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Effects of 2-deoxy-D-glucose on the immune system of seabream (*Sparus aurata* L.)

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ABSTRACT

Stressful situations are a major problem in aquaculture because they affect the immune system. 2-Deoxy-D-glucose (2-DG) is a derivative of a glucose analogue that reduces the availability of energy, thereby inhibiting cell metabolism so that it is unable to enter the glycolysis pathway. In this paper, 2-DG has been administered in order to study if the immune function is compromised during metabolic stress. Blood glucose level was measured as an indicator of the inhibition of glycolysis, and the effects of intraperitoneal administration of 2-DG on the main parameters of the humoral (complement, IgM levels and peroxidase activity in blood plasma) and cellular (respiratory burst, intracellular peroxidase level and phagocytosis activity) immune parameters of gilthead seabream (*Sparus aurata*, L) were evaluated. Furthermore, the expression levels of immune-associated genes (CSF-1R, NCCRP-1, Hep, TCR-β, IgM_H, MHC-IIα, C3 and IL-1β) were analyzed by real-time PCR in head-kidney. A total of 5 intraperitoneal injections were performed at 48 h intervals. Three experimental groups were established: a control group injected with phosphate buffer saline, group 2-DG 500 and group 2-DG 750 injected with 500 mg kg^{-1} and 750 mg kg^{-1} 2-DG, respectively ($N = 15$). After the third and fourth injection, some specimens of both DG-treated groups died. Following the first and third injection, the blood glucose levels of both 2-DG treated groups increased to a statistically significant extent with respect to the control group. While the humoral immune parameters were not significantly affected as a consequence of 2-DG administration, the cellular activities of leucocytes were. The injection of 500 mg kg^{-1} 2-DG provoked up- or down-regulation of the immune-relevant genes analyzed, while the injection of 750 mg kg^{-1} always caused down-regulation of these genes. The results suggest that 2-DG provokes metabolic stress, which reduces the activities carried out by immune cells (leucocytes) and induces down-regulation of the immune-relevant genes analyzed when the energy available to the cell decreases.

1. Introduction

The ultimate objective of aquaculture is to obtain the highest throughput in the smallest space possible by making the cultivation of aquatic organisms in reduced spaces that recreate the natural habitat. These conditions create a situation of stress that causes homeostatic changes, reduces resilience and directly affects the immune system responsible for regulating the primary system of global imbalances [1–3].

2-deoxy-D-glucose (2-DG) is a derivative of a glucose analogue in which the 2-hydroxyl radical is substituted by hydrogen and is therefore unable to enter the glycolysis pathway, so generating a situation of abnormal glucose deficiency in cells. Briefly, 2-DG is phosphorylated to 2-DG-6-PO_4, which competes with glucose-6-PO_4 for the same phosphoglucoisomerase, and thus blocks the pathway. Therefore, 2-DG establishes a situation of severe hypoglycemia and energy deficiency within the cell, creating a situation of stress [4,5]. There are numerous studies which show that energy reserves play an important role in regulating the functions of the vertebrate immune system [6,7], since the reduced availability of energy can act as a stressor, leading to the abolition or reduction of some functions due to the competition that may be established between them [7–9]. In fish, the effects of 2-DG remain practically unknown because, to the best of our knowledge, there are only two studies that have evaluated the effects of 2-DG on certain physiological parameters and, furthermore, neither study assesses the resulting effects on the immune system [10,5].

Taking into account all these considerations, the aim of the present work was to evaluate the effect of a possible stressful situation produced by the intraperitoneal administration of 2-DG on the main immune parameters, as well as on the function of leucocytes, in order to inhibit cellular metabolism and the expression of different immune relevant genes in specimens of gilthead seabream (*Sparus aurata* L.), a major aquaculture species of

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the Mediterranean. The consequences of 2-DG administration on seabream immune function are discussed.

2. Materials and methods

2.1. Animals

Forty-five specimens (99.83 ± 4.09 g and 18.10 ± 0.21 cm) of the hermaphroditic protandrous seawater teleost gilthead seabream (*Sparus aurata* L.), obtained from Culmarex S.A. (Murcia, Spain), were kept in running seawater aquaria (flow rate 1500 l h^{-1}) at 28‰ salinity, 20 °C and a 12 h light: 12 h dark photoperiod. The animals were fed with a commercial pellet diet (Skretting, Burgos) at a rate of 2% body weight per day. Fish were randomly distributed into three groups ($n = 15$) and acclimatized for two weeks to the laboratory aquaria conditions prior to the experiment. The Bioethical Committee of the University of Murcia approved the studies.

2.2. Experimental design and sampling

The experimental period lasted 9 days and involved five intraperitoneal injections at 48 h intervals. Three groups were established: a) a control group, in which fish were injected with 1 ml phosphate buffer saline (PBS); b) a second group, which was injected with 1 ml of 2-DG diluted in PBS at a concentration of 500 mg kg^{-1}; c) a third group, in which fish were injected with 1 ml of 2-DG diluted in PBS at a concentration of 750 mg kg^{-1}. After the first, third and fifth injection, five specimens from each group were anesthetized with MS222 (Sandoz, 100 mg ml^{-1} water) [11] and sampled. Blood samples were collected from the caudal vein with an insulin syringe. A drop of blood from each specimen was placed on a test strip, and the level of glucose was measured with a glucometer (Glucocard g meter, A. Menarini Diagnostics). The remaining blood samples were left to clot at 4 °C for 4 h and later the serum was collected after centrifugation (10,000 × *g*, 5 min) and stored at −80 °C until use.

Head kidney (HK) leucocytes were isolated from each fish under sterile conditions, according to Esteban et al. [12]. Briefly, the HK was excised, cut into small fragments and transferred to 8 ml of sRPMI [RPMI-1640 culture medium (Gibco) supplemented with 0.35% sodium chloride (to adjust the medium's osmolarity to gilthead seabream plasma osmolarity of 353.33 mOs), 2% foetal calf serum (FCS, Gibco), 100 i.u. ml^{-1} penicillin (Flow) and 100 mg ml^{-1} streptomycin (Flow)] [12]. Cell suspensions were obtained by forcing fragments of the organ through a nylon mesh (mesh size 100 μm), washed twice (400 × *g*, 10 min). HK cell suspensions were layered over a 34–51% Percoll density gradient (Pharmacia) and centrifuged at 400 × *g* for 25–30 min at 4 °C. After centrifugation, the bands of leucocytes above the 34–51% interfaces were collected with a Pasteur pipette, washed twice, counted (Z2 Coulter Particle Counter) and adjusted to 10^7 cells ml^{-1} in sRPMI. Cell viability was higher than 98%, as determined by the trypan blue exclusion test. All the cellular immune functions were performed only in viable cells.

2.3. Serum and leucocyte peroxidase activity

The peroxidase activity in serum or leucocytes was measured according to Quade and Roth [13]. Briefly, 15 ml of serum were diluted with 135 μl of HBSS without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. 50 μl of 20 mM 3,3′,5,5′- tetramethylbenzidine hydrochloride (TMB) (Sigma) and 5 mM H_2O_2 were added. To determine the leucocyte peroxidase content, 10^6 HK leucocytes in sRPMI were lysed with 0.002% cetyltrimethylammonium bromide (Sigma) and, after centrifugation (400 × *g*, 10 min), 150 μl of the supernatants were transferred to a fresh 96-well plate containing 25 μl of 10 mM TMB and 5 mM H_2O_2. In both cases, the colour-change reaction was stopped after 2 min by adding 50 μl of 2 M sulphuric acid and the optical density was read at 450 nm in a plate reader. Standard samples without serum or leucocytes, respectively, were used as blanks.

2.4. Natural haemolytic complement activity

The activity of the alternative complement pathway was assayed using sheep red blood cells (SRBC, Biomedics) as targets [14]. Equal volumes of SRBC suspension (6%) in phenol red-free Hank's buffer (HBSS) containing Mg^{+2} and EGTA were mixed with serially diluted serum to give final serum concentrations ranging from 10% to 0.078%. After incubation (90 min, 22 °C), the samples were centrifuged (400 × *g*, 5 min, 4 °C) to avoid unlysed erythrocytes. The relative haemoglobin content of the supernatants was assessed by measuring their optical density at 550 nm in a plate reader. The values of maximum (100%) and minimum (spontaneous) haemolysis were obtained by adding 100 μl of distilled water or HBSS to 100 μl samples of SRBC, respectively. The degree of haemolysis (Y) was estimated and the lysis curve for each specimen was obtained by plotting $Y/(1-Y)^{-1}$ against the volume of serum added (ml) on a log–log scaled graph. The volume of serum producing 50% haemolysis (ACH_{50}) and the number of ACH_{50} units ml^{-1} obtained for each experimental group were determined.

2.5. Serum IgM level

Total serum IgM levels were analyzed according to Cuesta et al. [15], using the enzyme-linked immunosorbent assay (ELISA), serial dilutions of seabream serum (from 1/1 to 1/1000) and the commercial monoclonal antibody as indicated by the manufacturer's instructions. The 1/100 serum dilution gave an OD in the linear range of the serum dilution versus absorbance curve and was chosen to compare the total IgM level in different serum samples. Thus, 20 μl per well of 1/100 diluted serum were placed in flat-bottomed 96-well plates in triplicate and the proteins were coated by overnight incubation at 4 °C with 200 μl of carbonate–bicarbonate buffer (35 mM $NaHCO_3$ and 15 mM Na_2CO_3, pH 9.6). After three rinses with PBT (20 mM Tris–HCl, 150 mM NaCl and 0.05% Tween 20, pH 7.3) the plates were blocked for 2 h at room temperature with blocking buffer containing 3% bovine serum albumin (BSA) in PBS, followed by three rinses with PBT. The plates were then incubated for 1 h with 100 ml per well of mouse anti-gilthead seabream IgM monoclonal antibody (Aquatic Diagnostics Ltd.) (1/100 in blocking buffer), washed and incubated with the secondary antibody anti-mouse IgG-HRP (1/1000 in blocking buffer). After exhaustive rinsing with PBT the plates were developed using 100 μl of a 0.42 mM TMB solution, prepared daily in a 100 mM citric acid/sodium acetate buffer, pH 5.4, containing 0.01% H_2O_2. The reaction was allowed to proceed for 10 min and stopped by the addition of 50 μl of 2 M H_2SO_4 and the plates were read at 450 nm. Negative controls consisted of samples without serum or without primary antibody, whose OD values were subtracted for each sample value.

2.6. Respiratory burst activity

The respiratory burst activity of gilthead seabream HK leucocytes was studied by a chemiluminescence method [16]. Briefly, samples of 10^6 leucocytes in sRPMI were placed in the wells of a flat-bottomed 96-well microtiter plate, to which 100 μl of HBSS containing 1 μg ml^{-1} phorbol myristate acetate (PMA, Sigma) and 10^{-4} M luminol (Sigma) were added. The plate was shaken and