	4.1 ± 0.96 ^{a}	2.9 ± 0.33 ^{cd}	2.4 ± 0.66^{e}	3.1 ± 1.45 ^{c}	3.7 ± 1.20 ^{ab}
Eosinophil (%)	7.6 ± 2.4	7.5 ± 2.02	7.4 ± 0.66	7.8 ± 0.66	7.8 ± 0.88

Values within the same row, not sharing common superscript letters are significantly different (p<0.05).

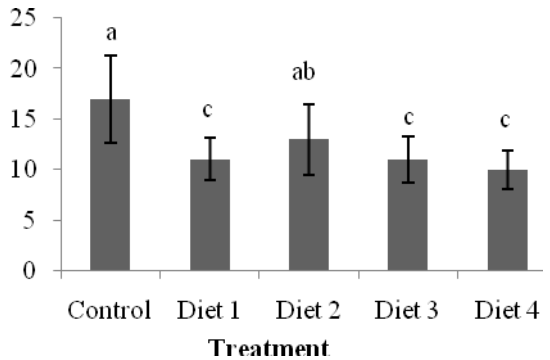

Figure 1: Lysozyme activity of juveniles' beluga *(Huso huso)* fed different dietary levels of isoflavone-genistein during 12 weeks of experiment. Values with different superscript letters are significantly different (p<0.05).

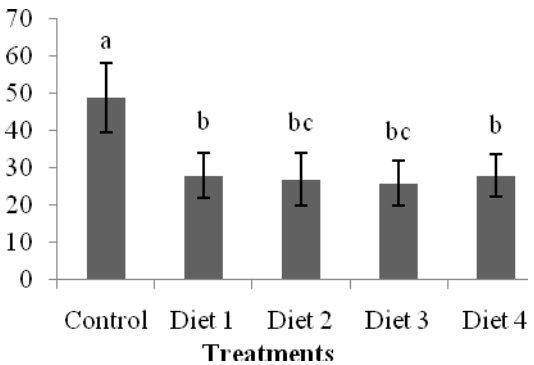

Figure 2: IgM concentration in serum of juveniles' beluga *(Huso huso)* fed different dietary levels of isoflavone-genistein during 12 weeks of experiment. Values different superscript letters are significantly different (p<0.05).

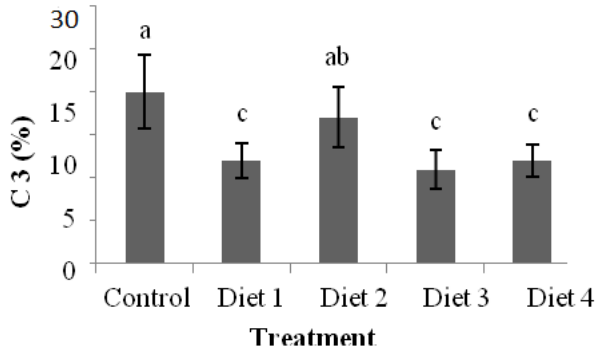

Figure 3: C3 concentration in serum of juveniles' beluga *(Huso huso)* fed different dietary levels of isoflavone-genistein during 12 weeks of experiment. Values with different superscript letters are significantly different (p<0.05).

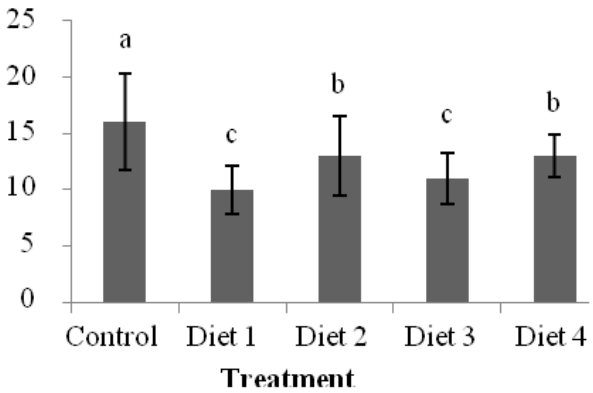

Figure 4: C4 concentration in serum of juveniles' beluga *(Huso huso)* fed different dietary levels of isoflavone-genistein during 12 weeks of experiment. Values with different superscript letters are significantly different (p<0.05).

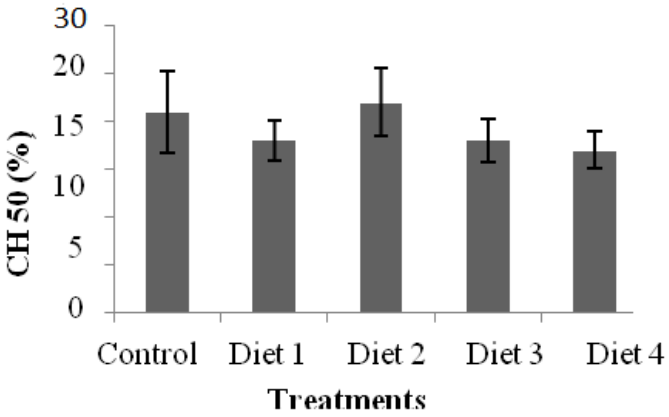

Figure 5: CH50 concentration in serum of juveniles' beluga *(Huso huso)* fed different dietary levels of isoflavone-genistein during 12 weeks of experiment. Values with different superscript letters are significantly different (p<0.05).

Discussion

To our knowledge, this is the first study showing the effects of dietary isoflavone-genistein on hematological and immunological parameters in fish. Isoflavones have a similar structure and function to estrogen and are known to exert several estrogen-like biological effects in animals. Several researches have reported estrogenic effects of genistein in fish. In yellow perch *Perca flavescens*, for example; Ko *et al*. (1999) reported that genistein induced vitellogenin (egg yolk protein) synthesis by the liver, and had a growth promoting effect similar to that of estradiol-17β (E2) (Malison *et al*., 1985). Genistein diets also increased plasma vitellogenin concentrations in Siberian sturgeon, *Acipenser baerii* (Pelissero *et al*., 1991) and juvenile striped bass, *Morone saxatilis* (Pollack and Ottinger, 2003). As far as we know, the biological effects of genistein on hematological and immunological parameters have not been reported in fish. In the present study, the inclusion of different levels of genistein in the diets did not alter haematological parameters. However, MCHC and neutrophil values significantly changed in those fish fed dietary genistein. Increasing levels of genistein in diets resulted in a decrease in hematological parameters in beluga, although not statistically significant. In fish, like other animals, one role of the spleen is to mature red blood cells (Press and Evensen, 1999). In many fish, the size of some body organs such as the spleen might increase when they fed diets containing soybean which has phytoestrogens such as genistein (Rycyzyn *et al*., 1998). The effect on the spleen may be related to the reduction in the volume of the erythrocyte cells in fish fed the soybean meal (Hemre *et al*., 2005). An increased organ index might be related to the activation and subsequent proliferation of leukocyte populations, which are generally the first immune responses against foreign antigens (Rycyzyn *et al.*, 1998). It can be speculated that trapping of damaged erythrocytes in the spleen because of a foreign agent might lead to a compensatory release of immature and smaller erythrocytes, resulting in reduced MCV. Differences in the response to the genistein may be due to differences in the fish species ability to metabolize or utilize a particular substance to which it has been exposed in the diet. Soy isoflavones have been shown to possess antioxidant activity (Wei *et al*., 1995; Ruiz-Larrea *et al*., 1997). It has been demonstrated that soy isoflavones decrease the concentrations of free radicals in plasma, liver, brain, testes, and kidney of male rabbits (Yousef *et al*., 2004). Cai and Wei (1996) suggesting that dietary genistein, one of the two major components of soy isoflavones, enhances the activities of antioxidant enzymes in various organs. Leukocytes are one of the affecting factors on immunity in fish. It has been found that leukocytes are the site of production of some complement proteins and an alteration of their function caused

by oxidation of membrane lipids can change the synthesis of these proteins (Obach *et al*., 1993). Lysozyme is liberated by leukocytes and plays a crucial role in antimicroorganism activity. The innate immune system of fish is considered to be the first line of defense against a broad spectrum of pathogens and is more important for fish as compared with mammals. Alexander and Ingram (1992) stated that this enzyme works in conjunction with complements. Lysozyme level is an important index of innate immunity in fish and is ubiquitous in its distribution among living organisms (Saurabh and Sahoo, 2008). In the present study, lysozyme and IgM concentrations in fish fed different levels of genistein showed significant differences. As the scientific research clearly demonstrates, dietary levels of genistein disrupt and damage the immune system, and also cause a variety of health problems including a variety of cancers, and DNA damage (Sabra *et al*., 2002). When mice were fed the "plant estrogen" genistein, which is found in soy products, levels of several immune cells dropped and the thymus, a gland where immune cells mature, shrank (Yellayi *et al*., 2002). Moreover, in mice that consumed genistein, thymus also became smaller (Curran *et al*., 2004). Nevertheless, the exact mechanism of action of genistein on the immune system is not well understood. The complementary activity is an important component of nonspecific immune defense in fish and has already been shown to be influenced by feed ration (Montero *et al.*, 1998). It has been understood that leucocytes are the places of production of some complementary proteins. Hence, changes in leucocytes membrane due to different intakes of feed could modulate the complementary activity in fish. Our results may not be comparable to other studies, due to the lack of data on the effects of genistein on complementary activities. Therefore, it is not clear to exactly what extent genistein affected complementary activities in this species at these levels.

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