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Research paper

# *In vivo* induction of human chorionic gonadotropin by osmotic pump advances sexual maturation during pre-spawning phase in adult catfish ☆

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ABSTRACT

Gonadal maturation is a critical event wherein gonads, under the influence of several hormones and factors, undergo cyclic morphological and physiological changes to produce functional gametes during the spawning phase. However, artificial induction can be effectively used to advance the maturation of gonad vis-à-vis spawning like behavior in seasonal breeders during the off-breeding season. In the present study, osmotic pumps loaded with 5000 IU of human chorionic gonadotropin (hCG) or saline as control were implanted intraperitoneally for 21 days during the pre-spawning phase (May–June) in catfish *Clarias batrachus* and *C. gariepinus*. Significant increase in gonado-somatic index and sperm motility, and in the levels of certain sex steroids were observed in the hCG treated catfish when compared to control while estradiol-17β ($E_2$) was low. Histological analysis in hCG treated testis revealed densely packed sperm and/or spermatids inside the lumen wherein the control testis displayed normal characteristics of the pre-spawning phase. In females, histological analysis showed a significant increase in post-vitellogenic full-grown immature follicles as seen in the spawning phase. In accordance with this, the steroid hormone profile correlated well with steroidogenic shift from $E_2$ to 17α,20β-DP indicating oocyte maturation. However, in the control ovaries of *C. batrachus*, perinucleolar and pre-vitellogenic oocytes were seen to be predominant. In addition, when compared with the control, the hCG treated group displayed a significant increase in the transcripts of several genes associated with gonadal growth. Taken together, artificial induction by slow release of hCG is an effective strategy to advance sexual maturation in catfish in a programmed manner.



## 1. Introduction

Artificial induction of reproduction is one of the commercially important techniques to enhance fertilization and survival of the offspring. It is practiced worldwide not only for quality seeds but also for high yield and constant production rate in aquaculture. Catfish, one of the economically important edible fishes, does not spawn spontaneously since the environmental conditions are not favorable in fish farms (Huisman and Richter, 1987). In India, several attempts have been made in catfish breeding with or without hormone stimulation to achieve a successful fertilization rate. In 1970, after the discovery of GnRH, spawning induction methods were extended to various species. Subsequently, several combination of synthetic agonists of GnRH with dopamine antagonists were tested and demonstrated in successful fertilization (Goos et al., 1999; Peter et al., 1988).

Miura et al. (1991) for the first time reported that a single injection of human chorionic gonadotropin (hCG) can induce all stages of spermatogenesis in the Japanese eel, yet milt volume was found to be low. As hCG shares the same receptor as the luteinizing hormone (LH) as demonstrated by Vischer et al. (2003), it is often used to induce ovulation due to its commercial availability as a recom-

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binant. Though hCG diverges in signaling pathways, it ensures excellent LH activity in the growing follicle due to its longer half-life and high affinity to receptors (Choi and Smitz, 2014). Procedures involving hCG or ovaprim for continuous administration at specific intervals often promote gonadal development and maturation in teleosts (Kumar et al., 2007). On the other hand, several methods were developed for the delivery of sustained hormonal release in fish to have a high yield in breeding. In *Salmo salar*, LH-releasing hormone (LHRH) was administered in a single cholesterol pellet which resulted in effective spermiation (Weil and Crim, 1983). Further, Matsuyama et al. (1993) described that copolymer containing LHRH induced sexual maturation in *Pagrus major*. In our laboratory, *in vitro* fertilization was performed after intraperitoneal injection of hCG in adult catfish during the spawning phase (Raghuveer et al., 2011). Different methods of hormone delivery systems successfully induced steroidogenesis, gonadal maturation and ovulation or spermiation in reproductively dysfunctional cultured fish (Zohar and Mylonas, 2001) yet advancing sexual maturation seemed difficult.

In view of this difficulty, the sustained-release of hCG through osmotic pump has been shown to be a reliable method to induce vitellogenesis and ovulation in females, and spermatogenesis and spermiation in males (Kagawa et al., 2009, 2013). Similarly, induction of oocyte maturation for *in vitro* fertilization during non-breeding season has been done in the honeycomb grouper, *Epinephelus merra* using osmotic pumps loaded with GnRH analogues (Kanemaru et al., 2012). Considering these reports, the present study aim at advancing sexual maturation in catfish using recombinant hCG instead of GnRH analogues for the purpose of cost effectiveness (Kagawa et al., 2009, 2013). Advancement of sexual maturation in catfish will benefit breeding as the normal process of spawning usually occurs during monsoon, which may fail at times due to rainfall delay and other environmental factors (Ramaswamy and Sundararaj, 1956). In addition, the average spawning time for catfish is relatively short (July–August), especially in India, and therefore the advancement of maturation in the breeding process will be significant for fish farming.

Pituitary gonadotropins regulate gametogenesis by controlling gonadal steroidogenesis. With respect to gametogenesis and steroidogenesis, several genes having central role were identified in teleosts and interestingly most of them are regulated either directly or indirectly by gonadotropins (see Sudhakumari and Senthilkumaran, 2013). In view of this, *in vitro* and/or *in vivo* induction of hCG have been demonstrated to evoke upregulation of various genes in several teleosts. In the Nile tilapia, hCG induction, *in vitro* resulted in the expression of *20β-hsd* in post-vitellogenic immature follicles (Senthilkumaran et al., 2002). Further in the Japanese eel, a single injection of hCG induced all the stages of spermatogenesis (Miura et al., 1991). In male goldfish, milt amount and sex steroid levels were increased dramatically after hCG injection (Kobayashi et al., 1986). Considering these reports, hCG seems to be an ideal hormonal supplement for spawning induction. In line to this, the present study is the first attempt in a seasonally breeding fresh water inhabitant teleost to examine the effect of continuous and slow induction of gonadotropin by implanting an osmotic pump loaded with 5000 IU of hCG in catfish for 21 days during the pre-spawning phase. Our results have been substantiated by analysis of gonado-somatic index (GSI) and sperm motility test in saline- (for control) or hCG-treated adult catfish, *Clarias batrachus* and *C. gariepinus*. Further, we have quantified, by real time PCR (qPCR) analysis, the expression levels of several key genes involved in steroidogenic pathway (*3β-hsd*, *cyp11a1*, *11β-hsd2*, *11β-h*, *star*, *17β-hsd1* and *12*, *cyp19a1a*, *P450c17* and *20β-hsd*), transcription factors/nuclear receptors (*dmrt1*, *sox9a*, *wt1*, *ad4bp/sf-1*, *gata4*, *pax2*, *foxl2*, *sox9b* and *erα*), and signaling molecules (*wnt4* and *wnt5*) involved in gonadal development. In addition, to determine the status of sexual maturation, we analyzed histological changes in testis and ovary, and measured steroid hormones such as testosterone (T) and 11-ketotestosterone (11-KT) in males and estradiol 17-β ($E_2$) and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) in females.

## 2. Materials and methods

### 2.1. Animals

Adult catfishes, *C. batrachus* and *C. gariepinus*, about 21 months old, were utilized for the experiment. Male catfishes were used from both species to compare our results among the species while females were chosen only from *C. batrachus*. Fishes (n = 5 of male and female each) were reared in fresh water tanks under ambient photothermal conditions (20 ± 3 °C; 12 h L/12 h D) and were fed with live tube worms (*Tubifex tubifex*), *ad libitum*. The experiment was performed during the pre-spawning phase (May-June) to easily observe the spawning behavior of fishes. They were housed in separate glass tanks throughout the experiment and divided into two groups irrespective of species and sex, one group (n = 5) was implanted osmotic pumps filled with hCG (5000 IU) while the control (n = 5) received saline. Feed, behavior, antibiotic treatment, temperature, and pH were monitored and water was replenished every day. Care was taken at each step and no mortality was observed until the end of the treatment period of 21 days.

### 2.2. Experimental procedure and sampling

Osmotic pumps (ALZET® osmotic pumps, Cupertino, CA, USA) loaded either with 5000 IU of hCG (Trade name: Pubergen; Sanzyme Ltd., Shameerpet, TS, India) dissolved in 100 µl of saline or 100 µl of saline (used as control) was used. The fishes were briefly anesthetized with 100 mg/L of ethyl 3-aminobenzoate methanesulfonate (MS-222; Sigma, St. Louis, MO, USA) in ice-cold water and an incision of approximately 8 mm in length was made using a sterile scalpel in the ventral region. Osmotic pumps loaded with saline or hCG were implanted into the peritoneal cavity adjacent to the gonads. The dissected ventral region was sutured using sterile 30 mm catgut to avoid back flow of osmotic pump from the body. After the procedure, the fishes were kept under observation for 3 days in glass water tanks and then maintained carefully for an additional 18 days. According to the manufacturer's manual, the osmotic pump used for this experiment can release approximately 5 µl of solution/day, when the fishes are maintained at an ambient water temperature (20 ± 3 °C). After 21 days, the fishes were sacrificed and samples were taken for analysis. GSI was calculated (based on the formula, [Gonad Weight/Body Weight] × 100) and blood was drawn (described in Section 2.5) to obtain serum from both the groups to measure sex steroids. Further, testis and ovary from the respective groups were dissected to prepare samples for histology (fixed in Bouin's solution; 15 parts of saturated picric acid:5 parts of formalin:1 part of glacial acetic acid) and qPCR analysis (snap frozen using liquid nitrogen and stored in −80 °C). Sacrifice of animals was done by briefly anesthetizing them with 100 mg/L of MS-222 (Sigma) in mild ice-cold water and, samples were dissected out by following the general guidelines of the Institutional Animal Ethics Committee, University of Hyderabad.

### 2.3. Sperm motility

The assessment of sperm motility was performed as per the method described by Kagawa et al. (2009). As gentle squeezing of abdomen did not release milt in catfish, testes were dissected out and minced to obtain milt. One µl of milt was diluted in 1 ml

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