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Effects of cholesterol, FSH and LH on steroidogenic activity of cat granulosa cells cultured *in vitro*

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Abstract

The aim of this study was to examine the effects of 22R-hydroxycholesterol (22R-HC), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) on estradiol and progesterone production by cat granulosa cells. Granulosa cells from follicles were collected and cultured for up to 5 days in 24 well plates containing Dulbecco's Modified Eagle's Medium (DMEM)/HAM F-12 supplemented with 10^{-7} M androstenedione, 0.1% ITS premix and 0.1% bovine serum albumin, in the presence or absence of 22R-HC (10 µg/ml), FSH or LH (10, 100 ng/ml each) on first and third day. Additionally, 5% fetal calf serum was added into the culture medium for the first 24 h. Treatment of cells with 22R-HC resulted in an increase ($P < 0.05$) in progesterone and estradiol production on days 3 and 5 of the culture. Incubation of cells with FSH (10 and 100 ng/ml) resulted in significant stimulations of progesterone ($P < 0.001$) whilst incubation had no effect on estradiol production. None of the LH doses (10 and 100 ng/ml) had any effect on progesterone production by granulosa cells during the culture time. With the inclusion of 22R-HC into the culture system, progesterone synthesis was enhanced ($P < 0.001$) in the presence of all FSH doses.

Keywords: cat, estradiol, FSH, granulosa cell culture, LH, progesterone.

Introduction

The female cat is described as being seasonally polyestrous and an induced ovulator (Tsutsui and Stabenfeldt, 1993). Puberty in cats occurs between 4 and 12 months. This period changes depending on the breed, body weight and photoperiod (Scott, 1970; Jemmett and Evans, 1977; Lofstedt, 1982). The follicle plays a fundamental role in the female reproductive processes by producing steroids. For the most part, ovarian follicles in cats are similar to the other mammalian species. The estrous cycle generally consists of four stages, which are proestrous, estrous, diestrous and anestrous (Bristol-Gould and Woodruff, 2006). Follicular development coincides with the onset of the proestrous stage. A rise in serum estradiol levels secreted by the ovarian granulosa cells occurs when the follicles are enlarging (Shille *et al*., 1979). As the estrous stage develops, three to seven follicles become more dominant while the other developing follicles undergo atresia (Wildt *et al*., 1981; Feldman and Nelson, 1996).

Follicular growth and development are primarily regulated by gonadotropins, which are important for the follicles to achieve successful ovulatory size (Shille *et al*., 1979; Wildt *et al*., 1986). The follicle-stimulating hormone stimulates the synthesis of estradiol by granulosa cells (Richards *et al*., 1987). Estradiol hormone is synthesized from androstenedione, which converts from pregnenolone in theca cells. Pregnenolone is converted from cholesterol (22R-HC), which can be derived from either cellular synthesis or plasma lipoproteins (O'Shaughnessy and Wathes, 1985; McKee and McKee, 2003; Harvey *et al*., 2005). Estradiol and FSH stimulate granulosa cell proliferation and formation of LH receptors on granulosa cells. This LH receptor formation on the granulosa cells provides the responsiveness of the aromatase synthesis induced by both FSH and LH, and this leads to more estradiol production (Richards, 1994). Estradiol synthesis is an important sign that the follicle cells reached maturity (Hsueh *et al.*, 1984). Estradiol facilitates the effect of FSH and LH by stimulating proliferation of granulosa cells in the follicles (Richards, 1980). Additionally, progesterone is synthesized at low levels by granulosa cells in pre-antral folicles in response to FSH. Follicle-stimulating hormone provides this effect by increasing the secretion of the P450 enzyme (Richards *et al*., 1995; Hillier, 2001). A member of the cytochrome P450 superfamily of enzymes is the cholesterol side-chain cleavage (SCC) that plays a role to convert from cholesterol to pregnenolone, which is one of the first steps in mechanisms of progesterone synthesis. However, FSH also stimulates the synthesis of the 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme in the endoplasmic reticulum for converting pregnenolone to progesterone (Hsueh *et al*., 1984; Richards and Hedin, 1988). Thus, steroidogenic granulosa cells from selected dominant follicles can be identified by testing for 3β-HSD activity (Bao *et al*., 1995). Luteinizing hormone stimulates androgen secretion, which is the substrate for the production of estradiol. A threshold LH stimulation is required for adequate follicular development and maturation. The LH receptors can be detected in granulosa cells around the time of selection of the

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dominant follicles that can utilize LH for continued growth (Hillier, 2001; Webb *et al*., 2004). At the advanced follicular development, prior to onset of the mid-cycle LH surges that trigger ovulation and tonic stimulation of mature granulosa cells by LH mimic the FSH (Robker and Richards, 1998).

The relationship between these endocrine and ovarian changes *in vivo* is complex. Cell culture provides an approach through which the actions and interactions of specific factors can be determined. There are studies published on the steroidogenic responses of granulosa cells to FSH and LH under *in vitro* culture conditions in human and several animal species such as bovine, rats, rabbit and pigs (Bréard *et al*., 1998; Greisen *et al*., 2001; Shanmugam *et al*., 2010, 2013).

There has been lack of study on the culturing of cat granulosa cells. Therefore, the aims of this study were to establish a cell culture protocol for granulosa cells isolated from queens and investigate the effects of 22R-HC, FSH and LH on steroid production by cat granulosa cells.

Materials and Methods

Animals

Eighteen female adult animals who were submitted for ovariohysterectomy were borrowed from cat fanciers after they were informed regarding all treatments in this study. The animal care and use protocol was reviewed and approved by the Ethics Committee of the Kirikkale University (28.04.2010/03). Animals were randomly divided into three groups for three different protocols, each having six cats. Each protocol was repeated with six cats, which mean six different cultures. Estrous and superovulation were induced using a protocol that includes intramuscular administration of 250 IU equine chorionic gonadotrophin (eCG; Folligon, Intervet, The Netherlands) on day 1, and followed with 120 IU on day 2. Following the onset of estrous behaviours (counted as day 0) such as crouching, treading, affection, vocalization, rolling and lordosis (Lein *et al*., 1982), the ovaries were collected on day 5 of the estrous cycle by laparotomy operation in the all group of animals, respectively.

Isolation of granulosa cells

All chemicals used in cell preparation and incubation were obtained from Sigma Chemical Company (Sigma-Aldrich, Co., Munich, Germany). The ovaries were transferred to the laboratory under sterile conditions in DMEM/HAM F-12 medium containing antibiotic after laparotomy operation. The ovaries were kept at 4ºC until the collection of granulosa cells was completed. Afterwards, granulosa cell recovery was processed with some modifications of the method described by Glister *et al*., 2001. Briefly, the follicles were dissected from the ovaries. Then the larger follicles (2.5-3.5 mm) were hemisected in a petri dish containing phosphate buffered saline (PBS) following the aspirating of follicular fluid with a 24 - gauge needle attached to a 2 ml syringe. Granulosa cell layers were removed from the follicle wall by gently disrupting it with the aid of 0.1 µl inoculation loop. Cells harvested were pooled, pelleted by centrifugation at 400 x g for 10 min, and re-suspended in 1 ml PBS. Double-distilled water (1 ml) was added, and the cells were agitated for 10 sec to lyse red blood cells. Then, isotonicity was quickly restored by the addition of 4 ml of 0.1 M PBS. Granulosa cells were pelleted again by centrifugation at 400 x g for 10 min followed by removal of the supernatant. Granulosa cells were washed twice by re-suspending the pellet in 10 ml of 0.1 M PBS, centrifuging for 10 min at 400 x g followed by removal of the supernatant. Following the second 0.1 M PBS wash, granulosa cells were re-suspended in 1.2 ml of medium (DMEM/HAM F-12 supplemented with 10^{-7} M androstenedione, 0.1% ITS premix and 0.1% BSA, containing 5% FCS).

Cell number and viability were determined in a hemocytometer using trypan blue exclusion, and varied between 20 and 30% among the different experiments.

Incubation of granulosa cells

Granulosa cells were cultured in 24 well culture plates, in DMEM/HAM F-12 medium: supplemented with 10^{-7} M androstenedione, 0.1% ITS premix (1.0 µg/ml insulin, 0.55 µg/ml transferrin ve 0.5 ng/ml sodium selenite) and 0.1% BSA, containing 5% FCS. Between 10 and 20 x 10^3 viable cells in 50 µl were seeded into each well containing 450 µl of pre-equilibrated medium. The cells were then incubated in a humidified incubator containing 95% air and 5% CO_2 and cultured for up to 5 days. Cells were allowed to attach for the first 24 h when the medium was first replaced. Thereafter, the medium was changed every 48 h. Following the first day of the incubation, cells were incubated in serum-free culture medium throughout the rest of the culture.

The treatment of the cells was started with the first change of culture medium after 24 h. Three different protocols were used in the cell treatment. In the first protocol, the cells were treated with 10 µg/ml 22R-HC (Sigma-Aldrich, Co., Munich, Germany). In the second protocol, the cells were treated with 10 ng/ml and 100 ng/ml FSH (Sigma-Aldrich, Co., Munich, Germany) with and without 10 µg/ml 22R-HC. In the third protocol, cells were treated with 10 ng/ml and 100 ng/ml LH (supplied by the National Hormone and Pituitary Program of the NIDDK, CA, USA) with and without 10 µg/ml 22R-HC. Each protocol had double untreated control wells on the plates. Treatment doses were determined according to the previous studies

(Rouillier *et al*., 1996; Silva and Price, 2000; Arikan and Yigit, 2009). Six different cultures were repeated for each protocol, in which each treatment also consisted of two separate cell wells. The medium used was stored frozen at -20ºC until assayed for progesterone and estradiol by radioimmunoassay (RIA).

Concentrations of progesterone and estradiol in the conditioned medium were measured by radioimmunoassay using a RIA kit (Biosource Europe SA, Nivelles, Belgium) following manufacturer's instructions. The intra- and inter-assay coefficients of variation were <9.0%, respectively.

Staining of granulosa cells

At the end of 5 days incubation, cells on the culture dishes having steroidogenic activity were shown by staining them for 3β-HSD activity, as described previously (Bao *et al*., 1995; Arikan and Yigit, 2009). Briefly, after media was removed from the plates, the cells were incubated in paraformaldehyde (1%) for cell fixation for 20 min. Thereafter, the paraformaldehyde was then removed and added to 500 µl staining solution (0.1 M PBS containing 0.1% BSA, 1.5 mM NAD, 0.25 mM nitro blue tetrazolium and 0.2 mM 5-androstene-3-ol-17 one, which was prepared from 8 mM stock solution in ethanol). Finally, the plate was then incubated for 4 h in the dark, in a metabolic shaker at 37ºC (Julabo, Labortechnik GmbH, Seelbach, Germany).

Statistical analysis

All results were reported as means ± SEM of six different cultures for each group and were considered statistically significant when $P < 0.05$. Progesterone synthesis was expressed as ng /10.000 cells per 48 h. Estradiol production was expressed as pg/10.000 cells per 48 h. The number of cells was based on the cell numbers added to the culture at the beginning of each experiment. All statistical analysis was carried out using the SAS 8.02 statistical software (Inst. Cary NC, USA). Interactions were expressed in doses × time and the differences between times and hormone doses in absence or presence of cholesterol were assessed by General Linear Model (GLM) procedure followed by a Fisher's least significant difference (LSD) test for multiple comparisons when appropriate.

Results

Cell Staining

Incubated granulosa cells were stained before and after 5 days culture to show the steroidogenic activity. Following the colour reaction with 3β-HSD enzyme, the cells having steroidogenic activity were stained as black/blue dye formation. The degree of staining (size and darkness of black/blue deposits) was subjectively evaluated between control and 22R-HC group. The cells subjected to the 22R-HC were stained darker than control group (Fig. 1), which is consistent with a high progesterone concentration in the group treated with 22R-HC in Figure 2.

Additionally, this staining process helped us to monitor the cell attachment and growth during the incubation. As well as cell border, the nucleus of cells was also monitored. The round shape of cells was changed to elliptical during cell growth on the attached surface. Cell membrane protruded through the nearest cells during the cell growth (Fig. 1). In case of any cell damage, poor cell development could easily be monitored on the stained samples.

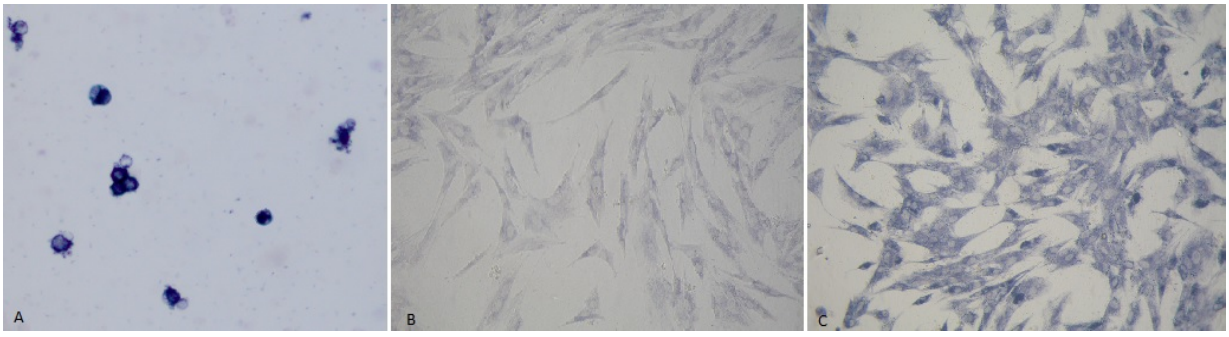

Figure 1. Cells stained for 3β-HSD activity. A: Before incubation B: Control group after 5 days incubation C: Cholesterol group after 5 days incubation on culture plate.

Cell treatments

Basal progesterone production by granulosa cells was not affected significantly ($P < 0.05$) from days 3 to 5 of the culture (Fig. 2). Basal estradiol production was found to be significantly higher ($P < 0.05$) on day 5 than on day 3 of the culture (Fig. 3).

Treatment of cells with 22R-HC resulted in an increase ($P < 0.05$) in progesterone and estradiol production on days 3 and 5 of the culture (Fig. 2, 3). When 22R-HC was used at a concentration of 10 µg/ml on days 3 and 5 of the culture, it resulted in 9.1 and 13.5 fold increase in basal progesterone production, and a 3.6 and 4.7 fold increase in basal estradiol production was

determined, respectively on days 3 and 5. Both estradiol and progesterone production was higher on day 5 than on day 3 of the culture of cells which were treated with 22R-HC (Fig. 2, 3)

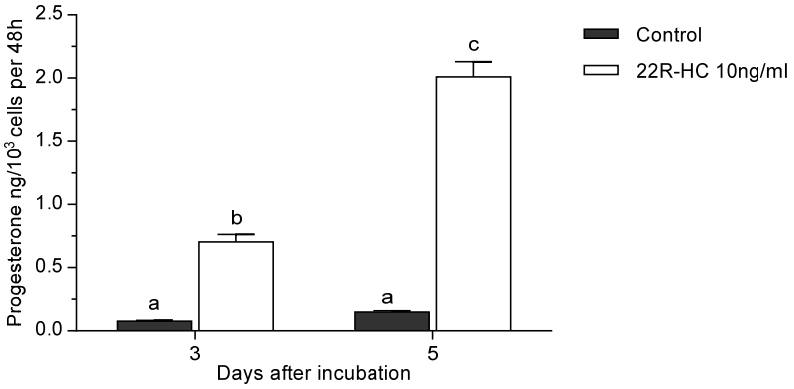

Figure 2. Effect of 22R-HC on bazal progesterone production by granulosa cells (Mean ± SEM). Groups with different letters are significantly different ($P < 0.05$).

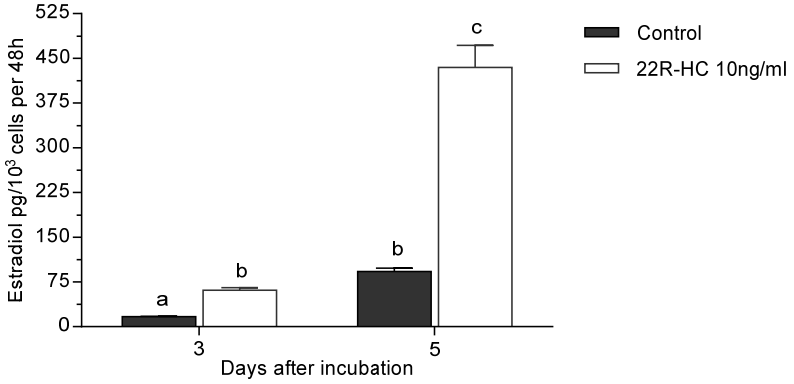

Figure 3. Effect of 22R-HC on bazal estradiol production by granulosa cells (Mean ± SEM). Groups with different letters are significantly different ($P < 0.05$).

Incubation of cells with both concentrations of FSH (10 and 100 ng/ml) resulted in significant stimulations of progesterone ($P < 0.05$) on day 3 and 5 while having no effect on estradiol production (Fig. 4, 5). With the inclusion of 22R-HC into the culture system, both doses of FSH further enhanced progesterone but not estradiol production ($P < 0.05$). Both progesterone and estradiol production in the cells treated with FSH and 22R-HC were higher ($P < 0.05$) on day 5 than on day 3 (Fig. 4, 5).

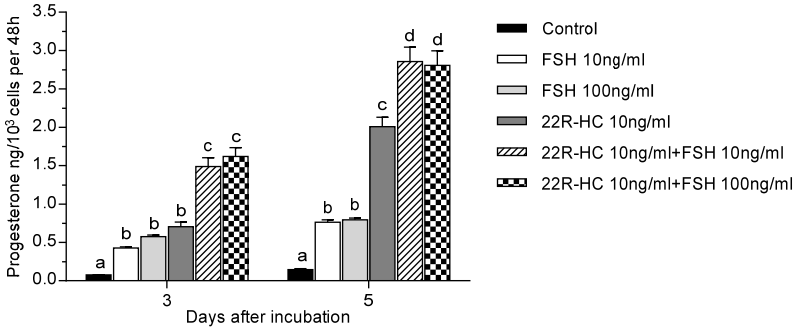

Figure 4. Effect of FSH on progesterone production by granulosa cells (Mean ± SEM). Groups with different letters are significantly different ($P < 0.05$).

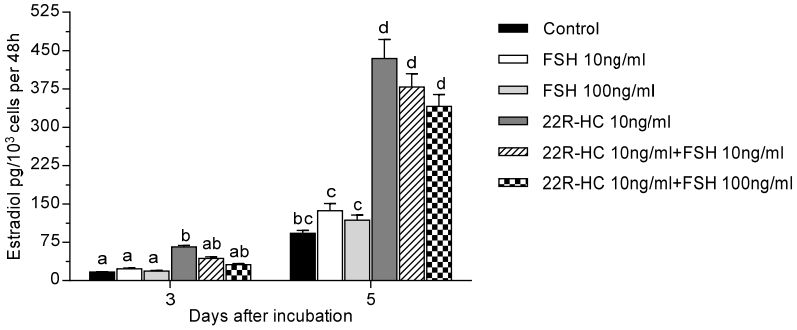

Figure 5. Effect of FSH on estradiol production by granulosa cells (Mean ± SEM). Groups with different letters are significantly different ($P < 0.05$).

None of the LH doses had any effect on estradiol production on day 3, or on progesterone production on day 3 and 5 by granulosa cells from the follicles (Fig. 6). However, on day 5, the higher dose of LH (100 ng/ml) resulted in significant stimulation on estradiol production (Fig. 7). With the inclusion of 22R-HC into the culture system, neither doses of LH further enhanced progesterone or estradiol production on day 3 and 5 ($P < 0.05$). Progesterone and estradiol production in the cells treated with LH and 22R-HC were higher ($P < 0.05$) on day 5 than on day 3 (Fig. 6, 7).

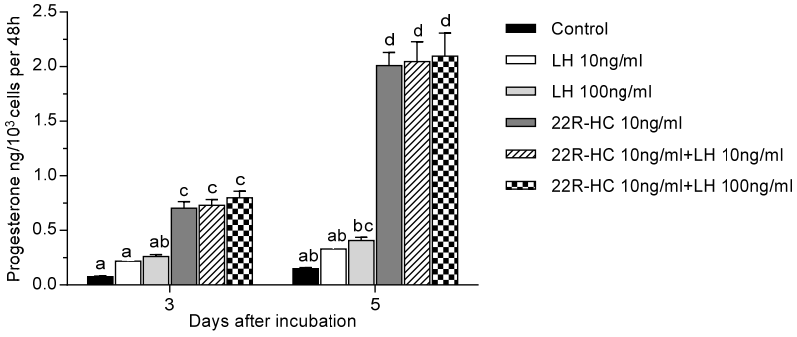

Figure 6. Effect of LH on progesterone production by granulosa cells (Mean ± SEM). Groups with different letters are significantly different ($P < 0.05$).

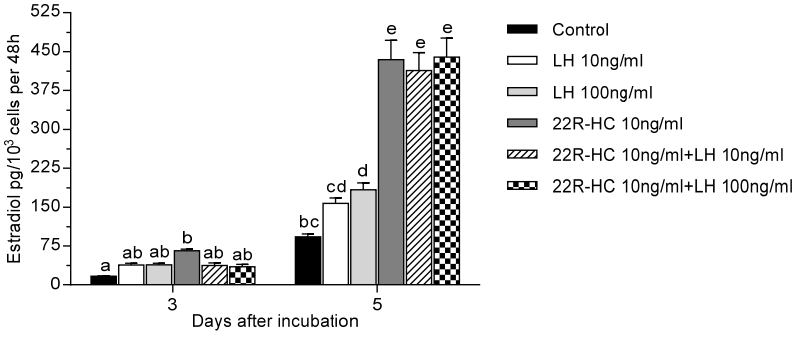

Figure 7. Effect of LH on estradiol production by granulosa cells (Mean ± SEM). Groups with different letters are significantly different ($P < 0.05$).

Discussion

To our knowledge, this is the first report on progesterone and estradiol production in short-term culturing of cat granulosa cells. In this study the effect of cholesterol, FSH and LH on steroidogenetic activity

in cat granulosa cells was evaluated.

In the present study, the level of basal estradiol production increased significantly during the culture procedures, which is not similar to the results of many preceding studies (Berndtson *et al*., 1995; Rouillier *et al*., 1996; Yang and Rajamahendran, 1998). There was not a decrease problem on estradiol production with time, unlike other authors reported before (Gutierrez, 1997; Shanmugam *et al*., 2010). The basal progesterone production was not affected significantly during the procedures of granulosa cell culture. This is not in accordance with the results of the earlier studies on granulosa cells collected from follicles in buffalo (Bhushan *et al*., 2004) and cattle (Silva and Price, 2000). The results in progesterone secretion can show us that granulosa cells were not in luteinisation process as designated by accompanying a constant increase in estradiol secretion during the course of culture.

Cholesterol is the precursor of steroid hormones so cholesterol supply is obviously a factor in the controlling of steroidogenesis. There is no report on the effect of 22R-HC on cat granulosa cells. This study demonstrates that incubation of cat granulosa cells with 22R-HC is effective to continue a high-level estradiol and progesterone secretion during short-term granulosa cell culture. The concentration of 22R-HC used in this study increased significantly ($P < 0.01$) the estradiol and progesterone secretion. Similarly, the effect of 22R-HC on progesterone secretion was reported in luteal cell culture studies carried out on feline (Arikan and Yigit, 2009), bovine (Arikan and Rodway, 2000) and ovine (Fitz *et al*., 1993). On the other hand, there are no studies in other species showing that 22R-HC was increased the estradiol production in granulosa cells. This could be due to a combination effect of 22R-HC and androstenedione used in estradiol production by granulosa cells, which needs to be clarified with a further molecular study.

Previous *in vitro* studies have shown that different doses of FSH had stimulated progesterone production by granulosa cells (Spicer *et al*., 1993; Silva and Price, 2000; Hooda and Yaday, 2002; Bhushan *et al*., 2004). The results of this study are consistent with Spicer *et al*. (1993), who reported that FSH-stimulated progesterone synthesis increased on day 4 of the culture in bovine granulosa cells. In another study, stimulatory effects of FSH (100 ng⁄ml) on progesterone production were showed by Basini and Tamanini (2000) at 48, 96 and 144 h in culture by granulosa cells. It has been found that progesterone production by bovine granulosa cells from all follicle size categories increased in response to FSH at dose levels of 1, 10 and 100 ng⁄ml in a 48 h culture, reported by Jimenez-Krassel and Ireland (2002).

In this study there was no effect of FSH on estradiol secretion by cat granulosa cells on days 3 and 5 of the culture in absence or presence of 22R-HC. There are not many studies reported on FSH-stimulated estradiol secretion by granulosa cells, whereas Gutierrez *et al*. (1997) reported a threefold increase in estradiol production after the addition of FSH to the granulosa cell culture from the bovine small follicles. However, Jimenez-Krassel and Ireland (2002) did not note any effect of FSH on estradiol production for short time culture. Similarly, there was not an effect of FSH in a study reported by Yang and Rajamahendran (1998).

A lack of effect of LH on estradiol on day 3 of culture and progesterone production on day 3 and 5 by cat granulosa cells is in agreement with a previous report on bovine granulosa cell culture (Yang and Rajamahendran, 1998). However, on day 5, the higher dose of LH (100 ng/ml) increased the estradiol production significantly, which is consistent with a study reported by Shanmugam *et al*., 2013. Although there was constant increase in estradiol secretion during the course of culture in this study, cat granulosa cells did not behave as *in vivo* responding to FSH and LH in terms of steroid production except on day 5 of culture. Equine chorionic gonadotrophin treated granulosa cells from mature selected follicles would be expected to respond less to FSH than to LH. It is intriguing that FSH enhanced progesterone but not estradiol production, whereas a high dose of LH (100 ng/ml) stimulated only estradiol production. If this dose was similar to the LH surge, then a stimulating effect on progesterone production would have been predicted. Treatment of eCG used for superovulation may have caused this responsiveness of gonadotropins during the culture, and the late response for LH on day 5 of the culture in terms of steroid production.

In conclusion, it is the first time a cell culture protocol was established for granulosa cells isolated from queens. Although the level of basal progesterone production was not affected with time, the basal estradiol production increased significantly during five days of culture of granulosa cells. This evaluation was a success compared to the some other studies reported in different species although there is a lack of responsiveness of FSH in estradiol production and a lack of effect of LH in steroid production. Thus, this culture system might be useful for further molecular studies to investigate these responsiveness problems in gonadotropins, which will be helpful to investigate a variety of aspects of granulosa cell function in female cats.

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