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Preliminary assessment of slow release implant model for immunosuppression in juvenile
pacu (Piaractus mesopotamicus)

Short Communication

Preliminary assessment of slow release implant model for immunosuppression in juvenile pacu (*Piaractus mesopotamicus*)

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ABSTRACT. Prolonged stress hampers immune function and lessens disease resistance of fish, causing economic losses. Attention is thus been centered in the study of fish immunology. The main 'in vivo' models used in immunological studies are: stimulation of immune response (by immunostimulant molecules); induction of inflammation; induction of immunosuppression by chronic stress; or administration of drugs. This trial aimed at evaluating existing protocols for immunosuppression by drugs in fish, adapted to slow release implants model, using hydrogenated vegetable fat (HVF), with the intention of set a controlled immunodeficiency state model for advanced studies. The implant model was not efficient in reducing the immune response in a controlled manner. Evidence of self, down-regulation in fish immune system was found in implanted fish, what should be further investigated using molecular tools.

Keywords: *Piaractus mesopotamicus*, homeostasis, immunomodulation, inflammation, leukocytes, stress, lymphocytes,

The negative relationship between prolonged stress and immune function is a well-known phenomenon in fish farming systems, and the cause of economic losses mainly in intensive systems (Wedemeyer, 1996). Although stress response is important to maintain homeostasis in adverse situations, a long-term activation of hypothalamic-pituitary-interrenal axis may cause higher blood cortisol levels, which can hamper fish defenses against pathogens (Ellis, 1981; Tort, 2011).

The recent interests in fish immunology came along with environmental problems resulting from intensification of farming systems. Moreover, teleost fish are in an interesting evolutionary position regarding immunological development, thus sprouting the current use of many species as animal models in immunology studies (Whyte, 2007). Immunologic response in laboratory fish may be studied through the modulation of the immune system. The main *in vivo* models used in immunological studies are: stimulation of immune response, by injection or administration of immunostimulant molecules in the diet (Anderson, 1992; Siwicki *et al.*, 1998; Sakai, 1999; Nayak, 2010; Hai, 2015); induction of inflammation by pathogens, antigens or inflammatory agents (Russell *et al.*, 2006;

Novoa *et al.*, 2010; Novoa & Figueras, 2012); induction of immunosuppression by chronic stress (Yin *et al.*, 1995; Tort *et al.*, 1996; Sadhu *et al.*, 2014); or administration of drugs by any ordinary process or via (Hickman-Davis *et al.*, 2001; Walsh *et al.*, 2002; Kumari & Sahoo, 2005; Cortes *et al.*, 2013). This short trial aimed at evaluating existing protocols for inducement of immunosuppression by drugs in fish, adapted to slow release implant model, aiming the setup of controlled immunodeficiency state for further immunological studies. The pacu was used as an experimental model by being a native fish of interest to Latin America aquaculture. In addition, is a less domesticated species and more sensitive to stress. Juvenile pacu (65.6 ± 15.6 g) were randomly stocked in five, 60 L aquaria set up in an open, continuous water flow system, constant aeration and natural photoperiod (13.5 h), temperature $25 \pm 1.2°C$. Fish were anesthetized in benzocaine solution (0.1%), individually weighted and injected intraperitoneally (1.0 mL syringes; 24G×3/4" needles) with the tested solutions or suspensions (Table 1). Implants were obtained using liquefied partially hydrogenated vegetable fat from soybean oil (Bunge) (HVF) as vehicle, warmed to 50°C, the minimum temperature necessary for melting,

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**Table 1.** Treatments used in immunosupression implants in juvenile pacu.

Treatment	Dose	Solution (SL)/Suspension (SP)
Negative control (NC)	X	X
Control (C)	X	Vehicle (HVF)
Cyclophosphamide (CF)	200 mg kg^{-1}	SP - 37.5 mg mL^{-1}
Dexamethasone (DX)	25 µg g^{-1}	SL - 10 mg mL^{-1}
Hydrocortisone (HC)	100 µg g^{-1}	SL - 20 mg mL^{-1}

and applied at 30°C, the nearest of the ambient temperature that was not too hot to harm the fish and, at the same time, still sufficient liquid for injection. This procedure resulted in a slow release implant, based on the methodology described by Specker *et al.* (1994). The immunosuppression drugs and dosage were cyclophosphamide (CYP), based on data reported by Kumari & Sahoo (2005) for Asian catfish (*Clarias batrachus*); dexamethasone (DX), based on data reported by Walsh *et al.* (2002) for clearnose skates (*Raja eglanteria*), and hydrocortisone (HC), based on data reported by Specker *et al.* (1994) for Atlantic salmon (*Salmo salar*).

Blood samples were drawn by puncture of caudal vein (3.0 mL; heparinized syringes) from eight fish per treatment four days after administration of drugs. The sampling was performed on 4th day after implant injection based on the time necessary to achieve immunosuppression, based on Kumari & Sahoo (2005), which used multiple drug injection over the days. In this trial, it was performed as a single injection in slow release implant model. Sampled blood were dispensed in microtubes for leukocytes respiratory activity assay. All procedures were performed in accordance with the ethical principles of animal experimentation, adopted by the Conselho Nacional de Controle de Experimentação Animal (CONCEA).

Leukocytes respiratory activity assay was carried out according to Anderson & Siwicki (1995) protocol, after adaptation of Biller-Takahashi *et al.* (2013). Data were tested for normal distribution (Cramer-von Mises test) and homoscedasticity (Brown-Forsythe test), transformed to log$_{10}$ base and submitted to one-way ANOVA and *post-hoc* analyses (Duncan's multiple range test) to detect differences ($\alpha = 0.05$) between treatments.

Leukocytes respiratory activity of drug-treated groups did not differ from negative control group, whilst leukocytes respiratory activity of control group was smaller than that of the remaining treatments (Fig. 1). A bacterial outbreak was registered four days after blood sampling with 100% mortality in groups treated with dexamethasone and hydrocortisone. No disease signals were registered for any other treatments. No mor-
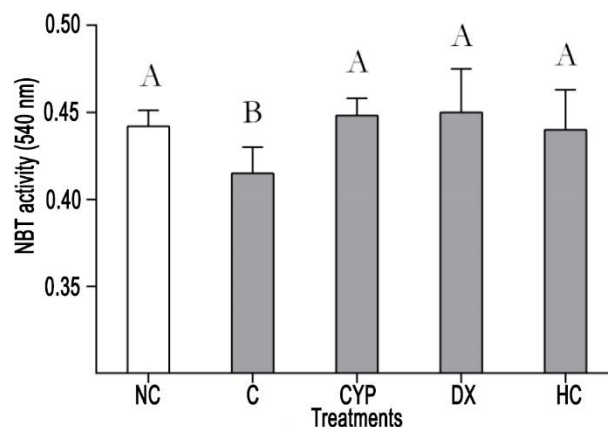
Figure 1. Leukocytes respiratory activity (NBT activity) (± SD) of pacu juveniles four days after treatments: Negative control (NC) without manipulation, control (C) with intraperitoneal injection of hydrogenated vegetal fat (HVF), cyclophosphamide suspension (CYP) and dexamethasone (DX) and hydrocortisone (HC) solutions in HVF. Capital letters indicate statistical difference (Duncan 0.05).

tality or disease was recorded in the period between implant procedure and blood sampling.

The capability of phagocytosis and oxidation by leukocytes, measured indirectly by NBT reduction assay is a reliable parameter for accessing the innate immune function of fish (Treves-Brown, 2000), tropical species included (Biller-Takahashi *et al.*, 2013). Therefore, the NBT activity was set as immunological status parameter.

The slow release implant model was chosen for because elicits reduced handling of fish, *i.e.*, requires a single injection, is less time-consuming and minute amount of drugs as compared to oral administration (Lovy *et al.*, 2008). The negative control group was set as a baseline value for NBT activity in laboratory conditions, eliciting direct comparison with vehicle control and drug-implanted fish. Expectations were obtaining a HVF group with values near to NC, and lower values to CYP, DX and HC groups. However, an inverse response was registered. Low NBT activity in control group could be related to inflammatory response mechanism and a possible down-regulation of immune system.

Inflammatory response is a physiological, protective process resulting from diseases or injuries. Although the inflammatory response varies to a great extent with the nature of the etiological agent, a general known pattern involves a well-established chain of events: vasodilation, increase of blood perfusion, migration of leukocytes to the inflammatory site, disease resolution and cleaning of cellular debris

(Suzuki & Iida, 1992). To prevent the extension of the inflammatory response beyond pathogen clearance ability and to avoid further damages to healthy tissues by chronic inflammation, the immune system releases inhibitory signs to down-regulate the immune response and reach homeostasis (Vigano *et al.*, 2012). Mahta *et al.* (2014) reported out of *in vitro* and *in vivo* study with mice, *de novo* synthesis of steroid (pregnenolone) by lymphocytes T-helper (th2), resulting in reduction of cell proliferation and immunosuppression. Although the information regarding lymphocyte functions are scarce in teleost fish (Castro *et al.*, 2011), Wang & Secombes (2013) inferred that the presence of lymphocytes T-helper in fish (salmonids) with different cell populations is similar to that found in mammals, and Wang *et al.* (2016) identified four interleunkin (IL-4/13) genomic loci in the salmonids genome, and the cloning of three active genes, IL-4/13A, B1 and B2, cytokines related to Th2-type lymphocytes. It is therefore possible that self-regulating mechanisms of the immune system are, somehow, present also in teleost fish. Once the "implant factor" (HVF) is a common condition to control group and drug-treated fish, and an opposite reaction was found in this comparison, there is an evidence that CYP, DX and HC, although had no difference between each other, had effect over leukocytes respiratory activity when compared to control group, that only received the implant vehicle.

During infection, hematopoietic tissue releases neutrophils in the blood stream; therefore, the increase of this cell type can be indicative of pathogen activity (Kindt *et al.*, 2007). In addition, the production of bactericidal reactive oxygen species (ROS) by macrophages and neutrophils can indicate host-pathogen interaction (Ellis, 2001). The increased leukocytes activity in DX- and HC-treated fish, added to bacteriosis and mortality after blood sampling stress, is a solid indication that the immune function, in this case, was reduced before the blood sampling at day four post implant, and the drug-treated fish had a latent infection by opportunistic pathogens, which advanced to a pathological condition after stress. Another indicative of this condition was the higher standard deviation in DX and HC treatments data, once there is an individual variation in this type of response. The standard deviation was 6 and 5% of leukocytes respiratory activity mean in DX and HC, respectively, against 2% of the same parameter in CYP group, in which was not recorded disease and mortality. Intraspecific, individual variation may occur in many physiological processes, including immune function (Crawford & Oleksiak, 2007). Studies with rainbow trout showed individual variation in head kidney

lysozyme concentration (Grinde *et al.*, 1988) and natural killer cells activity (Yoshinaga *et al.*, 1994), which leads to the assumption that immune response of experimental fish during pathogen infection may vary as a result of individual predisposition.

In what regards the CYP treatment, although similar results have already been reported with other drugs, no signals of disease were recorded and low individual variation was found. CYP is known to cause acute damage in blood-forming tissues and direct damage in lymphoid system (Hickman-Davis *et al.*, 2001). It is thus possible that, in this protocol of administration, CYP affected the immune response blocking the down regulation of the inflammatory process against the implant, what can explains the difference to control group.

In conclusion, the slow release implant model, using the tested drugs through HVF vehicle, was not efficient in reducing the immune response in a controlled manner. It is thus fair to state that more complex pharmacologic tests associated with pathogen-controlled experimental units should be used to transform the existing immunosuppression protocols in slow release implants. There is evidence of self, down-regulation in fish immune system when implanted only the vehicle, and that should be further investigated with the aid of more specific immune parameters, especially molecular tools.

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