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[Research]

Primary culture of ovarian follicular cells of Sterlet, *Acipenser ruthenus* to develop an *in vitro* system

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ABSTRACT

The aim of the present study was to develop an *in vitro* system for functional investigation of ovarian follicular cells in Sterlet, *Acipenser ruthenus*. Oocytes for the primary culture were obtained from the ovaries of a 6 years old Sterlet 729 g in weight and 47 cm in total length. The oocytes were in advanced vitellogenesis stage (PI >10). A part of the ovary (containing about 300 follicles) was removed, ovarian follicles isolated by manually removing those from the interstitial tissue and washed with sterile phosphate buffered saline (PBS) containing antibiotics and Amphotericin B. Follicular cells were separated by treating oocytes with 0.25% trypsin-EDTA in Ca^{2+} and Mg^{2+} free PBS and cultured in medium L-15 supplemented with 20% FBS, streptomycin sulphate (Gibco, 100 $\mu g.ml^{-1}$), penicillin G potassium (Gibco, 100 $IU.ml^{-1}$) and Amphotericin B (Gibco, 2.5 $\mu g.ml^{-1}$) at 22 °C. The concentrations of Testosterone (T), Estradiol-17β (E2), Progesterone (P4) and 17α-hydroxyprogestron (17αOHP) in the medium were measured at days 3, 5 & 7 by the Enzyme-Linked Immunosorbent Assay. According to the results, the ovarian follicular cells of Sterlet proliferated in L-15 medium were steroidogenically active as expressed by the secretion of T, E2, P4 & 17αOHP. Testosterone was the dominant hormone secreted by cultivated follicular cells, which was correlated closely with the end of vitellogenesis in the isolated oocytes. Decrease in production of these hormones was greater at days 3 & 4 in comparison with those at days 5 & 6. By successfully culturing ovarian follicular cells of Sterlet in L-15 culture medium, an *in vitro* system was developed which enables functional studies to be carried out similar to the *in vivo* situation in the ovarian follicles.

Key words: Sterlet, *Acipenser ruthenus*, Primary culture, Follicular cells, *In vitro*

INTRODUCTION

Fish oocytes, like other vertebrates, are surrounded by follicular cells (Unal *et al.* 2005). These cells play a vital role in fish reproduction as well as in the synthesis of steroid hormones, growth and final maturation of oocytes (Nagahama 1994). Several studies have evaluated the synthesis and secretion of steroid hormones by follicular cells in fish (Nagahama *et al.* 1982; Lessman 1991; Venkatesh *et al.* 1992; Kime *et al.* 1992). It has also been documented that the fish follicular cells remained alive and continued to secrete hormones in culture medium (Stoklosowa & Epler 1985; Petrino *et al.* 1989 a, b; Salamat *et al.* 2010). The primary culture of ovarian follicular cells throughout the year can serve as a valuable tool to study the seasonal variations in hormones secreted by these cells (Galas *et al.* 1999). Reproduction in sturgeons is regulated by the hypothalamus-

pituitary-gonadal (HPG) axis (Dettlaff *et al.* 1993; Doroshov *et al.* 1997). Gonadotropin releasing hormone (GnRH) is secreted from the hypothalamus under the influence of environmental factors (such as photoperiod and temperature). This hormone regulates the synthesis and release of gonadotropins (GTH I and GTH II) by the pituitary (Lescheid *et al.* 1995; Moberg *et al.* 1995; Querat *et al.* 2000). These hormones are transported to the follicular cells through bloodstream and induce them to secrete steroid hormones (Tyler *et al.* 1991). These hormones are involved in vitellogenesis, growth and final maturation of oocytes (Moberg *et al.* 1995).

With regard to the drastic decline in natural stocks of sturgeons during the recent years, the artificial breeding of these fish provides effective means for the restoration and sustainable use of sturgeon stocks. Artificial breeding of sturgeons first started with Sterlet, *Acipenser ruthenus* (Webb & Doroshov 2011). The Sterlet, belonging to the family Acipenseridae, is a native species in Volga River. It is also found in riverine regions of the Black, Azov and Balkan Seas. In their natural habitats, females attain sexual maturity between 4 and 7 years which can be reduced to 3 or 4 years under artificial rearing conditions. The eggs of Sterlet are 1.91 to 2 mm in diameter (Chebanov & Galich 2013). This species adapts easily to rearing conditions and does not require more special attention. It also feeds on a wide range of food items (Holcik 1989). Success in artificial breeding programs of sturgeon depends on the selection of female breeders that respond to hormone injection and produce the suitable quality post-vitellogenic oocytes, understanding of the factors that significantly affect the growth and maturation of oocytes, gamete quality, synthesis and secretion of the related hormones. Follicular cells are the key of the success in the sturgeon aquaculture. The present study was aimed at developing an *in vitro* system for functional studies of ovarian follicular cells in order to achieve suitable guidelines for the success in artificial breeding programs of sturgeons in the future. In this regards, the purpose of the present experiment was to culture the oocytes follicular cells in *in vitro* condition to study the viability and secretion of these cells.

MATERIALS AND METHODS

A sixty years old sterlet sturgeon (729 g in weight and 47.6 cm in length), was provided from International Sturgeon Research Institute, (Rasht, north of Iran) in March 2015. Fish was anesthetized by clove powder (200 ppm), abdominal area was cleaned with 70% alcohol and dissected to remove a part of the ovary (containing about 300 follicles). The ovary was attained at advanced stage of vitellogenesis (PI>10) according to Chapman *et al.* (2007). The protocol developed by Stoklosowa and Epler (1985) was followed for isolating of ovarian follicular cells with some modifications. The follicles were washed in a petri dish containing 5 ml sterile phosphate-buffered saline (PBS), streptomycin sulphate (200 $\mu g.ml^{-1}$), penicillin G potassium (Gibco 200 $IU.ml^{-1}$) and Amphotericin B (5 $\mu g.ml^{-1}$). After a minute the follicles were transferred to another petri dish containing 5 ml L-15 Medium (Sigma), streptomycin sulphate (Gibco, 200 $\mu g.ml^{-1}$), penicillin G potassium (Gibco, 200 $IU.ml^{-1}$) and Amphotericin B (Gibco, 5 $\mu g.ml^{-1}$). After 10 minutes incubation at 22 °C, the culture medium was drained and 5 ml of fresh culture medium of the same composition was added to the petri dish.

The culture medium was drained after 10 minutes and 5 ml of fresh culture medium of the same composition was added to the petri dish. The interstitial connective tissue of the oocytes was removed manually under a stereomicroscope (Nikon). The culture medium was drained and the connective tissue free follicles were washed in PBS solution containing streptomycin sulphate (200 $\mu g.ml^{-1}$), penicillin G potassium (200 $IU.ml^{-1}$) and Amphotericin B (5 $\mu g.ml^{-1}$) for three times. PBS was replaced by 5 ml 0.25 % Trypsin-EDTA (Gibco) in Ca^{2+} and Mg^{2+} free PBS (Gibco). Trypsinization was carried out at the optimum temperature of 37 °C (Galas & Epler 2002).

The process of trypsinization and the state of follicular envelop were monitored by an inverted microscope (Nikon, Model Eclipse-Ti). Trypsinization was carried out twice for 25 minutes and once for 10 minutes (2 × 25' and 1 × 10'). After each incubation time, supernatant fractions (containing the follicular cells) were pooled and centrifuged, then the supernatant was discarded and 1 ml Fetal Bovine Serum (FBS) was added to the sediment to inhibit trypsin action. After the third trypsinization and centrifugation, supernatant was removed and the deposited follicular cells were re-suspended in the medium L-15 and then diluted to a concentration of 1.25×10^6 cells.ml^{-1} culture medium. After assessing viability of cells by trypan blue (0.4%) exclusion test (Doyle & Griffins 1998), the follicular cells were cultured (1.25×10^6 cells.ml^{-1}) in medium L-15 supplemented with 20% FBS, streptomycin sulphate (Gibco, 100 µg.ml^{-1}), penicillin G potassium (Gibco, 100 IU.ml^{-1}) and Amphotericin B (Gibco, 2.5 µg.ml^{-1}) and then transferred into an incubator at 22 °C. Culture medium was collected on days 3, 5 and 7, centrifuged and supernatant was stored at -20 °C (Epler *et al*. 1997) for further analysis of steroid hormones produced including testosterone (T), progesterone (P4), 17α-hydroxyprogesterone (17αOHP) and estradiol-17β (E2). Steroids analyses were carried out by Enzyme-Linked Immune Sorbent Assay (ELISA).

After determining the concentrations of the steroids, the data were analyzed by One - Way analysis of variance (ANOVA) test (Holm 1979) and the significance of the differences between the means was determined using LSD test (Fisher 1935) performed by SPSS (Version 17) at 0.95% confidence level. Differences were considered significant at $p<0.05$ for all analyses. Graphs were plotted using Excel 2007 software package.

RESULTS

By removing the interstitial connective tissue, separated oocytes surrounded only by the follicular layers were obtained (Fig. 1), and follicular cells were observed as a thin layer when oocytes were examined under an inverted microscope (Fig. 2). Oocytes and the follicular layers were expanded after first trypsinization (Fig. 3).

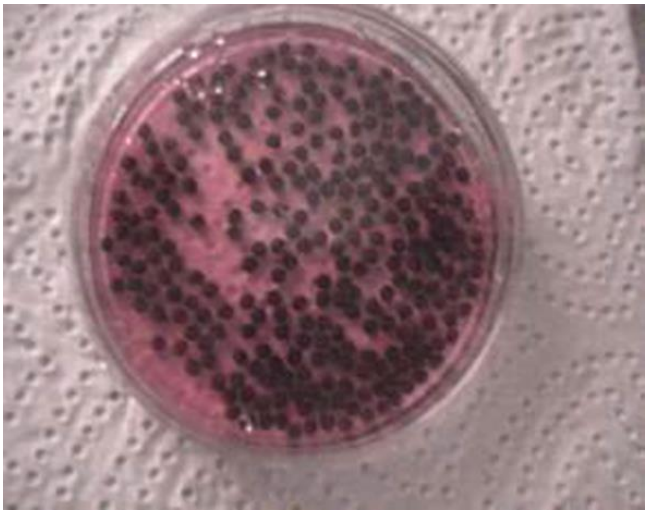

Fig. 1. Separated oocytes from interstitial tissue of the ovary in L-15 Medium.

Further trypsinization resulted in detaching of follicular layers from the rest of the oocytes and formation of the cell suspension containing theca and granulosa cells. The isolated follicular cells were observed under an inverted microscope as separated cells (Fig. 4A) and Sometimes formed clumps (Fig. 4B). The number of obtained follicular cells was 1.25×10^6 cells.ml^{-1}, and the viability of cells, determined by the trypan blue exclusion test, was 92%. After 48 hours, follicular cells were attached to the bottom of the culture vessel and

proliferated. The cells were mostly fibroblast-like in shape (Fig. 5).

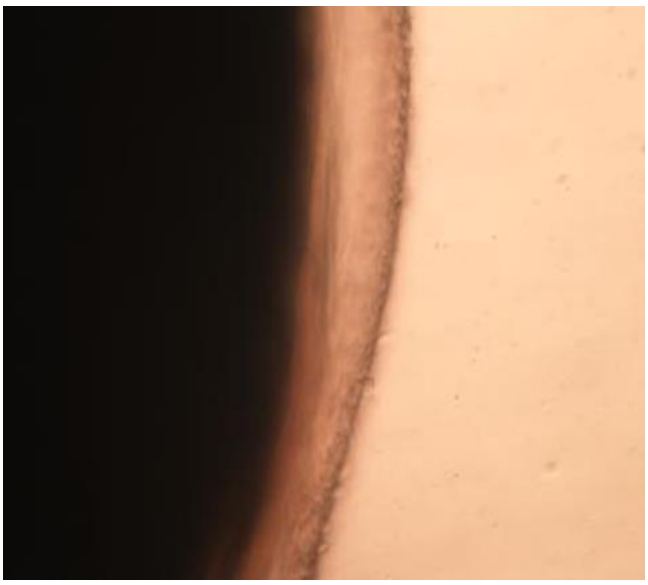

Fig. 2. Sterlet oocyte (× 600).

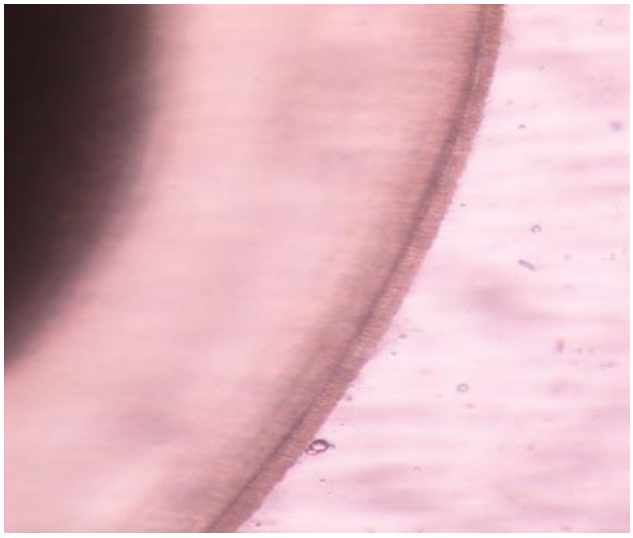

Fig. 3. Trypsinized oocyte of Sterlet (× 600).

The propagation of cells increased in the following days (Fig. 6) and after one week the cultured cells had almost completely covered the bottom of the culture vessel (Fig. 7). Based on the results, the concentrations of T secreted by cultivated follicular cells after 2, 4 and 6 days were 15.93 ± 1.57, 10.30 ± 0.20 and 9.63±0.24 ng.ml^{-1}, respectively (Fig. 8). According to Fig. 8, T secretion in the culture medium decreased over time. T levels on days 5 and 7 were significantly lower than that on day 3. However T level on days 5 and 7 did not differ significantly. E2 concentration in culture medium after 2, 4 and 6 days were recorded as 443.33 ± 12.01, 170.0 ± 11.54, and 130.0 ± 15.8 pg.ml^{-1}, respectively (Fig. 9). E2 levels gradually decreased in the culture medium and were significantly lower on days 5 and 7 than on day 3, although no significant differences were detected in E2 levels between days 5 and 7.

Levels of P4 in the culture medium increased gradually from 260.0 ± 17.32 pg.ml^{-1} on day 3 to 303.33 ± 34.80 pg.ml^{-1} on day 5 and further increased to 330.3 ± 36.05 pg.ml^{-1} on day 7, although no significant differences were

recorded in P4 levels between at the beginning and at the end of the culture (Fig. 10).

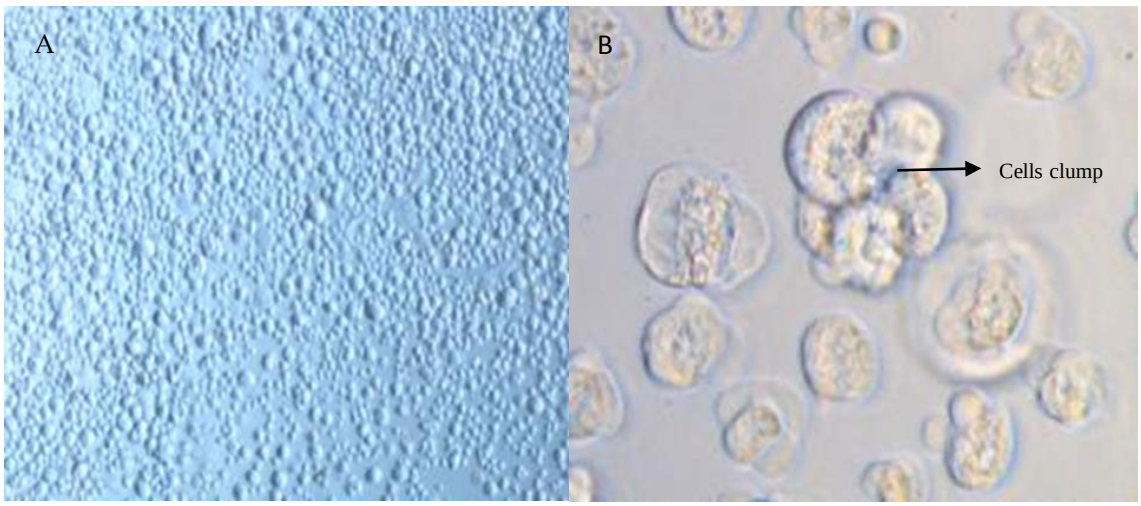

Fig. 4. Follicular cells of Sterlet oocytes 1 h after culturing (A: × 120; B: × 1200).

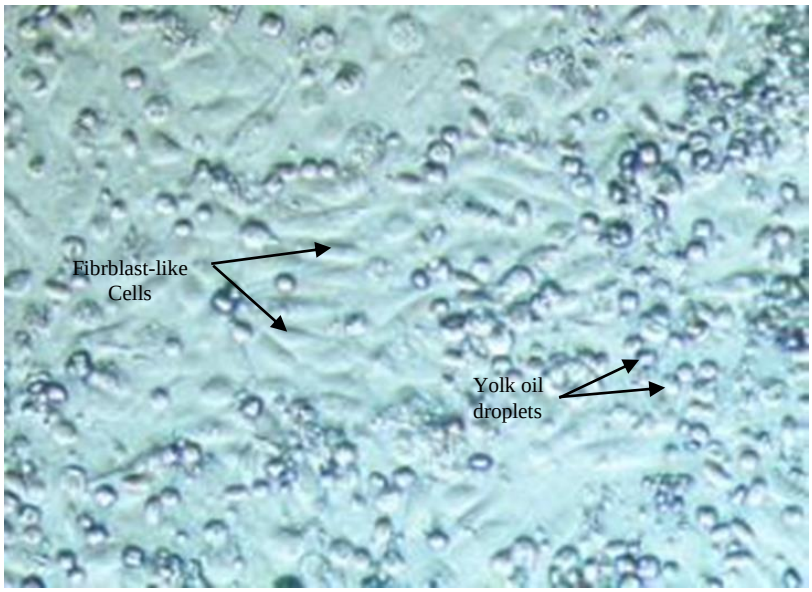

Fig. 5. Fibroblast- like cells after two days of plating (× 300).

Similarly, 17αOHP levels in the culture medium also increased after days 2, 4 and 6. 17αOHP level was 106.67 ± 8.81 pg.ml^{-1} on day 3 which significantly increased to 180.0 ± 5.78 and 193.33 ± 12.01 pg.ml^{-1} on days 5 and 7, respectively (Fig. 11). No significant differences were detected between 17αOHP levels on days 5 and 7. Based on the results obtained from this study, cultivated follicular cells of Sterlet in L-15 medium, containing 20% FBS, streptomycin sulphate (100 µg.ml^{-1}), penicillin G potassium (100 IU.ml^{-1}) and amphotericin B (2.5 µg.ml^{-1}) at 22 °C, responded and adapted to the culture, attached to the bottom of the culture vessel, proliferated and secreted steroids, T, E2, P4 and 17αOHP.

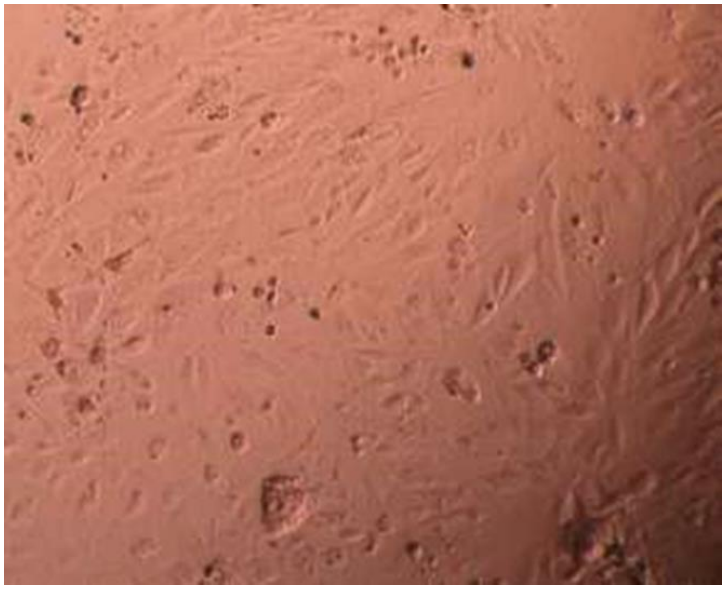

Fig. 6. Fibroblast - like cells after four days (× 300).

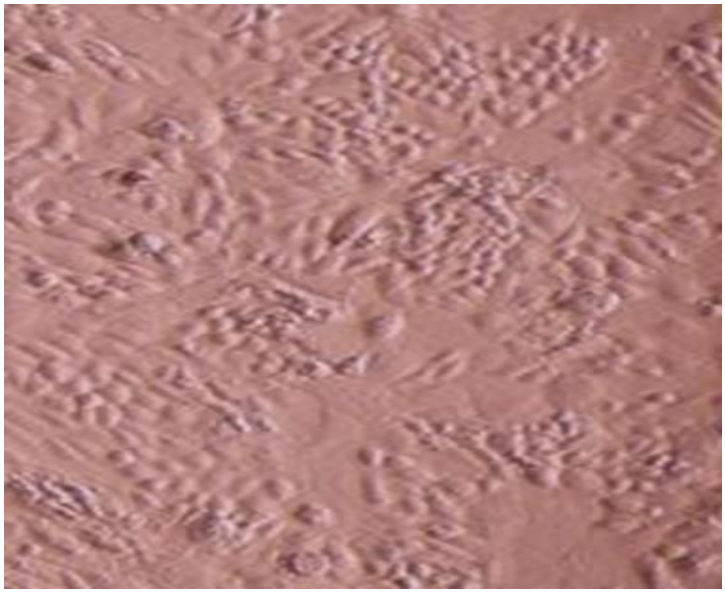

Fig. 7. Fibroblast - like cells after a week (× 120).

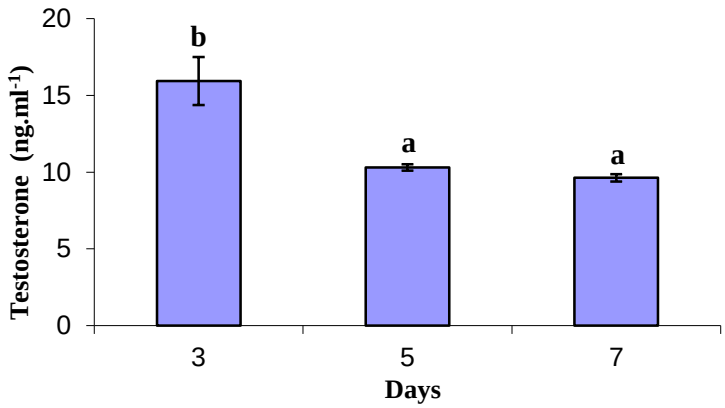

Fig. 8. Concentrations of T in the follicular cells culture medium (as mean ± SEM; P =0.700).

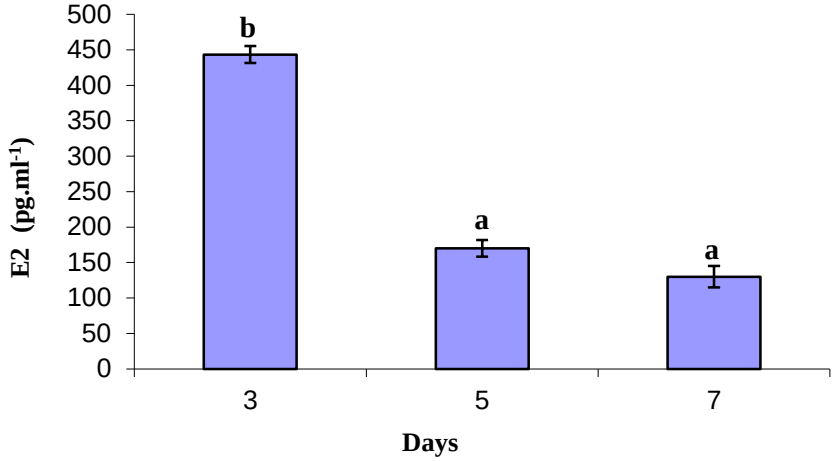

Fig. 9. Concentrations of E2 in the follicular cells culture medium (as mean ± SEM; P =0.001).

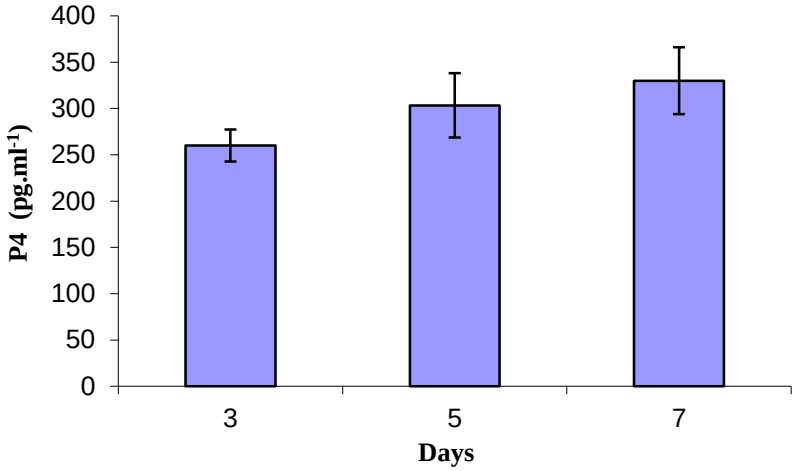

Fig. 10. Concentrations of P4 in the follicular cells culture medium (as mean ± SEM; P =0.010).

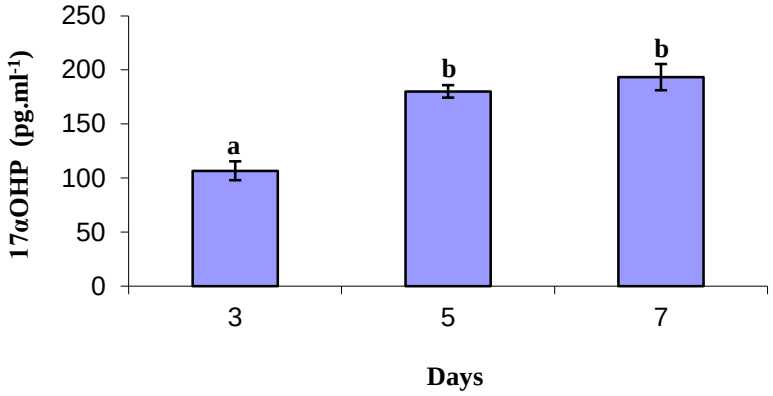

Fig. 11. Concentrations of 17α-OHP in the follicular cells culture medium (as mean ± SEM; P=0.287).

DISCUSSION

Primary culture of ovarian follicular cells was previously reported in common carp, *Cyprinus carpio* (Epler *et al.* 1997; Galas *et al.* 1999; Salamat *et al.* 2010) and European sea bass, *Dicentrarchus labrax* (Crespo *et al.* 2012). Epler *et al.* (1997) cultured interstitial cells of carp ovary and follicular cells isolated from the carp oocytes in

medium M199 and compared the levels of secreted hormones, P_4, E_2 and androgens (A_s) in the two seasonal periods pre-spawning, active time (April), and resting time (December). In another study Galas *et al.* (1999) cultured the follicular cells of common carp in culture medium 199 after isolating them and compared the seasonal variation of steroid secretions by the dispersed carp ovarian follicular cells as influenced by carp pituitary homogenate (CPH), human chorionic gonadotropin (HCG) and 17α, 20β-dihydroxy-4-pregnen-3-one (17,20β-P) and without the influence of these factors. Similarly Salamat *et al.* (2010) successfully cultured follicular cells of common carp in M199 and reported that isolated follicular cells continued to grow and proliferate in the culture medium and also E2 and 17αOHP were the main steroids secreted by the cultivated cells. Crespo *et al.* (2012) isolated ovarian follicular cells of European sea bass and set up a procedure to culture them to study their steroidogenic capacity at difference temperatures. All of these studies made employing of the medium 199 for culturing the follicular cells of the fish under study. However, in the present study, L-15 medium (Sigma) was employed to support follicular cells growth in medium without carbon dioxide equilibration. According to several researchers L-15 is the ideal culture medium for fish cell growth (Fernandez *et al.* 1993a; Sathe *et al.* 1995). In the present study, the isolated Sterlet ovarian follicular cells were successfully cultured and the cultivated cells secreted higher levels of T in the culture medium than the other hormones. In fish, T hormone is produced by theca cells. In most fish, follicular cells are made up of two layers, an outer thecal layer and an inner granulosa layer. These two layers of cells are separated by the basement membrane. The cells of these two layers show aromatase activity and the aromatizing potential of the granulosa cells is higher than that of theca (Kagawa *et al.* 1982b). Nagahama (1994) found that the theca and granulosa cells are the major sites of the production of steroid hormones. However it is difficult to recognize and differentiate these two types of cells in the culture medium (Galas *et al.* 1999; Galas & Epler 2002). Crespo *et al.* (2012) reported that granulosea cells were darker than theca cells. Stoklosowa & Epler (1985) after tripsinizing the ovarian follicles of common carp identified both types of cells, theca and granulosa, in the cell suspension. Darker cells with stronger aromatase activity were identified as granulosa cells, and lighter cells with weaker enzyme activity were reported as theca cells. In the present study, although large and small cells were observed among the follicular cells, it was not possible to clearly distinguish theca cells from granulosa cells in the culture medium. The production of T by follicular cells in the culture medium was more than that of other hormones. At the beginning of vitellogenesis, T is initially synthesized by the theca cells, and is later transformed to E_2 in the granulosa cells under the influence of the enzyme P_{450} aromatase (Kagawa *et al.* 1983). This hormone is then transported to the liver in the bloodstream where it is transformed into vitellogenin. From the liver, vitellogenin circulates in the bloodstream and reaches the ovaries where it is incorporated in the developing oocytes (Patino & Sullivan 2002). Early vitellogenesis is accompanied by an increase in T secretion by follicular cells. Plasma T levels in fish increase during this stage and decrease at the end of vitellogenesis. However plasma T levels remained higher than other steroid hormones until the end of vitellogenesis, before oocyte maturation (Webb *et al*. 2001; Barannikova *et al.* 2002a), and even until the beginning of follicular atresia and oocyte degeneration (Webb *et al.* 2001; Barannikova *et al.* 2002b; Linares- Casenave *et al.* 2002). In addition to being the precursor to E2, Testosterone has a feedback effect on the H-P-G axis (Bukovskaya *et al.* 1999; Safi *et al*. 1999; Bayunova *et al.* 2002; Ceapa *et al*. 2002), and also plays an important role in inducing maturation (Kime, 1993) and germinal vesicle breakdown (GVBD) in sturgeon (Semenkova *et al.* 2006). Therefore the plasma levels of this hormone after vitellogenesis have been reported to be

higher than those of other sex steroids including E2 in sturgeon as well as in white sturgeon (Webb *et al.* 2001; Webb *et al.* 2002).

It appears that testosterone remains stored in follicular cells before employing (Epler *et al.* 1997).

Sampling of Sterlet oocytes in the present study was carried out in late March 2015 when oocytes were at the end of vitellogenesis (post vitellogenesis) or before final maturation (PI>10) and this may explain the higher levels of T as compared to E2 in the culture medium. In the present study, the levels of T and E2 at the first 48 h were higher than at the following days.

However the levels of these hormones decreased from day 3, with T secretions decreasing faster than E2. In addition, decrease in hormone secretions on days 3 and 4 were greater than that on days 5 and 6. In all other teloests as in vertebrates (Stoklosowa & Epler 1985; Benninghoff & Thomas 2006) the secretions of T and E2 by theca and granulosa cells gradually decreased from the beginning of primary culture due to the decreased aromatase potential of follicular cells (Stoklosowa *et al.* 1982).

Similar results were reported in common carp by Epler *et al*. (1997) who found that the aromatase activity of follicular cells decreased from day 4 through 6 of culture.

They believed that follicular cells dedifferentiated and lost their characteristic secretory pattern.

Increased levels of P4 and 17αOHP in the culture medium from day 3 onwards seems to be associated with the role of these hormones in inducing oocyte maturation.

Research on several sturgeon species has shown that P4 exhibits a strong potential to induce oocyte maturation *in vitro* (Lutes 1985; Distaff *et al.* 1993; Williot *et al.* 1997; Mojazi Amiri 1999a). Semenkova *et al.* (2006) reported P4 to be the most effective sex steroid hormone in inducing final oocyte maturation in sterlet sturgeon with levels reaching 3.3 times the initial level during final maturation.

Galas *et al.* (1999) reported ovarian follicular cells of common carp in culture secreted steroid hormones for 48 hours *in vitro* in a manner reflecting *in vivo* pattern at the time of cell harvest, similar to the data obtained from the culture cells in mammals (Szoltys *et al.* 1982). Similarly, Galas *et al.* (1999) reported that the *in vitro* changes of steroid fluctuations by ovarian follicular cells in common carp during different seasons were similar to the annual fluctuations described in the blood plasma and ovarian tissue homogenates by Galas & Bieniarz (1989). The ovarian follicular cells isolated in the present study belonged to post vitellogenesis and hormonal data suggest that the steroid secretions by follicular cells in culture *in vitro* are similar to the pattern reported *in vivo*.

The secretion of T and E_2 was high for 48 hours and decreased from day 3, while secretion of T remained higher than the other sexsteroid hormones. However, levels of P4 and 17αOHP gradually increased after day 2.

Although several studies have been conducted on oocyte endocrinology and the effect of various factors including steroid hormones on the *in vitro* oocyte maturation in various sturgeon species including white sturgeon, *Acipenser transmontanus* (Webb *et al*. 2000); A hybrid sturgeon, Bester (Mojazi Amiri *et al.* 2001; Baranikova 2006; Smenekova *et al.* 2006; Goncharov *et al.* 2009; Azarine *et al.* 2013); Persian sturgeon *Acipenser persicus* (Taneh *et al.* 2012; Azarine *et al.* 2013), but there was no report on the primary culture of isolated ovarian follicular cells in sturgeons.

CONCLUSION

In the present study, the follicular cells of sterlet sturgeon cultured in the supplemented L-15 medium adapted well as an adherent monolayer culture, remained viable and continued to secrete steroid hormones into the culture medium.

These results indicate we have successfully developed an *in vitro* homologous system for follicular cells of oocyte in sterlet sturgeon that resembles the *in vivo* situation.

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کشت اولیه سلول‌های فولیکولی تاسماهی استرلیاد *Acipenser ruthenus* به منظور ایجاد یک سیستم آزمایشگاهی

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چکیده

هدف از انجام این تحقیق ایجاد یک سیتم آزمایشگاهی (*in vitro*) برای بررسی عملکرد سلول‌های فولیکولی تخمک تاسماهی استرلیاد *Acipenser ruthenus* بود. تخمک‌های مورد نیاز برای کشت اولیه از تخمدان یک عدد ماهی استرلیاد ۶ ساله پرورشی به وزن ۷۲۹ گرم و به طول ۴۷ سانتی متر به دست آمد. تخمک‌ها در مرحله پیشرفته ویتلوژنز بودند (PI < ۱۰). یک قسمت از تخمدان (دارای حدود ۳۰۰ عدد تخمک‌ها) از تخمدان خارج شد، تخمک ها از بافت های بنابینی جدا شدند و با محلول بافر نمکی فسفات (PBS) استریل حاوی آنتی‌بیوتیک‌ها و آمفوتریسین B شستشو شدند. سلول‌های فولیکولی از طریق تیمار تخمک‌ها با محلول trypsin-EDTA (۰/۲۵٪) حل شده در PBS عاری از یون‌های کلسیم و منیزیم جدا شدند و در محیط کشت L-15 حاوی ۲۰٪ سرم جنین گاو (FBS)، استرپتومایسین سولفات ($\mu g.ml^{-1}$ ۱۰۰؛ Gibco)، پنی‌سیلین پتاسیم G ($IU.ml^{-1}$ ۱۰۰؛ Gibco) و آمفوتریسین B ($\mu g.ml^{-1}$ ۲/۵؛ Gibco) تحت دمای ۲۲ درجه سانتی - گراد کشت داده شدند. غلظت هورمون‌های تستوسترون (T)، ۱۷- بتا استرادیول (E2)، پروژسترون (P4) و ۱۷- آلفا هیدروکسی پروژسترون (17αOHP) موجود در محیط کشت در روزهای سوم، پنجم و هفتم با استفاده از روش ELISA اندازه‌گیری شدند. بر اساس نتایج به دست آمده، سلول‌های فولیکولی تکثیرشده تاسماهی استرلیاد در محیط کشت L-15 از نظر تولید هورمون‌های استرویید فعال بودند بطوری که این موضوع با ترشح هورمون‌های T، E2، P4 و 17αOHP نمایان شد. هورمون T بیشترین مقدار هورمون ترشح شده توسط سلول‌های فولیکولی کشت داده شده بود که نشان دهنده اواخر مرحله ویتلوژنز در تخمک‌های جدا شده بود. کاهش تولید این هورمون‌ها طی روزهای سوم و چهارم بیش از روزهای پنجم و ششم بود. با کشت موفقیت‌آمیز سلول‌های فولیکولی تخمک تاسماهی استرلیاد در محیط کشت L-15 یک سیستم آزمایشگاهی (*in vitro*) ایجاد شد که انجام مطالعات عملکردی را مقدور می‌سازد و شبیه شرایط این سلول‌ها در تخمک‌ها است.

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