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Original Article

MODULATION OF GONADAL STEROIDS PRODUCTION BY TILAPIA PITUITARY EXTRACT: AN EVALUATION THROUGH *IN-VITRO* AND *IN-VIVO* STUDIES

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ABSTRACT

Objective: Endocrine regulation strategies are widely used for synchronization of fertility, but in some cichlids species, the treatments are not always effective. This study used tilapia (*Oreochromis niloticus*) as a model cichlid fish to evaluate homologous pituitary extract *in-vitro* and *in-vivo* bioassays.

Methods: In this study, guinea pig Leydig cells, tilapia follicular cells, and female tilapia were treated with tilapia pituitary extract (TP) to evaluate the ability of TP to modulate steroid production *in-vitro* and *in-vivo*. Sex steroid hormone quantification was performed using enzyme immunoassay (ELISA), and the relative vitellogenin (Vtg) level was measured using Western blot during the maturation cycle of female fish.

Results: Treatment with TP *in-vitro* significantly increased testosterone and estradiol (E2) levels in guinea pig Leydig cells and in tilapia follicular cells, respectively. *In-vivo* experiments showed a significant increase in plasma E2 and Vtg concentration in the TP-treated female. Interestingly, 40% oocyte maturation was observed in TP-treated adult female tilapia whereas, only 7% was observed in the control group. TP treatment is increased relative fecundity significantly, reaching a production of 15.7±5.8 oocytes/g of female.

Conclusion: The outcome of this study suggests that TP has a potential use in the control of cichlid fish reproduction and can be used as an alternative method for fish fry production.

Keywords: Cichlids, Tilapia pituitary, Estradiol, Vitellogenin.

INTRODUCTION

The supply of quality fish seed is a key factor in the expansion of fish farming. Currently, fish seed demand is very high and increasing, but supply is not up to the mark. There is always a need to look for alternative methods and sources that will help fulfill the fish seed requirements of this industry [1, 2].

Endocrine regulation strategies are widely used for synchronization of fertility and for enhancing fish, but treatment success varies, depending on the species. In this sense, carp pituitary extracts and gonadotropin-releasing hormone analogs (GnRH) are widely used in the critical stage of fish reproduction [2]. However, this treatment is not effective in many endemic cichlids species in Ecuador as "vieja azul" (*Andinoacara rivulatus*) and "vieja roja" (*Cichlasoma festae*). Moreover, these methods coupled with photoperiod affect the quality of oocytes [1, 3].

Reports suggest changes in the structure of glycosidic residues present in heterodimers constituting the follicle stimulating hormone (FSH), especially in the β-subunit that may alter the steroidogenic signal response when bound to its specific membrane receptor. This structure in tilapia is specific for the genus *Oreochromis* [4, 5]. Therefore, the use of tilapia pituitary extracts (TP) may have a synergistic effect on steroidogenic processes *in vitro* and *in vivo* bioassays.

This study used tilapia (*Oreochromis niloticus*), a highly productive performance fish, as a cichlid model, and homologous pituitary extract to treat i) cells *in-vitro* to determine the biological activity of TP in a dose-dependent manner, and ii) female tilapia, during their reproductive period, to determine the effect of TP on plasma estradiol (E2) and vitellogenin (Vtg) levels and their impact on the development and size of oocytes.

MATERIALS AND METHODS

In-vitro bioassays

The experiments with Leydig and follicular cell models were conducted according to the principles and procedures approved by Universidad de Chile. The pituitaries were obtained during the spawning period from 200 female tilapias. Fresh pituitaries were treated with acetone (96%), lyophilized and divided into aliquots. The biological activity of TP was compared to carp pituitary extract (CP) (Argent Labs, USA), in a concentration of 2.5 mg/ml of physiological solution.

Testis from guinea pigs (*Cavia porcellus*) with average weight 1123±172 g were dissected, washed with distilled water, and immersed in HAM medium (Sigma, USA) pH 7.4 to obtain Leydig cells. Subsequently, collagenase (0.25 mg/ml) from *Clostridium histolyticum* (Sigma, USA) was added and incubated at 37 °C with constant stirring for 15 min, followed by centrifugation at 350 g for 10 min at 27 °C. The supernatant was removed, and the pellet was suspended in 5 ml of HAM medium. The biological activity of TP and CP were measured after 12 h by triplicate. As control used Leydig cells, treated with physiological solution [6].

Follicular cells were obtained from ovaries adult females of tilapia (weight 80±5 g) and trout (weight 290±17 g). Ovaries were divided into uniformly sized fragments (20 mg each) and transferred to a 96-well culture plate containing HAM balanced salts, penicillin (100 IU/ml), streptomycin (0.1 mg/ml), nystatin (1.25 IU/ml) and 0.05% BSA (Sigma, USA) at pH 7.4 [7]. The gonads were washed three times for 1 h intervals and incubated at 26 °C. In the last wash, 0.2 mM theophylline was added together with the TP and CP treatments. Experiments were carried out in triplicates during 18 h. The supernatants were used to measure E2 levels and to build the

saturation curve [6-9]. Male, female plasma and young ovarian tissue, treated with a physiological solution, were used as a control.

***In-vivo* bioassays**

The experimental evaluation was conducted under real production conditions in Ecuador. Forty-eight adults tilapia (weight 319.8±38.6 g) both male and female (1:1) were divided randomised into 8 tanks, containing 1500 L water. Ideal rearing conditions, including water recirculation system, a temperature of 28±2 °C and natural light were maintained. Two different treatments by triplicate were established, the TP treatment and the control. Twelve female fish were treated with an intraperitoneal injection of pituitary extract (5 µg/g body weight). Post treatment, blood samples were drawn weekly from fish caudal vessels using a heparinized syringe, and plasma samples were isolated by centrifugation at 1500 g for 15 min and 1 mM of phenylmethylsulfonyl fluoride (PMSF) was added. Samples were stored at-80 °C until levels of E2 and Vtg in were analyzed. During spawning, ovaries were dissected from fish and parameters such as a gonad somatic index (GSI), relative fecundity (FR), weight and volume of oocytes were measured immediately.

Analysis of hormonal steroids

Quantification of testosterone and E2 levels from both *in-vitro* and *in-vivo* studies were performed by enzyme immunoassay technique (ELISA) using commercial kits protocols (Cayman Chemicals Company, MI, USA). All samples were analyzed in duplicate, and a separate standard curve was run for each ELISA plate. Steroid levels in samples were further validated by using different serial dilutions and ran in parallel to the relevant standard curve. The detection limit was 20 pg/ml.

Vitellogenin analysis

Vtg was quantified from stored plasma samples using quaternary ammonium anion-exchange columns procured from Sortobind MA Q-15 (Sartorius, Germany) [10]. Western blot with polyclonal anti-Vtg was used to validate the previous essay [11]. This detection was used as a reference to locate the protein in SDS-PAGE for subsequent densitometry image analysis, to calculate the relative concentration with image J free software.

Statistical analysis

Data from *in-vivo* assays for steroids and Vtg levels were monitored for homogeneity of variance and normality with the Levene and Shapiro-Wilk tests, respectively. An analysis of variance (ANOVA) was performed to determine the existence of significant differences among treatments. Differences in mean values were determined by Tukey´s test. Data from *in-vitro* assays were analyzed by the Kruskal-Wallis nonparametric test followed by the Mann-Whitney test. The results were considered significant if p 0.05. The data were expressed as mean±SEM with Info-stat 5.0 statistical software.

RESULTS

***In-vitro* assays**

The effect of CP on gonadotropins hormones is well documented in other fish species [2]. Hence, it is important to note that 12 h after treatments, secretion testosterone levels in Leydig cells increased from 0.54±0.2 ng/ml to 1.57±0.34 ng/ml in CP treatment (n=9; P=0.0001). However, in TP treatment also increased to 1.14±0.12 ng/ml (fig. 1). The intra assay coefficient of variation (CV), calculated by measuring replicates of the same sample within the assay, was estimated at 6.2%. The inter assay CV, calculated by measuring replicates of the same sample in different assays, was 9.3%.

Similarly, secretion E2 levels in trout follicular cells increased from 0.29±0.04 ng/ml to 3.26±0.19 ng/ml and 3.85±0.11 ng/ml with TP and CP treatments, respectively (n=21; P<0.0001). In the same experiment, E2 levels on tilapia follicular cells increased from 2.34±0.14 ng/ml to 3.195±0.34 ng/ml and 2.812±0.299 ng/ml with TP and CP treatments, respectively (Fig.2). These results may indicate that TP and CP have a biological activity in not only in trout cells but also in tilapia cells.

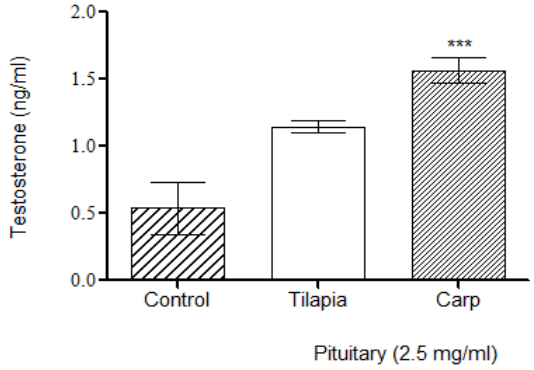

Fig. 1: Effect of tilapia and carp pituitary extracts on the levels of testosterone in guinea pigs Leydig cells. The results are expressed as mean±SEM, n= 9. ***p= 0.0001

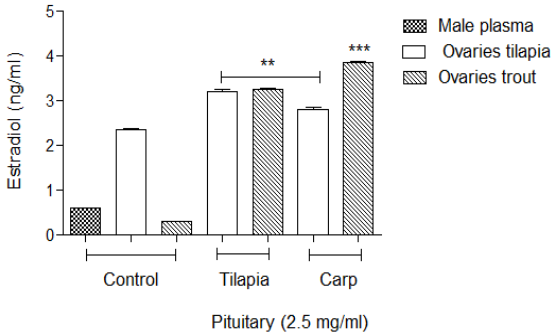

Fig. 2: Effect of tilapia and carp pituitary extracts on the levels of estradiol in tilapia and trout follicular cells. The results are expressed as mean±SEM, n=21. ***p = 0.0001; **p = 0.001

Moreover, in challenge tests of TP concentrations between 0.13 to 7.5 mg/ml. of culture cells, the E2 response with 2.9 to 3.8 ng/ml. on average. The E2 response was higher at low concentrations of TP, while a high extract concentration showed an equal or lowers effect. Significant differences were detected a dose of 0.38 mg/ml compared with tilapia male plasma (n=48; P<0.0001; fig. 3).

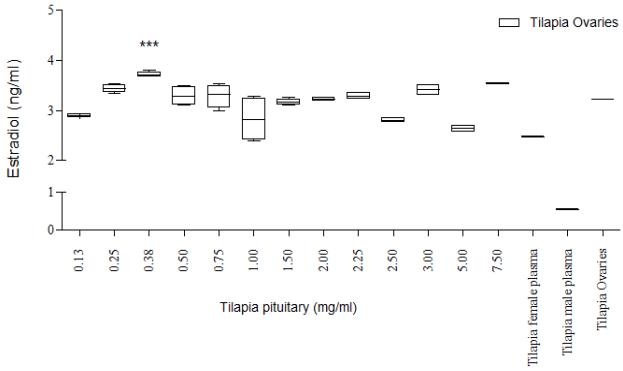

Fig. 3: Effect of tilapia pituitary extracts on the levels of estradiol in tilapia follicular cells in different concentrations. Controls were performed with ovaries, male and female plasma of the same species. The results are expressed as mean±SEM, n= 48. ***p = 0.0001

***In-vivo* assays**

The adult fishes showed a positive response to the TP treatment. The GSI was 3.5%, and FR showed 15.7 oocytes/g female, with

significant differences to the control (n= 24; P<0.005). The oocytes diameters and volume showed differences between TP and control treatments (fig. 4).

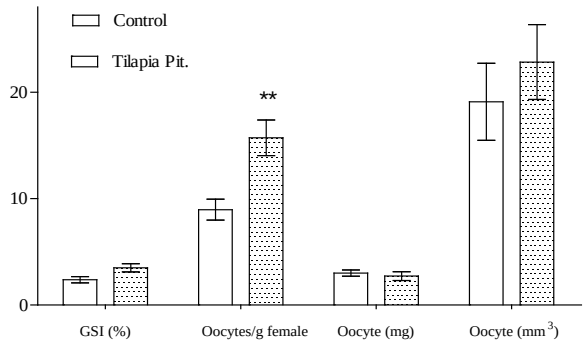

Fig. 4: Reproductive parameters of adult *O. niloticus* females after treatment with Tilapia pituitary. The results are expressed as mean±SEM, n= 24. **p = 0.001

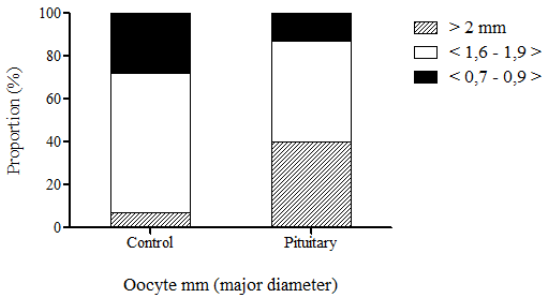

Fig. 5: The proportion of oocytes according to the diameter of the major axis (mm) and stage of development.<0.7-0.9 mm>pre vitellogenic; <1.6-1.9 mm>vitellogenic; >2 mm. mature oocyte. The results are expressed as %

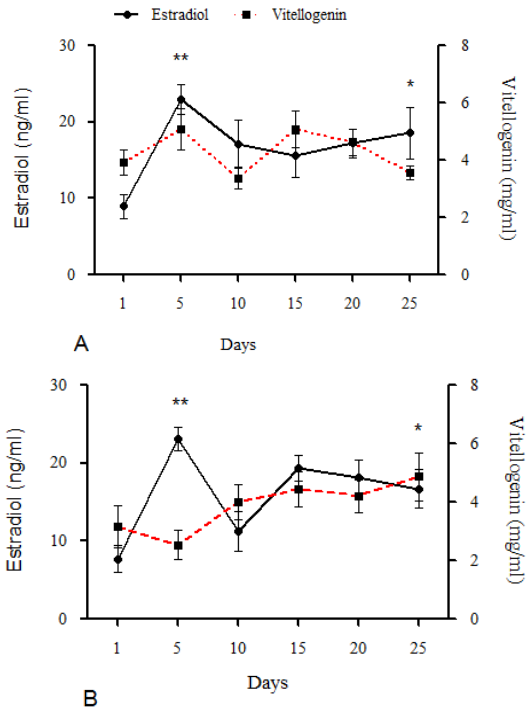

Fig. 6: Plasma estradiol and vitellogenin levels in adult females of *Oreochromis niloticus* under two experimental conditions: (A) control and (B) pituitary extract. The results are expressed as mean±SEM, n= 24. **p = 0.001; *p = 0.01

Oocytes volume and its symmetry analysis detected significant differences between control and TP treatment (n= 200; P<0.0009). It was noted that the treatment with pituitary extract have a higher proportion of mature oocytes (>2 mm) and symmetrically better formed in the early stages (<1.6-1.9 mm). The proportion of maturity of oocytes is more homogeneous in induced TP treatment (fig. 5).

The plasma E2 levels were between 8.9 and 22.9 ng/ml in the control and between 7.74 and 23.14 ng/ml after TP treatment in the first five days. In the next 20 d, the E2 levels reached at 18.5 ng/ml and 18.2 ng/ml with control and TP treatment, respectively. On the other hand, plasma Vtg levels during the reproduction period showed significant differences between days (n=24; P=0.0002). This variation ranges from 3.9 to 5.09 mg/ml in the control and between 3.17 and 2.5 mg/ml after TP treatment in the first five days. In the next 20 d, the Vtg levels reached at 2.5 mg/ml and 4.8 ng/ml with control and TP treatment, respectively. Vtg levels were different between treatments (n=24; P<0.05), in which TP generates a gradual and proportional increase in the reproduction time (fig. 6).

DISCUSSION

The biological activity of the tilapia pituitary extract was quantified in different experimental models such as guinea pig's Leydig cells and fish follicular cells. The results show statistical differences with CP as a commercial product. This variation could be explained by heterodimers' glycosidic structure changes, especially in tilapia FSH β altering the translation of the steroidogenic signal, given by the interaction with the receptor. The degree of glycosylation of the structure is different and represents 30-35% of the molecular mass, allowing the functionality of the hormone. The tilapia hormone structure is specific for the genera *Oreochromis* and *Tilapia* [5, 7]. Furthermore, the cell culture tests showed similar time response to generate steroid hormone and obtaining positive results at low concentrations of the homologous pituitary. Under these circumstances, homologous pituitary tilapia extracts may have a synergistic effect on steroidogenic processes involving reproductive tissues. Nevertheless, taking into consideration that, for *in-vivo* tests, some diseases may occur, and that results may be highly variable [2], it is necessary to develop and carry out more accurate *in-vitro* experiments. Pituitary extracts tend to go directly into the circulatory system where gonadotropins are released, generating a wider range of possible responses based on homology of receptor binding sites and the presence of co-activator and co-repressor proteins [6, 12].

The present study detected differences between TP treatment and control *in-vivo* bioassays. The system improves hormonal processes after the tenth day, increasing the production of Vtg levels, ovarian growth and reduced concentrations of E2. These findings suggest that each cell regulates its own receiver for a given response. Thus, when the hormones are released in pulsatile format lower doses, the response will be sustained by the receptor, but when the release is continuous at higher doses, the target cell will be saturated, and the receptors change their signaling and desensitize. Therefore, measuring the concentration of circulating hormone is an approximation in the phenomenon, in which the number of cellular receptors plays a pivotal role [6].

FR showed interesting differences between treatments, especially with the proportion of mature oocytes obtained in the control group, where the normal breeding range with Tilapia go from 1.3 to 7.2 eggs/g female [13, 14]. However, the results obtained by the provision of pituitary extract can generate double the capacity of the control, and improve the number of embryos in the production process.

Oocytes volume and its symmetry analysis detected significant differences between control and experimental treatment. It was noted that the treatment with pituitary extract has a higher proportion of mature oocytes and symmetrically better formed in the early stages. The stages of oocyte development have a direct relationship with the individual volume of the oocyte. The increase in production capacity, in this case, could be due to high availability of yolk precursors and environmental factors such as temperature and normal photoperiod in the region. Furthermore, reports defined

that the growth of the oocyte is directly related to the endocytosis of Vtg and other compounds in the retention period of meiosis in prophase I, until maximum growth, wherein the meiosis to metaphase II restarts [2, 15].

In summary, circulating gonadotropin homologous hormones due to inoculation of tilapia pituitary preparations should generate steroidogenic responses *in-vitro* and *in-vivo*. In this sense, the present work would be a contribution to increasing the fry production in some cichlid fish, such as tilapia and to improve the induction of maturation in several percid fishes (*Perciformes*). Availability of tilapia fish pituitary strongly supports the use of tilapia pituitary preparations, generating an alternative treatment as CP and GnRH.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

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