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In situ forming microparticle implants for delivery of sex steroids in fish: Modulation of the immune response of gilthead seabream by testosterone

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ABSTRACT

Current knowledge on the sensitivity of marine fish to androgenic environmental chemicals is limited, despite the growing interest in the effects of endocrine disrupting chemicals. To study *in vivo* the effects of testosterone (T) on the fish immune response, we used a microencapsulation implant technique, the *in situ* forming microparticle system, containing 1 mg T/kg body weight (T-ISM), in adult specimens of gilthead seabream (*Sparus aurata* L.), a species of great economic interest. We demonstrated that implants themselves (without T) have no significant effect on most of the parameters measured. In T-ISM implanted fish, T serum levels reached supraphysiological concentrations accompanied by a slight increase in 11-ketotestosterone and 17β-estradiol levels 21 days post-implantation (dpi). Liver and head-kidney samples were processed 7 and 21 dpi to assess T-ISM effect on (i) the mRNA expression of genes involved in the metabolism of steroid hormones and in the immune response, and (ii) phagocyte activities. The expression profile of cytokines, chemokines and immune receptors was altered in T-ISM implanted animals that showed an early pro-inflammatory tendency, and then, a mixed pro-/anti-inflammatory activation during longer exposure. Furthermore, the enhancement of phagocytic activity and the production of reactive oxygen species by leukocytes 21 dpi in T-ISM implanted specimens suggest fine modulation of the innate immune response by T. Taken together, these data demonstrate for the first time the feasibility of using ISM implants in an aquatic species, and provide new data on the role played by T on the immune response in fish.

1. Introduction

The effects that sexual steroid hormones exert on fish physiology are a prominent field of research, especially in view of the increasing levels of endocrine disrupting compounds (EDCs), including xenobiotic hormones, that are found in aquatic environments. Indeed, EDCs have been detected in these environments in concentrations capable of altering the reproductive physiology and behavior of fish [1,2]. Such studies highlight the complexity of the organism's responses to estrogenic EDCs; however, the effects of androgenic EDCs in fish have been less well studied and most of them have focused on the alterations of the reproductive physiology that they provoke, such as sex reversion [3] or abnormal mating behavior [4].

The modulation that estrogens exert on the immune competence and responses of fish has been extensively studied, and their ability to affect the maturation and function of leukocytes has been demonstrated [5–7]. These steroids primarily promote immunosuppressive and anti-inflammatory activities by reducing the production of reactive oxygen species (ROS) and phagocytic capabilities of leukocytes [6–8], as well as the inhibition of lymphocyte proliferation and hence the reduction of immunoglobulin (Ig)-M production [9,10]. However, they also show pro-inflammatory activities, depending on the concentration and exposure time in several species [11–13]. All this evidence points to a complex role for estrogens in fish inflammation, whose pro- or anti-inflammatory effects depend on the widely varying responses of immune and repair systems, as reviewed in mammals [14].

As regards the effect of androgens on fish immunity, the scarce literature available indicates that a complex role, similar to that described for estrogens, must be expected for them. Most of the studies in fish support the immune-competence handicap hypothesis (ICHH) which postulates a trade-off between the androgen-mediated sexual display and the immune functions which

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negatively affect both immune competence and response [6,7,15], as has also been described in several vertebrate models and in human cells [16,17]. However, some studies reject the idea of a testosterone (T)-immunosuppressive effect, while others conclude that T-immunosuppressive pattern in teleosts is not absolute, since it was unable to alter immune parameters such as the number of phagocytic cells [18] or Ig-M production, but increased the complement activity [9]. In addition, it was demonstrated that an immune activation leads to a decrease in T serum levels in bats, but T itself does not affect their immune competence [19].

In situ forming microparticle (ISM) implants have previously been tested in non-aquatic animals, where they were seen to be an efficient option for parenteral drug delivery with better performance than other methods. This improvement includes a reduced burst effect and extended drug delivery time that can be adjusted and facilitates the implantation process [20,21], thus reducing the animal stress provoked by handling, surgery and hormone release.

The gilthead seabream (*Sparus aurata* L., Actinopterygii, Teleostei) is a seasonally breeding, protandrous, hermaphrodite teleost of great commercial value in the Mediterranean area. In this specie, we observed that the administration of natural and synthetic estrogenic compounds, such as 17β-estradiol (E_2) and 17α-ethynylestradiol (EE_2), respectively, revealed the existence of a complex equilibrium between their anti- and pro- inflammatory effects [9,12], demonstrating the major role played by macrophages in such regulation [12,13]. Furthermore, we recently demonstrated that T and 11-ketotestosterone (11-KT) have different effects on professional phagocytes *in vitro*, modulating their activity and transcriptional profiles [22].

In this framework the present study try to demonstrate the feasibility of using ISM implants in an aquatic species and to examine the role of T *in vivo*, by using this system (T-ISM), in the innate immune response of the gilthead seabream. With this aim we have, first, compared some biometric parameters, steroid levels, gene expression and cellular activity related to the immune response between control animals (implanted animals without T) and non-implanted animals and, secondly, we analyzed the effect of T-ISM on the mRNA expression of genes involved in the metabolism of steroid hormones and in the immune response and on phagocyte activities.

2. Materials and methods

2.1. Animals

Healthy adult specimens (1100 ± 209 g mean weight) of gilthead seabream (*S. aurata* L., Perciformes, Sparidae) were obtained from a captive broodstock in the hatchery facilities of the Spanish Oceanographic Institute (IEO) of Murcia (Spain) and maintained in 14 m^3 running seawater aquaria (dissolved oxygen 6 ppm, flow rate 20% aquarium volume/h) with natural temperature and photoperiod, and fed twice a day with a commercial pellet diet (Trouvit, Spain) at a feeding rate of 1.5% fish biomass. Fish were treated and sampled during July–August (testicular involution stage) at the end of 2nd reproductive cycle. Animals were fasted for 24 h before sampling and weighed and measured. The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and the Bioethical Committee of the University of Murcia (Spain) for the use of laboratory animals.

2.2. In situ forming microparticle (ISM) implants

As the ISM had not previously been used in fish, we proceeded according to the methodology described for the emulsion preparation [20,21] and injection [23], and to adjust the implant composition to deliver up to 20% total of T during the 40 days after the implantation [20,21,24]. The polymer solutions were prepared by mixing poly-D,L-lactate-co-glycolate PLGA (P2066-1G, Sigma) with the solvent 2-pyrrolidone (Fluka Analytical) 20% (w/w) in glass vials until a clear solution was formed. T (Sigma) was dissolved in polymer solution, 10% (w/w), and the ISM implants were prepared by emulsifying the steroid-containing polymer solution into peanut oil (external oil phase) at a polymer to oil phase ratio of 0.1:1 in a sonication bath (Ultrasons 3000512, P Selecta) for 10 min at room temperature. Pluronic F127 (Sigma) 1% (w/w) based on the amount of the total formulation and aluminum monostearate (Fluka Analytical) 2% (w/w), based on the oil phase, were added to increase the stability of the emulsions. The final nominal concentration of T in the implants was 2 mg/ml.

T-ISM implants were placed by means of intramuscular injection of 550 μl, which is equivalent to an average dosage of 1 mg T/kg body weight, expecting to reach T serum concentrations similar or higher than to the highest physiological levels detected before that were close to 5 ng/ml [5,25]. Fish were anesthetized (1 ml clove-oil in 30 l of seawater), briefly handled with wet filter paper, injected and returned to clean water. Injections were applied approximately 2 cm below the dorsal fin using disposable medical grade 10 ml syringes coupled to 18G (1.20 mm OD) needles. Control animals were implanted following the same procedure with ISM implants prepared as previously described but without T. The effect of ISM implants themselves was previously evaluated by comparing biometric parameters, steroid levels, gene expression and cellular activity related to the immune response between control animals and non-implanted animals at the longest exposure time (21 days post-implantation, dpi).

2.3. Tissue sampling

Seven specimens for each experimental condition (T-ISM and control animals) were bled and dissected 7 and 21 dpi and liver, head-kidney and blood samples were collected. The liver and head-kidney were maintained in Trizol Reagent (Invitrogen) at −80 °C until they were processed for RNA extraction. Head-kidneys were also processed for cell isolation as described below.

2.4. Enzyme linked immunoabsorbent-assay (ELISA)

Serum levels of T, 11-ketotestosterone (11-KT) and E_2 were quantified by ELISA as we have previously described for gilthead seabream [5,26]. Steroids were extracted from 40 μl of serum in 600 μl of methanol (Panreac). T, 11-KT and E_2 standards, mouse anti-rabbit IgG monoclonal antibody (Ab) and specific anti-steroid antibodies (11-KT-Ab, T-Ab and E_2-Ab) and enzymatic tracers (steroid acetylcholinesterase (AChE) conjugate: 11-KT-AChE, T-AChE and E_2-AChE) were obtained from Cayman Chemical Company. Microtiter plates (MaxiSorp™) were purchased from Nunc International. A standard curve from 6.1×10^{-4} to 10 ng/ml was established for all the assays. Standards and extracted serum samples were run in duplicate. The intra-assay coefficients of variation were calculated for sample duplicates and inter-assay coefficients were used to compare between plates [5,25,26]. Details on cross-reactivity for T, 11-KT and E_2 specific antibodies were provided by the supplier, Cayman Chemical Company. The values obtained were also used to calculate the 11-KT/T, E_2/T and E_2/11-KT ratios, since sex-steroid ratios has previously been reported as being feasible markers of inflammation in mammalian tissues [27,28].

2.5. Analysis of gene expression

Total RNA was extracted from tissue samples with Trizol Reagent (Invitrogen) following the manufacturer's instructions and treated with DNase I (amplification grade, 1 U/μg RNA, Invitrogen).