Pituitary primary cell culture of Common carp (*Cyprinus Carpio*) and evaluation of its secretion effect on endocrine activity of incubated ovarian follicles

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Summary

In this study, five carp pituitary glands were collected and dispersed enzymatically and mechanically. Then, the cells were cultivated as monolayer in MEM (minimum essential medium Eagle). The culture media were collected after 72 h and frozen at -20°C. Carp ovarian follicles also were separated mechanically and incubated in BSS (basic salt solution) Cortland medium in 24-well microplates for 48 h at 20°C. Then, they were divided into two groups: control group which were incubated in BSS medium and experimental group which subdivided into three subgroups according to treatment with different concentration of collected pituitary secretion (50, 100 and 200 µl/ml). Follicles culture media were collected 24 h later and were analyzed for 17-β-oestradiol (E_2) and 17-α-hydroxy progesterone (P_4) content by radioimmunoassay (RIA). The results showed that adding low concentration (50 µl/ml) of collected pituitary secretion (CPS) increased steroid hormones (E_2 and P_4) secretion of incubated ovarian follicles significantly ($P<0.05$) but the high concentration of CPS (200 µl/ml) significantly decreased the secretion of E_2 and P_4 ($P<0.05$). Collected pituitary secretion at the concentration of 100 µl/ml had no significant effect on steroid hormones ($P>0.05$).

Key words: Pituitary, Cell culture, Steroids, Radio immunoassay, Common carp

Introduction

Common carp (*Cyprinus Carpio*) is a seasonal breeding fish with the definite fluctuations of reproductive function during annual cycle. It is well known that this process is controlled by hypophyseal gonadotropin hormones (GtH I and GtH II) which play a decisive role in gonadal development, ovulation and spermiation (Breton *et al*., 1998; Planas *et al*., 2000). Carp pituitary gonadotropins are also known to be responsible for oestradiol-17-β and 17α-20β-hydroxy progesterone secretions (Tyler *et al*., 1991) which in turn stimulate germinal vesicle break down in ovarian follicles during reproductive season (Degani and Boker, 1992). Seasonal changes of steroid levels which have been described in immature and mature carps are correlated with changes of blood gonadotropin (GtH) concentration during the annual cycle (Bieniarz and Epler, 1992; Galas *et al*., 1999).

Spawning induction in common carp and many other fish is currently carried out by the carp pituitary extract (CPH) in Iran, which is expensive with unpredictable activity and probability of pathogen transmission from the donor fish to recipient fish (Dorafshan *et al*., 2003). Therefore, the aim of the present study was to find the possibility of carp pituitary primary cell culture and effect of its secretion on incubated ovarian follicles endocrine activity.

Materials and Methods

Animals

The experiment was conducted in October 2007 in two stages. Five sexually mature common carp (2.6 ± 0.4 kg) were used in the first step (pituitary primary cell culture) and one 4-year-old female common carp spawner (3.6 kg) with gonadosomatic indices (GSI) 0.07 was used in the second step of the experiment (ovarian follicles incubation). The fishes were raised in the Shahid Maleki Fish Farm, Ahvaz, Iran. They were netted from the outdoor ponds in September when the water temperature was 30.7°C and placed in the flow-through basins containing 300 liter water at 18-20°C.

Reagents

MEM Eagle (minimum essential medium Eagle with non-essential amino acids, Earle's salts and glutamine), Hepes, collagenase H, BSA (bovine serum albumin), antibiotics (penicillin and streptomycin) and antimycotic (Fungizone) were obtained from Sigma-Aldrich, USA. The 96-well and 24-well microplates were from Nunc A/S, Denmark.

Pituitary primary cell culture

On the day of experiment, five sexually mature carp euthanized with MS222 (200 ppm) and their pituitary glands were collected aseptically and placed in sterile ice-cold MEM buffered with 15 mM Hepes and 9 mM bicarbonate. The medium had an osmotic pressure of 275 mOsmol/kg and pH of 7.7 (the standard values for common carp plasma) (Mikolajczyk *et al*., 2005). The enzymatic dispersion of the glands was performed according to the method described by Weil *et al*. (1986) for trout and adapted by Mikolajczyk *et al*. (1990) for carp.

The collected glands were chopped into small pieces and subjected to dispersion for 2-3 h at 20°C in medium containing 1% collagenase H and 1% BSA. Enzymatic dissociation was followed mechanically by aspiration of the cell clusters into a 10 ml syringe. The cells were harvested by 10 min centrifugation (200 g) at 20°C and washed twice with pre-incubation medium containing fatal bovine serum (FBS) (2%) and antibiotic-antimycotic (1%) solution.

Cell counting and cells viability test were carried out by trypan blue exclusion staining and Thoma haemocytometer. As a result of dispersion, 85% of live cells were obtained. They were resuspended in incubation medium and transferred into 96-well microplate (approximately 5×10^4 cells/250 µl of medium/well). The plate was incubated for 72 h at 22°C. The media then were collected after centrifugation (200 g for 10 min) and frozen at -20°C until use.

Ovarian follicles incubation

The ovaries of a 4-year-old female carp spawner were used for collection of follicles. The fish was euthanized with MS222 (200 ppm) and its ovaries were removed aseptically. Ovarian fragments were incubated for 48 h in 24-well microplates in BSS (basic salt solution) Cortland medium (pH = 7.6) at 20°C (approximately 120 ± 30 mature vitellogenic stage follicles/2 ml BSS medium/well). After 48 h, the ovarian follicles were divided into two groups:

A- Control group: ovarian follicles incubated only in BSS medium.

B- Experimental group: ovarian follicles incubated in BSS medium supplemented with different concentrations of collected pituitary secretion (CPS) (50, 100 and 200 µl/ml).

The plates were centrifuged after 24 hr (200 g for 10 min) and the ovarian follicle culture media were collected and frozen at -20°C for further steroid analysis.

Steroid analysis

17-α-hydroxy progesterone (P_4) and 17-β-oestradiol (E_2) were analyzed by radioimmunoassay described by Stoklosowa *et al*. (1982). The concentrations of steroids were computed in pg/ml of culture medium and were expressed as mean ± SE.

Oestradiol-17β was determined using [2, 4, 6, 7-^{3}H] estradiol (sp. Act. 100 Ci/mmol: Immunotech, Beckman Culture Company, France) as a tracer and an antiserum raised in human against oestradiol-17β-sodium azide, which it was highly specific for oestradiol. Extremely low cross reactivities (under 0.01%) were obtained against other

steroids (estrone, estriol, cortisol, cholesterol, progesterone, androsterone…). In a series of 15 experiments the coefficients of variations between and within assays were 11.2 and 12.1%, respectively. The limit of the standard curve was between 6-5000 pg/ml.

17-α-hydroxy progesterone was estimated using 17-α-progesterone (sp. Act. 100 Ci/mmol: Immunotech, Beckman Culture Company, France) as a tracer and an antibody raised in human against 17α-hydroxy progesterone-sodium azide-BSA. The limit of the standard curve was between 46-50000 pg/ml. Cross reactions for other steroids (hydroxylpregnenolone, progesterone, androsterone…) were under 1.3%. In a series of 15 experiments the coefficients of variations between and within assays were estimated to be 15.7 and 7.2%, respectively.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and the significance of the differences between the means were determined using Duncan's multiple range test.

Results

Culture of the common carp pituitary cells was carried out successfully in MEM medium supplemented with FBS which was formulated to support the growth of common carp cells. The majority of the cells had been attached to the bottom of the culture vessels 5 to 6 h after plating and they were observed in different shape (round, oval and rod like) in the first day of culture using inverted microscope. The cells were proliferated and became confluent rapidly. Cultures reached almost 40 to 60% confluences at the second day and 100% after 3 days. The carp pituitary culture media were collected at the third day of culture and then the subcultures were made immediately. Since the cells were sensitive to trypsinization, the passage of cells was carried out by pipetting. The cell proliferation was continued successfully in the second passage (eight day of culture), anyway we did not continue cell culture more than 15 days. Bacterial and fungal contaminations were not detected in cultures.

For ovarian follicles incubation, the large follicles (0.8-1.2 mm in diameter) with vitellogenic central oocyte and two surrounding layers, granulosa and theca cells were selected.

Results showed that the E_2 and P_4 secretion of control group incubated ovarian follicles were 750 ± 10.9 and 116 ± 5.42 pg/ml, respectively (Fig. 1). In the incubated ovarian follicles treated by 100 µl/ml of CPS, the E_2 and P_4 concentration were 752.17 ± 40 and 119.6 ± 6.87 pg/ml, respectively. There was no significant difference in comparison with the control group ($P>0.05$, Fig. 1). However, adding low concentration (50 µl/ml) of CPS, increased E_2 and P_4 secretion of incubated ovarian follicles, significantly (1099.07 ± 22 and 222.2 ± 45.3 pg/ml, respectively) ($P<0.05$, Fig. 1).

Result showed that E_2 and P_4 secretion of incubated ovarian follicles (474.27 ± 32 and 92.2 ± 11.17 pg/ml, respectively) decreased significantly by adding high concentration (200 µl/ml) of CPS ($P<0.05$, Fig. 1).

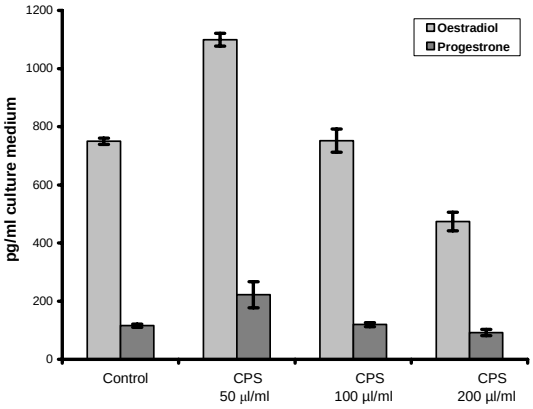

Fig. 1: Concentrations of E_2 and P_4 in the ovarian follicles incubation medium as mean ± SE

Discussion

To obtain a more definitive system for studying growth and activity potency of pituitary cells apart from the whole body, we used cell culture method. To establish carp pituitary cell culture, it was necessary to identify culture condition (osmotic pressure,

pH and temperature) that prevent or at least reduce the loss of viability, growth and proliferation of the isolated cells (Helmut, 1998). The enzymatic dispersion procedure, which was applied for carp pituitary cell dissociation in this study, was found to produce consistently a high yield of cells with excellent cell viability (as determined by trypan blue staining). The chemically defined medium influenced positively the conservation of cell morphology and proliferation for at least 15 days. Attachment of cultivated cells to culture vessels is a critical step for primary cultures (Freshney, 1994) which occurred in this experiment after 5 to 6 h of plating even without using poly-L-lysine. Ribeiro and Ahne (1982) reported that the cultivated cells appeared firstly as scatter clusters with different cell morphology and the overgrowth and confluention of pituicytes were observed during 3-5 days of primary culture which is in agreement with our finding. It was very difficult to distinguish different cultivated pituitary cell types and the situation was further complicated by existence of numerous cell clumps.

The carp ovarian follicles were thoroughly mixed before being distributed in microplates to ensure homogeneity of cellular response to various stimuli (Degani and Boker, 1992).

According to the results, E_2 was a main hormone secreted by incubated carp ovarian follicles (Fig. 1), which is correlated closely with vitellogenes stage. The results of this study are in agreement with the study done by Galas *et al*. (1999).

Kagawa *et al*. (1982) proposed two cell type model of E_2 secretion based on studies on the wall of salmon ovarian follicles where E_2 secretion results from the interaction between granulosa and theca cells. Clombo *et al*. (1982) and Stoklosowa and Epler (1985) reported the high level of E_2 in vitellogenic carp plasma (in the same stage of ovarian maturity as that of the female used in the present experiment). Drori *et al*. (1994) and Yaron (1995) observed that the action of pituitary extract on steroid secretion was depended on the stage of follicles maturation. This high content of E_2 obtained in vitellogenic stage fish may be correlated with the role of E_2 in the promotion of hepatic synthesis of vitellogenin (Sen *et al*., 2002).

In this study, CPS at the chosen concentration affected E_2 and P_4 secretion from incubated ovarian follicles depending on its dosage as well as maturity of follicles. Collected pituitary secretion at the lowest concentration (50 µl/ml) increased E_2 and P_4 secretion in comparison with the control group. Epler *et al*. (1989) reported that mature female carp injected with CPH (carp pituitary extract) to induce spawning showed a 20-fold increase in E_2 concentration and 10-fold increase in P_4 level in plasma. In another experiment, CPH stimulated mainly E_2 secretion by carp follicles (Galas *et al*., 1999). Theca cells at the vitellogenes stage produce and release testosterone in response to GtH, which then diffuses into granulosa cells layer and is converted to 17-β-oestradiol (E_2) by aromatase. E_2 released into blood stream and stimulates the hepatic synthesis of the vitellogenin (Ostrander, 2000).

According to the results of this study, adding 100 µl/ml of CPS had no significant effect on steroid secretion by carp ovarian follicles. The high dosage of CPS (200 µl/ml) decreased the steroid secretion significantly, which may be caused by down-regulation of gonadotropin hormones receptors on the ovarian follicle cells (Ostrander, 2000). When a hormone or neurotransmitter is present in excess (as the high concentration of CPS, which we used in this experiment) the number of active receptors generally decreased (down-regulation), whereas in the presence of a deficiency of the chemical messenger, the number of active receptors is increased (up-regulation) (Ganong, 1997).

Since carp pituitary cells can be alive and active *in vitro*, the pituitary primary cell culture could be a useful tool for investigating several aspects of the hypothalamo-hypophyseal system of ciprinid fish. Further work is needed to measure the amount of GtH and its extraction from pituitary cell culture media for spawning induction in fish.

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