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Growth hormone-insuline-like growth factor-I system in pejerrey *Odontesthes bonariensis* (Atheriniformes)

S.E. Arranz[1], A. A. Sciara1, P. Botta[1], P. Cerutti[3], M.Tobin[3], G.M. Somoza[2]

[1] *Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET) – Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Provincia de Santa Fe, Argentina.*
[2] *Laboratorio de Ictiofisiología y Acuicultura. IIB-INTECH (CONICET-Universidad de San Martín). Chascomús, Provincia de Buenos Aires. Argentina*
[3] *Cátedra de Histología y Embriología-Facultad de Ciencias Veterinarias- Universidad Nacional de Rosario, Rosario, Provincia de Santa Fe, Argentina.*

ABSTRACT- Using biotechnology to increase the growth rates of fish is likely to reduce production costs per unit of food. Among vertebrates, fish appear to occupy a unique position, when growth patterns are considered. With few exceptions, fish species tend to grow indeterminately, implying that size is never fixed. Both hyperplasia and hypertrophy contribute to post-larval muscle growth in fish. Growth hormone (GH) - Insulin-like Growth Factor I (IGF-I) is the most important growth axis in fish. Our experimental model, the pejerrey, *Odontesthes bonariensis* (Ateriniformes) is a South American inland water fish considered to be a promising species for intensive aquaculture. However, one major drawback to achieve this goal is its slow growth in captivity. In order to understand how growth is regulated in this species, our first objective was to characterized pejerrey GH- IGF-I axis. We first cloned and characterized pejerrey (pj) GH, IGF-I and the growth hormone receptors (GHRs) I and II. In addition to providing valuable data for evolutionary comparison of GH, investigation of GH action in teleosts is particularly important because of its potential application in aquaculture. GH can not only promote the somatic growth in fish but also lower dietary protein requirements. A prerequisite for providing sufficient amounts of GH for basic research and aquaculture application is a large-scale production of GH. For that purpose, recombinant pjGH was expressed in a bacterial system. Protocols for solubilization and proper folding were achieved. Activity of recombinant pjGH was assessed in fish by measuring the liver IGF-I response to different doses of GH. IGF-I transcript was measured in the liver after pjGHr *in vivo* stimulation by means of quantitative real-time PCR assays. A dose-dependent response of IGF-I mRNA was observed after pjGHr administration, and reached a 6 fold IGF-I maximum increase over control group when 2.5 µg pjGH /g-body weight were injected. Temporal analysis of hepatic IGF-I mRNA levels showed that administration of a single dose of pjGHr into juvenile pejerrey resulted in a significant increase ($P<0.02$) 9 hours post injection. These results demonstrates that recombinant pjGH could promote a dramatic response in liver, increasing the IGF-I mRNA level. We also study the effect of GH on muscle growth after oral administration. A significant association between GH doses and mean fiber area (MFA) was observed even with a caloric restrictive diet. MFA increased 3.7 μm^2 per each unit of GH supplied indicating that GH promoted white muscle hypertrophy. These preliminary data indicates that GH could be absorbed by the intestine in an active form and promote somatic growth.

Key words: growth, growth hormone, muscle hypertrophy, oral administration, recombinant protein

Among vertebrates, fish appear to occupy a unique position, when growth patterns are considered. With few exceptions, fish species tend to grow indeterminately, implying that size is never fixed. Both hyperplasia and hypertrophy contribute to post larval muscle growth in fish.

Fish growth is strongly regulated by environmental factors, such as food availability, temperature and photoperiod. This process is mainly regulated by GH and IGF-I, both acting through membrane bound receptors, the GH receptor and IGF-I receptor respectively, and modulated by serum binding proteins (Duan & Xu 2005;Le Roith et al., 2005). GH has important metabolic effects. A strong link between energy status and circulating plasma GH levels exists in fish. The loss of hepatic GH receptors and circulating IGF-I levels is a characteristic feature of catabolic states.

Our experimental model, the pejerrey, *Odontesthes bonariensis* (Atheriniformes) is a South

American inland water fish considered to be a promising species for intensive aquaculture. However, one major drawback to achieve this goal is its slow growth rate in captivity (Strüssmann et al., 1993). Although growth hormone, IGF-I and its receptors have been characterized in a number of fish there are no published data on the structure of these proteins in atherinid fish, except for *Odontesthes argentinensis* growth hormone (Marins et al., 2003).

During the last years the interest of the laboratory has been focused on the study of the growth physiology in pejerrey, not only from a basic point of view but also keeping attention to the potential application of the knowledge to increase the growth rates and allowing pejerrey aquaculture.

In this context, the goal of the present work was to summarize the findings on the characterization of the GH-IGF-I system in *Odontesthes bonariensis* and to produce a recombinant pejerrey GH for oral administration for growth rates improvement.

Growth hormone characterization and production of the recombinant protein

Growth hormone is an essential polypeptide required for normal growth and development of vertebrates (Forsyth & Wallis 2002). In addition to the well known effects of GH on somatic growth in vertebrates (Isaksson et al., 1987), it has also been shown to have roles in bony fish reproduction (Gomez et al., 1999;Le Gac et al. 1993) as well as in osmoregulation in euryhaline fish (Mancera et al., 2002).

The mature form of pejerrey growth hormone (pjGH) is a protein comprising a single polypeptide chain of 193 amino acids. It is found in the Proximal pars distalis of anterior pituitary gland (Sciara et al., 2006), like all GHs reported in vertebrates.

The homology of pjGH is high within members of Acanthopterigii superorder (from 75% in Pleuronectiformes to 85-92% in Perciformes and Beloniformes) but less homologous to members of Paracanpthoterigii and Protacanthopterigii superorders being the most divergent teleosts groups Cypriniformes and Siluriformes (53-66%). Pejerrey GH showed 28% identity with human GH (Sciara et al., 2006). Species differences in GH immunoreactivity are pronounced, and mammalian GH does not cross react in fish GH radioimmunoassay (Le Bail et al., 1993). Furthermore, the cross reactivity of polyclonal antibodies against fish GH is total with pituitary homogenates or GH preparations from fish of the same family, partial within the same order and very weak between orders. To overcome this limit, specific polyclonal antibodies against pjGH were produced (Sciara et al., 2006) as a prerequisite to assess the physiological role of GH in Atheriniformes.

The GH cDNA of various teleosts have been cloned and sequenced, and their recombinant GH has been shown to be potent in accelerating the growth rate of fish (Saito et al., 1985;Tsai et al., 1995). These findings make the use of GH in fish farming practical and promising because GH enhances appetite, feeding efficiency and growth rate (Donaldson et al., 1979).

Pejerrey recombinant GH was efficiently synthesized by *Escherichia coli* cells, although it exists in denatured form in inclusion bodies. Pejerrey GH, as all vertebrates GHs studied, has four cysteines that form two disulphide bridges. It was found that the large disulfide loop is essential for growth-promoting activity (Chen et al., 1992). This type of proteins undergoes conformational distortion, leading to aggregation and loss of function when they are express in bacterial systems that do not form disulfide bonds.

We have over-expressed recombinant growth hormone from pejerrey (*Odontesthes bonariensis*) in *E. coli* by a pET expression system. A 100-ml sample of pjGHr-producing *E. coli* produced 40 mg of inclusion bodies (dry weight) after 20 h induction by 20g/l lactose. The optimal solubilization conditions were 8 M urea and pH 12.5. Upon dissolution, the sample was diluted (1/10 dilution in 40 mM Tris-HCl pH 10.5), and the recombinant growth hormone subjected to a two-step equilibrium dialysis procedure (20 mM Tris-HCl pH 10.5 and 20 mM Tris-HCl pH 9 containing 50 mM L-arginine). Using this procedure, almost 100% hormone was recovered and no significant amounts of pjGHr were lost by precipitation of mis-folded polypeptides (Sciara et al, in press). Activity of pjGHr was tested *in vivo* by injecting juvenile fish with pjGHr and measuring IGF-I transcript by means of quantitative RT-PCR. Relatively high IGF-I mRNA levels are produce in the liver, which is the main endocrine source of serum IGF-I (Stewart & Rotwein 1996). Therefore, the liver was the organ of choice for an initial

investigation of the GH dependency and temporal pattern of IGF mRNA expression. Our data clearly demonstrate that the level of IGF-I transcript in the pejerrey liver was significantly increased by recombinant pjGH administration in dose-dependent manner. Synthesis and degradation of IGF-I mRNA was shown to be fast in pejerrey. IGF-I basal levels were almost reached 6 hours after peak level (Sciara et al., in press). Remarkably, this dynamic was resembled in rainbow trout and tilapia *in vivo* and in cultured hepatocytes but pejerrey showed the fastest synthesis and degradation rates. Plasmatic IGF-I levels should be analysed. If this high clearance of IGF-I mRNA is related with a low plasmatic IGF-I level, an initial explanation of the endocrine reasons influencing pejerrey low somatic growth would arise.

Insulin-like growth factor-I

In mammals, somatic growth was thought to be controlled by pituitary GH and mediated by circulating IGF-I expressed exclusively by the liver. With the discovery that IGF-I is produced by most, if not all, tissues, the role of autocrine/paracrine IGF-I *vs.* the circulating form has been hotly debated (revised in (Le Roith et al., 2001). In teleosts it has been established that GH is the major activator of the IGF-I system, as it stimulates IGF-I gene expression in both liver and other tissues as well as it raises plasma IGF-I levels (Moriyama 1995). However, the question of whether the action of GH is direct or indirect has not yet been resolved.

IGF-I is a 70 amino acid hormone belonging to a conserved polypeptide family structurally related to proinsulin. Among other functions, IGF-I is involved in the regulation of proteins, lipids, carbohydrates, mineral metabolism in the cells, cell differentiation and proliferation, and ultimately in body growth (Moriyama et al., 2000). IGF-I by itself can promote somatic growth as it has been proved in carp (Zhang et al., 2006) and salmon (McCormickn et al., 1992) after administration.

Cloning and characterization of multiple forms of IGF-I mRNAs has been achieved for several teleosts including Salmoniformes (Duan 1997), Pleuronectiformes (Tanaka et al. 1998), Siluriformes (Clay et al, 2005), Cypriniformes (Zhang et al., 2006) and Perciformes (Vera Cruz et al., 2007), some of them encode different pro-IGF-I molecules which are finally processed into the same mature peptide. These prohormones were found to be produced by alternative mRNA splicing generating different sized carboxy-terminal E domains. The predicted mature IGF-I protein from pejerrey has 68 amino acid residues with a calculated molecular mass of 7,4 kD that is codify by a single transcript (Sciara et al., in press). Although the liver always presents the highest levels of IGF-I expression in pejerrey fish, high IGF-I transcription was also detected in brain and posterior intestine (Sciara et al., 2008). The former could imply an elevate proliferation rate of neural precursors or neurogenesis (Ajo et al., 2003) a process that can also involve an inhibition of apoptosis (Chung et al., 2007). In this context, brain cell proliferation has been characterized in pejerrey brain, not only during larval development but also during adulthood (Strobl-Mazzulla et al. 2007). On the other hand, the physiology of intestine could manifest a difference in the physiology of intestine regions and their response to growth hormone. Differential absorption capacity of anterior and posterior intestine after GH chronic stimulation should be analyzed to give some answers to this question.

It was suggested that plasmatic IFG-I exerts a negative feedback effect over the hypothalamic control of GH production, as in mammals. It is also accepted that fasting abolishes the stimulatory action of GH on hepatic IGF-I synthesis and release (Duan 1998). Liver GH resistance is characterized by the loss of hepatic receptors and reduction of plasmatic IGF-I levels.

Research on GH axis in fish has yet to reach the level of mechanistic detail to understand its mode of action. Characterization of fish growth hormone receptors is a prerequisite for the study of mechanism governing the expression within each tissue and the signal pathways leading to the pleitropic effects of GH.

GH receptors and tissue distribution

Growth hormone exerts its functions interacting with a GH receptor on the cell membrane of the target tissues. GHR belongs to the cytokine receptor superfamily. It consists of a single extracellular domain involved in the GH-binding, a single transmembrane domain, and one intracellular domain with two

conserved sequences or boxes. The extracellular domain of each receptor contains several pairs of conserved cysteine residues which interact by disulfide bonds, and a single cysteine necessary for the receptor dimerization. Intracellular boxes are important for signal transduction of the receptor. The proline-rich Box 1 region is the site of Janus kinase 2 (JAK2) binding and is essential for the signaling functions of GHR (VanderKuur et al., 1995). Box 2 contains about 15 amino acid (aa) residues and is believed to be involved in the internalization of the receptor (Govers et al., 1999).

The GHR cDNA sequences of more than 13 fish species have been reported. In nearly all the orders examined two subtypes of GHRs were found in a single fish species that clustered into different clades when the aa sequences were aligned. GHR cDNAs corresponding to distinct clades were characterized in zebrafish (*Danio rerio*, Cypriniformes (Di Prinzio et al., 2007), Gilhead sea bream (*Sparus aurata*, Perciformes (Calduch-Giner et al., 2003), Japanese eel (*Anguilla japonica*, Anguilliformes,(Ozaki et al., 2006)) and catfish (*Silurus meridionalis,* Siluriformes (Jiao et al., 2006)). One of the two subtypes of receptors characterized both in salmonids (*Salmo salar*, salmoniformes (Fukada et al. 2005)) and medaka (*Oryzias latipes*, Beloniformes (Fukamachi et al., 2005)) was reported to be a Somatolactin receptor (SLR). Phylogenetic analyses classified these SLRs as ortologs of the type I GHRs of other fish. Further studies are needed to unravel these intricacies. One clade (GHRI), containing 6 to 7 extracellular cysteine residues, is structurally more similar to the unique form reported in tetrapods. The other clade (GHRII), containing only 4 to 5 extracellular cysteine residues, is unique to teleosts and is structurally more divergent from the non-teleost GHRs (Fukamachi et al., 2006).

Functional differences were observed between the two subtypes receptors in black seabream using a number of reporter transcription assays in cultured eukaryotic cells (Jiao et al., 2006). The two subtypes possess differences in their signal transduction mechanisms and in their relative expression patterns. *In vivo* expression of sbGHR1 and sbGHR2 in seabream liver under various hormonal stimulations (cortisol, testosterone, and estradiol) also suggest a different regulation of each GHR. These functional differences probably explain and justify the preservation and co-existence of both GHR subtypes during teleostean evolution after genome duplication.

The co-existence of both GHR I and GHR II were also demonstrated in pejerrey (*Odontesthes bonariensis*, Atheriniformes (Botta et al., 2007), an Atherinid fish. Both receptor cDNAs could be detected in all tissues studied implying multiple physiological roles of GH, as it is suggested in mammalian species. The presence of GHRs in muscle and adipose tissue reflects the importance of GH in promoting growth and energy homeostasis (Perez-Sanchez, 2000). Moreover, GH seems to play a major role in the control of adipocyte lipolysis (Albalat et al., 2005). Transcripts for both types of receptor were also detected in the pituitary of pejerrey, suggesting a possible role in negative feedback on its own secretion. However, the GHR-expressing cells were not determined yet in the pituitary gland of pejerrey. GH has been shown to directly inhibit GH secretion in rainbow trout pituitary perfusion system (Agustsson & Bjornsson 2000). Recently, a novel intrapituitary feedback loop regulating GH release and GH expression has been identified in fish (for discussion see Canosa et al. 2007;Wong et al. 2006). Moreover, Zhou and colleagues (Zhou et al., 2004) identified the presence of one type of GHR (GHRI) in isolated carp somatrotropes. Since fish GHRs functional partitioning was reported (Saera-Vila et al. 2005), both receptors may be included for further studies.

Plasma GH levels show a clear seasonal pattern in fish in association to gonadal maturation, showing a correlation between GH and reproductive function (Björnsson et al., 1994; Gomez et al., 1999; Weber & Grau, 1999; Einarsdottir et al., 2002). In contrast, there is no clear correlation between serum GH levels and growth rates, especially in catabolic states. This fact may be explained by changes in GH binding protein (GHBP), the disponibility of GHR or the levels of IGF binding proteins (IGFBPs). To understand growth regulation is crucial to characterized and study not only GH secretion but GHRs and IGF-I expression. *In vivo* expression of pjGHRII in pejerrey liver under pjGHr stimulations was investigated (Botta et al. 2007). Although a significant increase of IGF-I expression in the liver was found 9 hours post injection, GH could not elicit any changes in pjGHRII gene expression in the fish liver during the same period of time suggesting that

regulation of GHRII expression is more probably influence by nutritional status than to the plasma levels of GH.

Growth hormone oral administration

Studies were performed in order to determine whether recombinant pjGH could be absorbed by the intestine and promote growth in pejerrey. Pejerrey is a stomachless fish, so no special encapsulation was carried out in preliminary studies. It was reported that GH administration influences both hyperplasia and hypertrophy in white muscle fibers (Fauconneau et al., 1997). Oral doses of pjGHr were administrated once a week for 4 weeks at dosages of 2 (T1) and 20 (T2) µg pjGHr g^{-1} fish body weight juvenile pejerrey (50 mg average weight). The effect of pjGHr on muscle hypertrophy (mean fiber area, MFA) and hyperplasia (percentage of fibers with an area less than 500 μm^2, <500) is shown in Table I. A significant association between GH doses and MFA (P=0.0002) and F<500 (P<0.0001) was observed. MFA increased 3.7 μm^2 per each unit of GH administrated, indicating that GH promoted white muscle hypertrophy. On the other hand, F<500 was reduced in 0.05% per each unit of GH which suggests that the hormone inhibit hyperplasia or recruitment of new fibers in *O. bonariensis*.

Table 1. Mean (SEM) values of dependant morphological variables of different groups and time of sampling.

	14 days			28 days		
	Control	**T1**	**T2**	**Control**	**T1**	**T2**
MFA (μm^2)	1227 (78.8)	1437 (91.1)	1767 (217.1)	1405 (77.6)	1542 (48.0)	1672 (69.5)
F<500 (%)	7.7 (1.69)	3.7 (0.92)	1.0 (0.31)	1.6 (0.43)	1.3 (0.17)	0.8 (0.19)

Although more detailed field studies on the growth promoting effect of recombinant pjGH by oral administration are needed in the future, these results clearly demonstrated that the hormone can be absorbed in the intestine and is biologically active.

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