

## Isolation of common carp ovarian follicular cells and evaluation of their endocrine activity in primary cell culture

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### Abstract

To study viability and activity of isolated common carp, *Cyprinus carpio*, ovarian follicular cells (granulosa and theca cells), 17- $\alpha$ -Hydroxy progesterone (17 $\alpha$ -OHP) and 17 $\beta$ -Estradiol (E<sub>2</sub>) levels were estimated in the culture media of cultivated carp ovarian follicular cells, using radioimmunoassay (RIA). Oocytes were isolated from the ovaries of female carp. Interstitial tissue was manually removed in order to obtain single oocytes surrounded only by the follicular envelope. Such a preparation was trypsinized at room temperature. Follicular cell suspension contained both cells and cell clumps. It was the mixture of theca (T) and granulosa (G) cells. The cells were grown as monolayer in 24-well microplates in M199 medium supplemented with FBS. Culture media were analyzed for estrogen and progesterone content by appropriate radioimmunoassay. Trypsinization of ovarian follicles resulted in the formation of the cell suspension which contained a mixture of G and T cells. The follicular cells attached to glass and grew well during culture period. E<sub>2</sub> was the main steroid hormone secreted by cultivated cells. Estrogen secretion increased by 415.52  $\pm$  25 pg/mL at the first 3 days up to 530.25  $\pm$  55.8 pg/ml on day 5 and it didn't change significantly until the end of the experiment. 17 $\alpha$ -OHP secretion, however, gradually increased from 23.84  $\pm$  8.2 pg/ml at the beginning of culture up to 35.76  $\pm$  5.4 pg/ml at the end of cultivating. As the result of the present study the fish follicular cells grown in tissue culture were steroidogenically active cells as expressed by the secretion of E<sub>2</sub> and P<sub>4</sub> and The E<sub>2</sub> was a dominant hormone secreted by isolated follicular cells, which it correlated closely with vitellogenesis stage

**Keywords:** Granulosa cells, Theca cells, Steroid hormones, *Cyprinus carpio*, Cell culture.

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## Introduction

Common carp, *Cyprinus carpio*, is an economically important cyprinid species in Iran and its ovarian development stages were determined previously according to age and annual cycle (Carballo *et al.*, 2005). Teleost oocytes as in other vertebrates are surrounded by two major cell layers; an outer theca layer and an inner granulosa (Unal *et al.*, 2005). It is known from histochemical and ultrastructural investigations that follicular cells of fish oocytes show steroidogenic enzymes activity (Galas *et al.*, 1999) and are able to synthesize estrogens and Progestogens (Unal *et al.*, 2005). In many teleosts it has been indicated that plasma estradiol-17 $\beta$  levels increased during the vitellogenic stage but decreased during the maturation stage (Kime *et al.*, 1992; Erdogan *et al.*, 2002). E2 has been considered to be the main hormone of female fish and its role in the synthesis and secretion of hepatic vitellogenic protein is well established (Chaves-Pozo *et al.*, 2008; Kokokiris *et al.*, 2000). Progesterone reaches its maximum concentration at the ovulation or after ovulation (Tripathi & Verma, 2004). In some teleosts, 17 $\alpha$ -OHP may play a role as an indirect inducer on oocyte maturation (as a precursor of 17 $\alpha$ ,20 $\beta$ -Dihydroxy progesterone) (David & Lynn, 2000; Pavlidis *et al.*, 2000). Tissue culture of isolated follicular cells offers an opportunity to examine the ability of granulosa and theca cells to secrete steroid hormones (Stoklosowa *et al.*,

1982), therefore, the aim of the present investigation was to isolate ovarian follicular cells, cultivating them in culture medium and evaluation of the interaction of these cell types in E2 and P4 biosynthesis in vitro.

## Materials and methods

An experiment was conducted in February 2008 on five 4-years old female common carp spawners (3.6-4 kg). The fishes were raised in the Shahid Maleki fish farm, Ahvaz, Iran. They were captured from the outdoor ponds month before experiment and transferred to the flow-through basins containing 300 liter water at 18-20 °C.

At the day of experiment, fishes were anesthetized with MS222 (Tricaine) (200 ppm) and ovaries were aseptically removed and placed in a beaker containing sterile phosphate buffered saline (PBS). Isolation of follicular cells procedure followed the protocol used by Galas and Epler (2002). The connective tissue capsule of the ovary was manually removed and the tissue was transferred in to another beaker containing medium 199 (M199) (Sigma-Aldrich; USA) diluted 4:1 with sterile distilled water. Ovarian pieces were separated from all interstitial tissues so that single oocytes remained (Galas and Epler, 2002). The medium was decanted and then replaced several time to rinse free oocytes thoroughly. To obtain a suspension of single follicles, vigorous pipetting was applied using a pipette with a large orifice. Owing to such procedure, it was

possible to obtain separate oocytes surrounded only by the follicular layer composed of granulosa (G) and theca cells (T). Follicles free from connective tissue, were trypsinized by a 0.25% solution of trypsin (Sigma-Aldrich; USA) in  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free PBS for 30 min and then subsequently in three changes of solution at 10-min intervals at 37°C, the optimum temperature for obtaining the suspension of follicular cells (Galas and Epler, 2002).

Trypsin action was inhibited by the addition of FBS (fatal bovine serum) (0.5 mL after each trypsinization). The progress of trypsinization and the state of the follicular envelope were monitored using an inverted microscope. Supernatant fractions, containing released cells were pooled, centrifuged and the pellet resuspended in the medium 199 and diluted to a concentration of  $2 \times 10^6$  cell / mL culture media. Cell suspension may contain occasional fibroblasts, but these cells do not have any influence on steroid secretion.

The viability of cells, as assessed by the trypan blue exclusion test, was 95%. Efforts were made to count free cells using a hemocytometer. The cell suspension, however, contained not only single cells, but also numerous cell clumps. The isolated follicular cells were cultured according to a method described by Galas *et al.* (1999). Cells were grown as monolayers in 24-well microplate (Nunc A/S; Denmark) in medium 199 supplemented with 10% FBS and penicillin (120 IU/mL)

(Sigma-Aldrich; USA) and streptomycin (0.1 ng/mL) (Sigma-Aldrich; USA) (pH = 7.4) ( $2 \times 10^6$  cell / 1 mL culture media /well) at 20°C and 5%  $\text{CO}_2$  for 7 days (Galas *et al.*, 1999). The culture media were collected every day and frozen at -20°C for further steroid analysis.

17- $\alpha$ -OHP and 17- $\beta$ -estradiol ( $\text{E}_2$ ) 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 means  $\pm$  SEM.

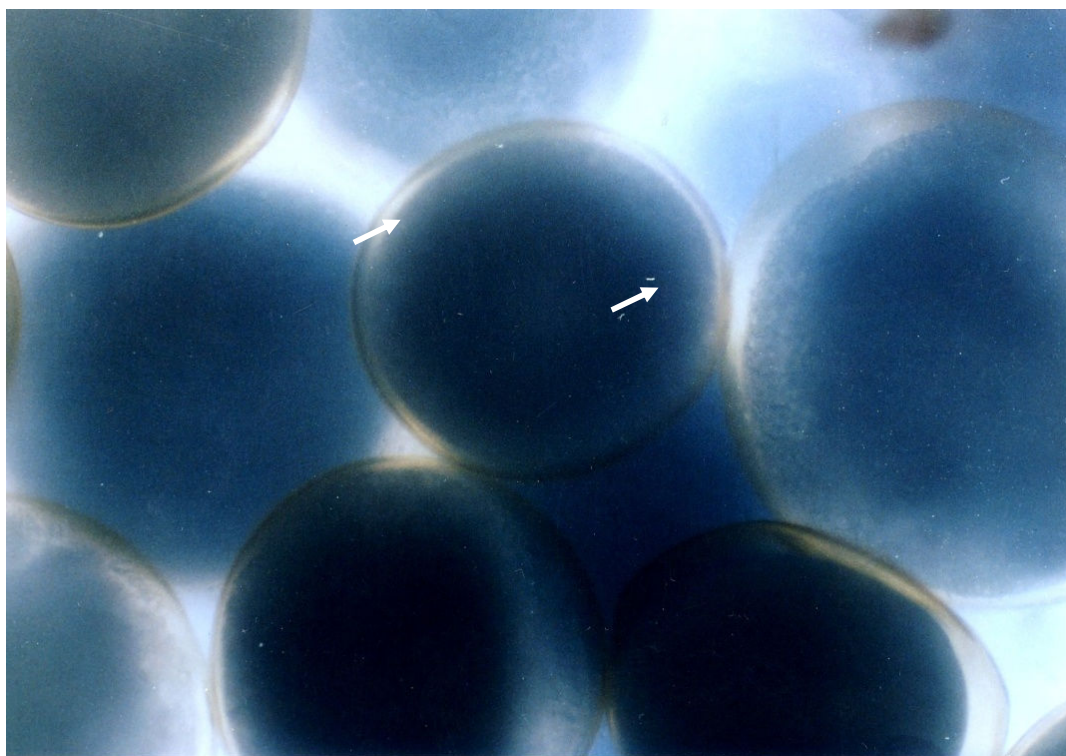
17- $\beta$ -estradiol was determined using [2, 4, 6, 7- $^3\text{H}$ ] Estradiol (sp. Act. 100Ci/mmol: Immunotech, Beckman culture company, France) as a tracer and an antiserum raised in human against estradiol-17 $\beta$ -sodium azide, which it was highly specific for estradiol. Extremely low cross reactivities were obtained against other steroids (estrone, estriol, cortisol, cholesterol, progesterone,...) (under 0.01%). 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 $\alpha$ -OHP was estimated using 17 $\alpha$ -OHP (sp. Act. 100Ci/mmol: Immunotech, Beckman culture company, France) as a tracer and an antibody raised in human against 17 $\alpha$ -OHP-sodium azide-BSA (bovine serum albumin). The limit of the standard curve was between 46-50000 pg/mL. Cross reactions for other steroids (hydroxylpregnenolone,

progesterone, etc) 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.

Data were analyzed by one way analysis of variance (ANOVA) and the significance of the differences between the means was determined using LSD test.

## Results

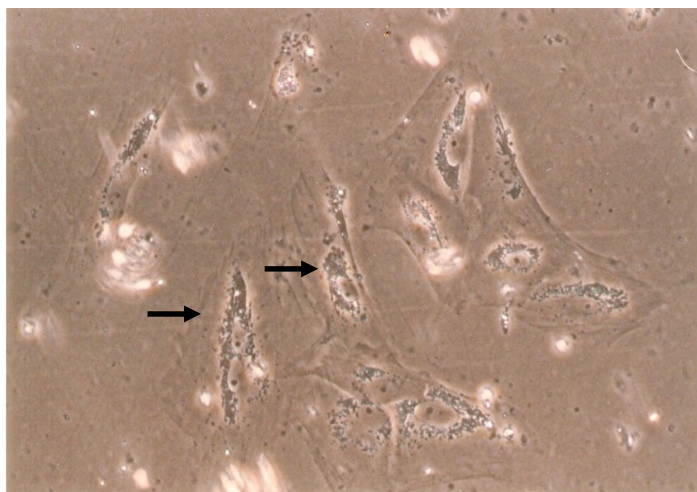
Endocrine follicular layer of carp oocyte is a very thin sheet of tissue composed of G and T cells embedded in connective tissue. After 15 min of trypsinization at 37°C, a kind of swelling of this layer was observed (Fig. 1). During the next 15 min its



**Figure 1:** Carp oocytes (white arrows) separated from interstitial tissue of the ovary. First step of trypsinization . Single oocytes show swollen follicular layer (×20)

detachment from the rest of the oocyte took place. Further trypsinization resulted in the formation of the cell suspension, which contained a mixture of G and T cells. In spite of gentle trypsinization many of the oocyte walls

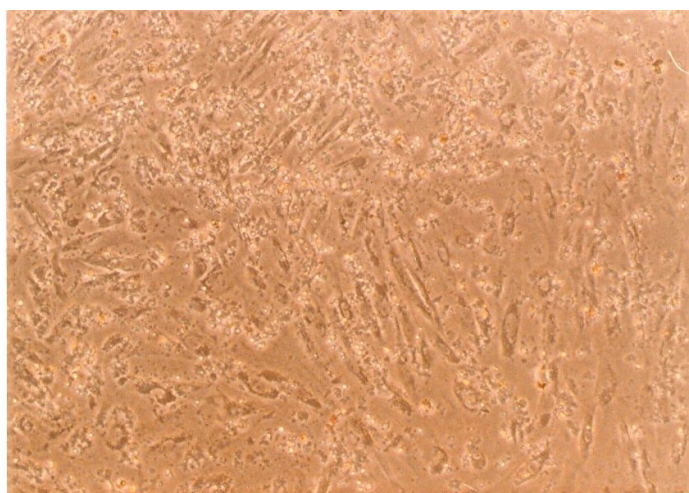
were damaged and the suspension became contaminated with the large amount of yolk. The cells were attached to the bottom of culture vessels after 24 hr of plating and the majority of cells had fibroblast- like shape (Fig. 2).



**Figure2:** The second day culture. The fibroblast- like cells (black arrows) were attached to the bottom of culture vessels after 24 hr of plating ( $\times 40$ )

The cells were proliferated and confluent fast. After 3 days in culture colonies of viable cells were seen.

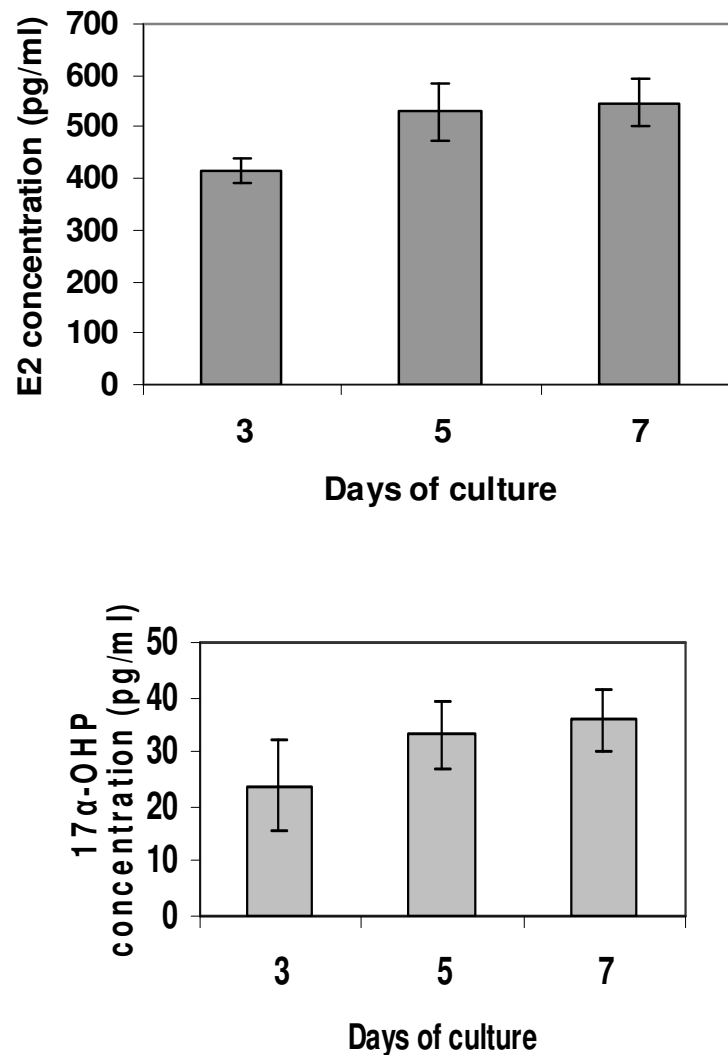
Figure 3 shows living cells of a 4-day culture.



**Figure 3:** A colony of follicular cells in 4-day culture ( $\times 20$ )

Cultures reached almost 100% confluences after 6 days. The secretion of two investigated steroid hormones is shown in Figure 4. Estrogen secretion increased by  $415.52 \pm 25$  pg/mL at the first 3 days up to  $530.25 \pm 55.8$  pg/mL on day 5 and it didn't change significantly until the end. 17-OHP

levels were very low. 17-OHP secretion, however, gradually increased from  $23.84 \pm 8.2$  pg/mL at the beginning of culture up to  $35.76 \pm 5.4$  pg/mL at its end.



**Figure 4:** Concentrations of E<sub>2</sub> and 17α-OHP in the follicular cells culture medium as mean  $\pm$  SEM (P<0.05)

Estrogen secretion increased by  $415.52 \pm 25$  pg/mL at the first 3 days up to  $530.25 \pm 55.8$  pg/mL on day 5 and it didn't change significantly until the end. 17-OHP levels were very low. 17-OHP secretion, however, gradually increased from  $23.84 \pm 8.2$  pg/mL at the beginning of culture up to  $35.76 \pm 5.4$  pg/mL at its end.

### Discussion

There are reports of cultures of various fish tissues (Jeserich & Stratmann, 1992; Ganassin & Bols, 1996; Helmut, 1998) including organ culture of ovarian fragments (Glasser *et al.*, 2004). The result of the present investigation showed that follicular cells adapted well in culture, attached to glass, and grew better at 20°C than at room temperature. The method of dispersion of follicular layer presented in this paper eliminated the ovarian interstitial tissue which is composed of connective tissue, endocrine cells and endothelial cells of capillary blood vessels. Prior to culture, the cell suspension contained mainly endocrine follicular cells and possibly some fibroblasts (a component of the follicular layer of the oocytes). However, during 7 days of primary culture no overgrowth with fibroblasts was observed. It was also true for cultures of follicular cells isolated from salmon ovarian follicles (Galas & Epler, 2002). The cell suspension resulting from trypsinization of the follicular layer composed of G and T cells which, according to Nagahama 1994, are the major sites of steroid synthesis in the teleost ovary. In culture, it is more

difficult to distinguish between the two cell types (G and T cells) because they don't show different characteristics, which it is true with other experiments (Galas *et al.*, 1999; Galas & Epler, 2002). Follicular cells grown in tissue culture were steroidogenically active cells as expressed by the secretion of E<sub>2</sub> and P<sub>4</sub>. Colombo *et al.*, 1982 reported the existence of steroidogenesis in vitellogenic carp ovary (in the same stage of ovarian maturity as that of the females used in present experiment). In the present experiment, isolated cells were cultured in high concentrations. The data suggest that the secretion took place during the culture period. Residual hormones were removed prior to culture by preincubation of cells during trypsinization and washing the cell preparation.

The E<sub>2</sub> was a dominant hormone secreted by isolated follicular cells, which it correlated closely with vitellogenesis stage. The result obtained previously by Colombo *et al.*, 1982 is in agreement with present study. Also Galas and Epler, 2002 reported the high level of E<sub>2</sub> in carp plasma in vitellogenesis stage. This high content of E<sub>2</sub> obtained in vitellogenic stage fish may be correlated with the role of E<sub>2</sub> in the promotion of hepatic synthesis of vitellogenin (Sen *et al.*, 2002). Vitellogenic ovarian follicular cells are ready for intensive androgen production and its aromatization (Galas *et al.*, 1999). At this stage theca cells produce and release testosterone in response to gonadotropin hormones, which then

diffuses into granulosa cells layer and is converted to 17- $\beta$ - estradiol ( $E_2$ ) by aromatase.  $E_2$  released into blood stream and stimulates the hepatic synthesis of the vitellogenin (Nagahama, 1994). On the other hand insignificantly change of  $E_2$  follicular cells secretion from day 5 of culture until the end was also a typical phenomenon observed in primary mammalian follicular cell culture and it may result from an insufficient amount of androgen precursor supplied by theca (Stoklosowa *et al.*, 1982).

Study of steroid hormones secretion during stimulation of isolated carp follicular cells by carp pituitary extract (CPE), in vitro secretion of carp pituitary cells (CPS) and human chorionic gonadotropin (HCG) is the subject of further experiments.



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