A Comparative study of synthetic carp GnRH and carp pituitary homogenate effects on in vitro steroidogenesis of oocytes in common carp (*Cyprinus carpio*)

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Abstract

Vitellogenic stage carp ovaries were collected aseptically and the cells of ovarian follicles were dispersed by trypsin treatment and cultivated as monolayer. Control cultures were grown in medium M199 alone. Media for experimental cultures were supplemented either with 100 µl ml^{-1} of CPH and CPS-carp GnRH (cultivated pituitary cell secretion affected by 10^{-6}, 10^{-8}, 10^{-10} M carp GnRH). The direct effect of these factors on 17-α-Hydroxy progesterone (17α -OHP) and 17β-Estradiol (E_2) secretion of isolated follicular cells in cell culture was assayed by appropriate Radioimmunoassay (RIA). According to the results the E2 was a main hormone secreted by cultivated carp granulosa and theca cells. Adding 100 µl $ml^{-1}$of 10^{-6} M CPS-carp GnRH had no significant effect on cultivated granulosa and theca cells endocrine activity ($P> 0.05$). On the other hand, carp granulosa and theca cells treated with the lower contents of CPS-carp GnRH (10^{-8}, 10^{-10} M) showed a 4.02 and 3.74-fold increase in E_2 concentration and 4.27 and 3.64-fold increase in 17α -OHP level, which was significantly higher than CPH ($P< 0.05$). Also, there was a significant increase of steroid secretion in group stimulated with 100 µl ml^{-1} of CPS and CPH, but there were no significant differences between them ($P> 0.05$).

Keywords: *Cyprinus carpio*, Cell culture, carp GnRH, Gonadotropin hormones, Steroid hormones

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Introduction

It is well known that the reproductive process in teleosts, as in other vertebrates is mainly regulated by the endocrine system, the brain- pituitary- gonad (BPG) axis. Gonadotropin releasing hormone (GnRH) produced in the brain governs the release of gonadotropin from the pituitary and is one of the primary factors in BPG axis (Kumakura et al., 2004). Two types of gonadotropins (GtH-I, GtH-II) regulate gonadal activity such as gametogenesis and steroiogenesis (Nagahama, 2000). Many fish exhibit reproductive dysfunctions when reared in captivity. Most commonly, females fail to undergo final oocyte maturation (FOM), and thus ovulation and spawning (Peter et al., 1993). Manipulations of various environmental parameters such as temperature, photoperiod, salinity, etc can not always improve the reliability of spawning, and hormonal treatments are the only means of controlling reproduction reliably (Yaron, 1995; Zohar and Mylonas, 2001). Using pituitary homogenate was the first method of spawning induction. Pituitary homogenate contains two fish gonadotropin (GtH-I, GtH-II) and is known to be responsible for estradiol-17 β production, stimulating in turn 17α, 20β OH-P secretion (Tyler et al., 1991) which stimulated germinal vesicle break down (GVBD) in Trichogaster ovaries during reproductive season (Degani and Boker, 1992). On the other hand, the success of this method is quite variable (Weil et al., 1986). The other approach is the stimulation of spawning by a synthetic superactive analogue, to release the endogenous gonadotropine (GtH) from the pituitary of treated fish (Zohar, 1989). To facilitate the GtH releasing activity of GnRHa, it is necessary to combine it with a dopamine receptor antagonist such as domperidone, pimozide or metoclopramide (Peter et al., 1988). Follicular cells around the oocyte are a good model for the investigation of hormonal events in the ovary. The objectives of the present experiment were to investigate the effects of carp GnRHa (synthetic carp GnRH) (containing domperidone) on cultivated pituitary cells secretion and sensitivity of ovarian follicular cells to these secretions compared to CPH (carp pituitary homogenate), expressed in terms of steroid secretion.

Materials and methods

An experiment was conducted in Medical Cell Culture Research Center of Jondi Shapour Medical Science University in February 2008. All fishes were raised in the Shahid Maleki fish farm, Ahvaz, Iran. They were netted from the outdoor ponds and transferred to the flow-through basins containing 300 liter water at 18-20 ^{o} C a month before the experiment.

Reagents

Synthetic carp GnRH (carp GnRHa ethylamide combined with dopamine antagonist, domperidone, diluted in propylene glycol as a solvent) was supplied by Samen pharmacy; IRAN, M199 (Medium 199), MEM Eagle (Minimum Essential Medium Eagle with Non-essential amino acids, Earle's salts and Glotamin), Hepes, trypsin, collagenase

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H, BSA (Bovine Serum Albumin), antibiotics (Penicillin and streptomycin) and antimycotic (Fungizone) were obtained from Sigma (Sigma-Aldrich; USA). The 96-well and 24-well microplates were from Nunc A/S; Denmark.

Pituitary primary cell culture

Pituitary primary cell culture was performed according to the method described by Weil et al. (1986) for trout and adapted by Mikolajczyk et al. (1990) for carp. At the day of experiment, five sexually mature male common carp (2 ± 0.5 kg, 2-3 years old) were euthanized with MS222 (200 ppm) and after taking off the roof of cranium and moving the brain and its surrounding fat, the pituitary glands were collected aseptically (under hood class II) and placed in sterile ice-cold MEM buffered with 15mM Hepes and 9mM bicarbonate (osmotic pressure of 275 mOsmol /kg and pH 7.7). The pituitary cells were dissociated by 1% collagenase H solution for 2-3 hr at 20^{o} C. The cells were harvested by 10 min centrifugation (200 g) at 20^{o} C and washed twice with pre-incubation medium containing FBS (2%) and antibiotic-antimycotic (1%) solution. The cells viability assessed by trypan blue exclusion test was 95%. They were re-suspended in incubation medium and transferred into a 96-well microplate (approximately 5×10^{4} cells / 250 µl of medium / well). The plate was incubated for 72 hr at 22^{o} C. Then the media called CPS (Cultivated pituitary cell secretion) were collected after centrifugation (200 g for 10 min) and frozen at -20^{o} C until use. Thereafter the cells were washed twice with 1 ml PBS/ well and the medium containing tested concentration of carp GnRH (10^{-10}, 10^{-8}, 10^{-6} M) was added. The wells containing control group received culture media alone. The cells were incubated for another 24 hr at 22^{o} C. At the end of this period the media were collected and frozen at -20^{o} C until use (Mikolajczyk et al., 1990).

Isolation of follicular cells

The procedure of ovarian follicular cells isolation followed the protocol used by Galas and Epler (2002). At the day of experiment, five 4-year old female common carp spawners (4.5 ± 0.5 kg in vitellogenic stage [the maturity stage of female carps was previously identified using histological methods [Fig. 1]]) were euthanized with MS222 (200 ppm) and ovaries were aseptically (using sterile dissection devices under hood class II) removed after abdomen dissection. Ovaries were placed in a beaker containing sterile phosphate buffered saline (PBS). The connective tissue capsule of the ovary was manually removed and the tissue was transferred into another beaker containing medium 199 diluted 4:1 with sterile distilled water. Ovarian pieces were separated from all interstitial tissues so that single oocytes remained. The medium was decanted and then replaced several times to rinse free oocytes thoroughly. Owing to such procedure, separate oocytes surrounded only by the follicular layer composed of granulosa (G) and theca cells (T) were obtained. Follicles free from connective tissue, were trypsinized by a 0.25% solution of trypsin in Ca^{2+} and Mg^{2+} free PBS for 30 min and three

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changes of solution at 10-min intervals at 37°C. Trypsin action was inhibited by the addition of FBS (fatal bovine serum). Supernatant fractions, containing released cells were pooled, centrifuged and the pellet was re-suspended in the medium 199 and diluted to a concentration of 2×10^{6} cell / ml culture media. The viability of cells was checked by the trypan blue exclusion test and the cells proved to be 90% viable. Cells were grown as monolayers in a 24-well microplate in medium 199 supplemented with 10% FBS and penicillin (120 IU ml^{-1}) and streptomycin (0.1 ng ml^{-1}) (pH = 7.4) (2×10^{6} cell / 1 ml culture media /well) at 20°C and 5% CO_2 for 72 hr. The culture media were collected and replaced (after washing) with the medium containing 100 µl ml^{-1} of CPH, CPS and CPS-carp GnRH (10^{-10}, 10^{-8}, 10^{-6} M). The wells containing the control group received culture media alone. The plates were incubated for 24 hr at 20°C and then the media were collected and frozen at -20° C for further steroid analysis (Galas and Epler, 2002).

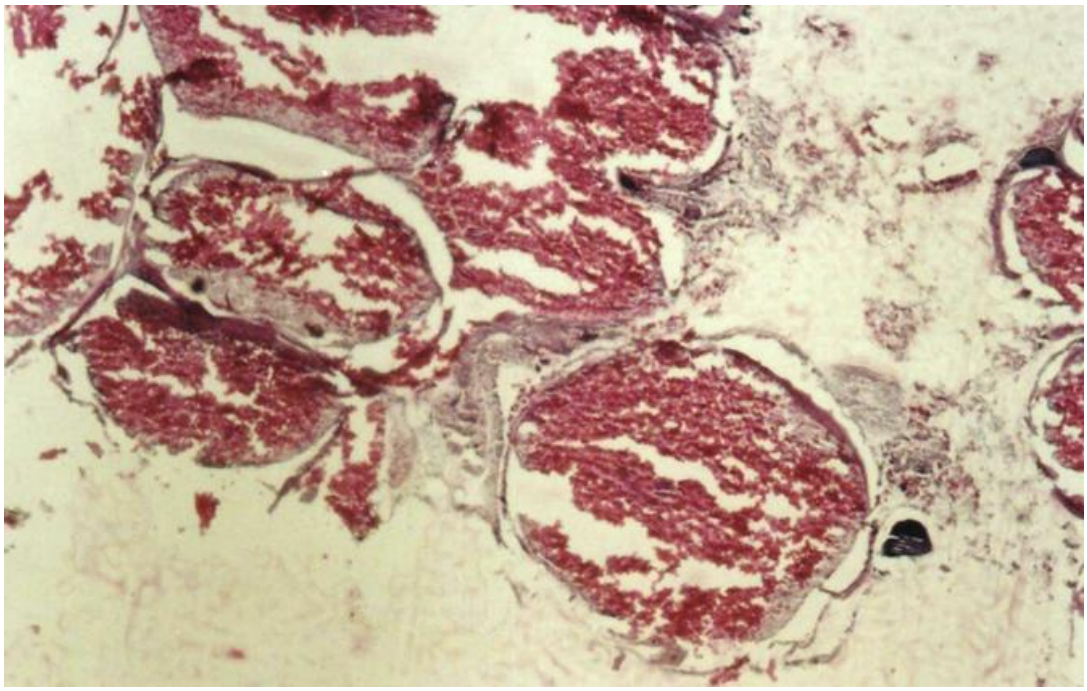


Figure 1: Common carp ovarian follicles in late vitellogenic stage (Hematoxilin & Eosin; ×20)

Steroid analysis

17-α-Hydroxy progesterone (17α-OHP) and 17-β-Oestradiol (E_2) were analyzed by radioimmunoassay described by Stoklosowa et al. (1982) in the Department of Physiology of Jondi Shapour Medical Science University, Ahvaz. The concentrations of steroids were computed in pg ml^{-1} of culture medium and were expressed as means ± SE.

17β-estradiol was determined using [2, 4, 6, 7-^{3}H] Estradiol (sp. Act. 100Ci/mmol: Immunotech, Beckman culture company, France) as a tracer and an antiserum raised in humans against oestradiol-17β-sodium azide, which was highly specific for oestradiol. Extremely low cross reactivity was obtained against other steroids (estrone, estriol, cortisol,

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cholesterol, progesterone, androsterone…) (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^{-1}. 17α-OHP was estimated using 17-α-hydroxy progesterone (sp. Act. 100Ci/mmol: Immunotech, Beckman culture company, France) as a tracer and an antibody raised in humans against 17α-hydroxy progesterone- sodium azide-BSA (bovine serum albumin). The limit of the standard curve was between 46-50000 pg ml^{-1}. 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 (Stoklosowa et al., 1982).

Statistical method

There were 6 groups all together: 1. The control group, 2. The group with CPH, 3. The group with CPS, 4. The group with CPS + carp GnRH 10^{-10} M, 5. The group with CPS + carp GnRH 10^{-8} M, 6. The group with CPS + carp GnRH 10^{-6} M. Two steroid hormones (17α-OHP and E_2) were measured in all groups. The normality of the data was determined using a test of normality (shapiro wilk) and then data were analyzed by a one way analysis of variance (ANOVA) and the significance of the differences between the means was determined using an LSD test.

Results

In order to perform ovarian follicular cell culture, the large follicles (1- 1.5 mm in diameter) with vitelogenic central oocyte and two surrounding layers, granulosa (G) and theca (T) cells, were selected. During trypsinization, follicular theca and granulosa layer easily detached from the follicles and relatively few follicles were damaged during enzyme treatment, therefore the contamination of cell suspensions with yolk was negligible. 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). The cells were proliferated and quickly became confluent. Cultures reached almost 100% confluences after 3 days (Fig. 2).

Results showed that the E_2 and 17α-OHP secretion of ovarian follicular cells were 350.8 ± 6.208 and 41.720 ± 3.622 pg ml^{-1} in the control group, respectively (Fig. 3). Adding 100 µl ml^{-1} of CPH increased E2 and 17α-OHP secretion of ovarian follicular cells significantly in comparison with the control group (976.2± 9.645 and 92.1742 ± 4.915 pg ml^{-1}, respectively) ($P<0.05$, Fig. 3). In the cultivated granulosa and theca cells treated by 100 µl ml^{-1} of CPS, the E_2 and 17α-OHP concentrations were 1005 ± 58 and 88.875 ± 8.3 pg ml^{-1} respectively, which didn't cause any significant difference compared to the CPH treated group ($P>0.05$, Fig. 3).

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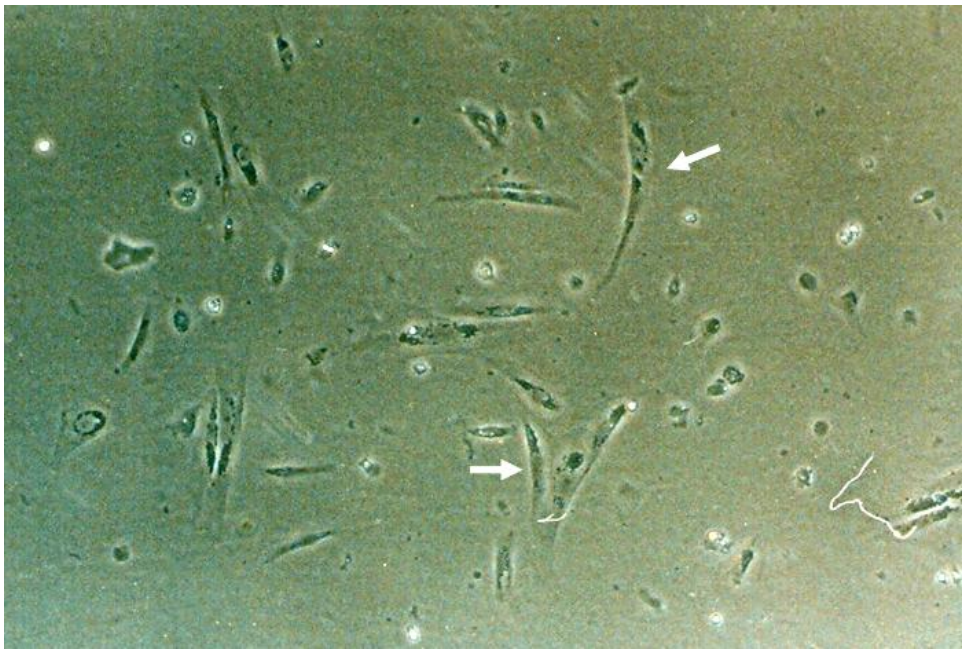


Figure 2: The second day culture. The fibroblast- like cells were attached to the bottom of culture vessels after 24 hr of plating (×20)

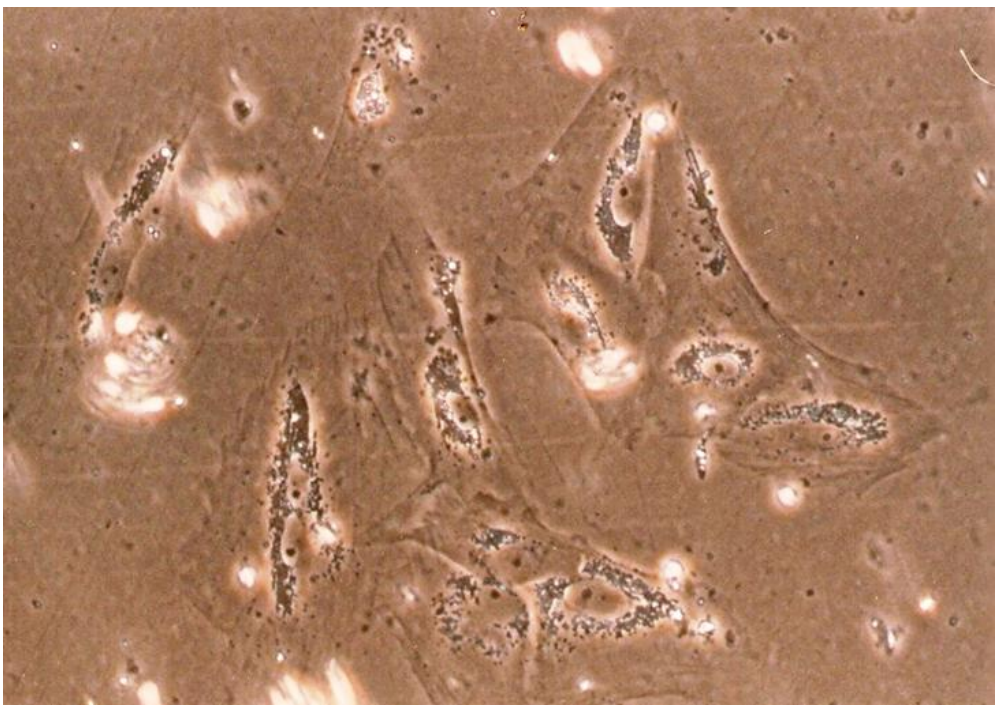


Figure 3: The third day culture. The fibroblast- like cells proliferated and reached almost 100% confluences after 72 hr of plating (×40)

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The addition of 10^{-6} M CPS-carp GnRH (CPS treated with 10^{-6} M carp GnRH) had no effect on endocrine activity of cultivated granulosa and theca cells (P>0.05, Fig. 3). On the other hand, the lower contents of carp GnRH (10^{-8}, 10^{-10} M) caused the significant increase of E_2 and 17α-OHP levels in comparison with the other groups (1413 ± 24.392 and 178.44 ± 6.271 pg ml^{-1}, 1312.6 ± 44.136 and 152 ± 11.903 pg ml^{-1}, respectively) (P<0.05, Fig. 3), but had no significant difference with each other (P>0.05, Fig. 3).

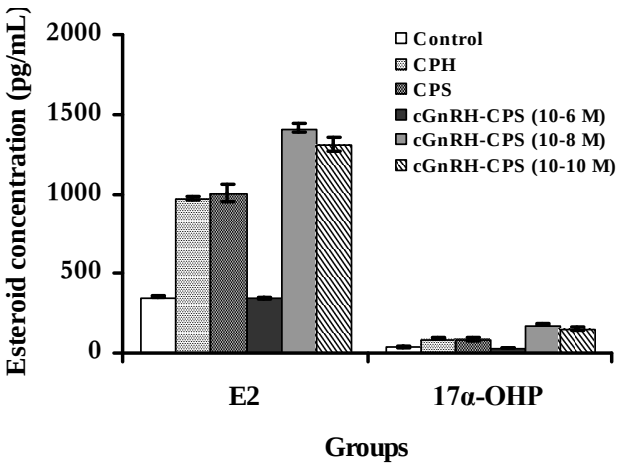


Figure 4: Concentrations of E_2 and 17α-OHP in the follicular cells culture medium as mean ± SE.

Discussion

The present study allows the investigation of secretory potency of isolated components of the ovary apart from the complexity of the organ. On the basis of the studies which showed that cultivated ovarian follicular cells secreted steroid hormones (Nagler and Idler, 1992: Mugnier et al., 1997; Galas et al., 1999) in vitro steroid secretion by isolated carp ovarian follicular cell was studied.

In the present study E_2 was a dominant hormone secreted by carp ovarian follicular cells to the culture medium. This high level of E_2 was probably connected with ovulation. Also Galas et al., (1999) showed E_2 secretion by carp follicular cells was especially significant in February, May, September and December, which closely related with the spawning and vitellogenesis periods. As about 17α-OHP, an important metabolite in steroidogenesis, its maximum often occurred in April and May, which might be related to its intense conversion to 17α, 20βOH-P essential for final oocyte maturation (Venkatesh et al., 1992). The necessity of using an inducing agent such as CPH, HCG and GnRHa for spawning induction has been demonstrated in cyprinid fish such as common carp (Peter et al., 1988). Results showed 2.7-fold increase in E_2 and 2.2-fold increase of 17α-OHP secreted from follicular cells treated by Carp pituitary homogenate

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(CPH). The results obtained previously by Galas et al., (1999) are in agreement with this reported in the present work. The crude carp pituitary contains both gonadotropins GtH-I and GtH-II (Quesnel and Breton, 1995) and is extensively used for inducing spawning in fish hatcheries especially in developing countries. On the other hand, use of ground pituitaries is associated with various drawbacks, the most important ones being (a) expensive, (b) not always readily available, (c) having unpredictable activity (Drori et al., 1994). Furthermore, there is a possibility of diseases transmission from the donor fish to recipient broodstocks (Zohar and Mylonas, 2001).

The higher steroid secretion rate was seen in cultivated carp follicular cells which received CPS-carp GnRH compared to CPH treated cells. This result was in accordance with that obtained by Behl (2006) who reported that the increased level of steroidal secretion can be taken to be an indicator of enhanced steroidogenesis in carp follicular cells treated with GnRH- pituitary homogenate in vitro.

However, as it seems, carp GnRH in the highest concentration (10^{-6} M) blocked the gonadotropine secretion from cultivated pituitary cells, because adding 100 µl ml^{-1} of 10^{-6} M CPS-carp GnRH had no significant effect on cultivated granulosa and theca cells endocrine activity. It may be caused by down-regulation of GnRH hormone receptors on the pituitary cells (Ostrander, 2000). When a hormone or neurotransmitter is present in excess (as the high concentration of carp GnRHa, which we used in this experiment) the number of active receptors generally decreases (down- regulation), whereas in the presence of a deficiency of the chemical messenger, the number of active receptors is increased (up-regulation) (Ganong, 1997). According to our results, carp granulosa and theca cells treated with the lower contents of CPS-carp GnRH (10^{-8}, 10^{-10} M) showed a 4.02 and 3.74-fold increase in E_2 concentration and 4.27 and 3.64-fold increase in 17α-OHP level, which was significantly higher than CPH. Dorafshan et al. (2003) reported the higher spawning rate in fish receiving GnRHa + domperidone compared to CPE. Also, Szabo et al. (2002) pointed out that using GnRHa + domperidone in order to induce ovulation in nase (*Chondrostoma nasus*) resulted in a higher fertilization rate as compared to pituitary injected fish.

Using GnRH agonists for hormone therapies, repairs the endocrine disruption that results in the failure of captive fish to undergo FOM, ovulation and spawning, by inducing the release of endogenous LH (Sherwood et al., 1994; Zohar and Mylonas, 2001). On the other hand, to facilitate the GtH releasing activity of GnRHa, it is necessary to combine it with a dopamine receptor antagonist such as domperidone (Peter et al., 1988) which is known as the Linpe method.

In summary, this study demonstrated that the culture of ovarian follicular cells with new Iranian made

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GnRHa (carp GnRH) containing domperidone resulted in a more pronounced effect on in vitro ovarian steroidogenesis and it could be a reliable procedure for in vivo induction of ovulation and spawning. However it is necessary to conduct more research to examine the effects of carp GnRH on spawning rate, latency period, working fecundity and embryo viability.

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مطالعه مقایسه ای اثرات carp GnRH سنتزی و مخلوط هموژن هیپوفیز ماهی کپور بر استروئیدوژنز اووسیت های کپور معمولی *Cyprinus carpio* در شرایط آزمایشگاهی

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چکیده

تخمدانهای ماهی کپور معمولی در مرحله ویتلوژنی در شرایط استریل خارج و سلولهای فولیکولهای تخمدانی با استفاده از تریپسین جداسازی شده و به صورت تک لایه کشت داده شد. سلول های کشت داده شده گروه کنترل در محیط M199 کشت داده شدند. محیط کشت گروه های آزمایش توسط $\mu l\ ml^{-1}$ ۱۰۰ از CPH و carp GnRH (ترشحات سلول های هیپوفیز کشت داده شده با M carp GnRH $۱۰^{-۶}$، $۱۰^{-۸}$ و $۱۰^{-۱۰}$) غنی سازی شد. اثر مستقیم این فاکتورها بر ترشح ۱۷-آلفا- هیدروکسی پروژسترون (17α -OHP) و ۱۷بتا-استرادیول (E_2) توسط سلول های فولیکولی جداشده در محیط کشت سلولی با استفاده از روش رادیوایمونواسی مورد بررسی قرار گرفت. با توجه به نتایج E_2 هورمون اصلی ترشح شده توسط سلول های گرانولوزا و تکا کپور در محیط کشت بود. افزودن $\mu l\ ml^{-1}$ ۱۰۰ از محلول $۱۰^{-۱۰}$ مولار CPS-carp GnRH تاثیر معنی داری بر فعالیت آندوکرینی سلول های گرانولوزا و تکا کپور کشت داده شده نداشت ($P> 0.05$). از طرف دیگر، سلول های گرانولوزا و تکا کپور متاثر شده توسط غلظت های کمتر CPS-carp GnRH (M $۱۰^{-۶}$، $۱۰^{-۸}$) افزایش ۴/۰۲ و ۳/۷۴ برابری در غلظت E_2 و افزایش ۴/۲۷ و ۳/۶۴ برابری در غلظت 17α -OHP را نشان دادند که به طور معنی داری نسبت به CPH بیشتر بود ($P< 0.05$). همچنین ترشح استروئیدی گروه تحریک شده توسط $\mu l\ ml^{-1}$ 100 از CPS و CPH نیز افزایش معنی داری داشت ولی بین این دو گروه تفاوت معنی داری مشاهده نشد ($P> 0.05$).

واژگان کلیدی: *Cyprinus carpio*، کشت سلول، carp GnRH، هورمون های گنادوتروپینی، هورمون های استروئیدی

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