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REPRODUCTIVE PHYSIOLOGY AND DISEASE

Felis catus ovary as a model to study follicle biology in vitro

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Abstract
Purpose The current study was designed to evaluate the response of individual intact antral follicles from adult female domestic cats to a luteinizing hormone (LH) stimulus in vitro by assessing cumulus-oocyte expansion (C-OE) and steroid production.
Methods C-OE and steroid levels (estradiol [E2] and progesterone [P4]) obtained from individual antral feline follicles (n=366 follicles; n=56 cats) were analyzed after 12 or 24 h of culture in the presence or absence of LH (low [3.4 ng/ml] or high [100 ng/ml]).
Results At the end of the culture, the highest percentage of expanded cumulus-oocyte complexes (COCs) was observed in the LH groups at 12 or 24 h in comparison to their controls (p<0.001). There was a significant increase in expanded COCs when comparing LH concentrations (high vs. low) at 12 or 24 h. Higher levels of both E2 and P4 were observed in the media from antral follicles after 12 and 24 h of culture in the presence of LH (both concentration, p<0.05). There was no association between hormone levels and follicle diameter; high variability was observed in the steroid levels produced by antral follicles within all treatment groups.
Conclusions These data indicate, for the first time, that feline antral follicles (0.5–2 mm) from different stages of the natural estrous cycle can be cultured and will respond to an LH stimulus, based on an increase in steroid levels as well as C-OE after 12 or 24 h in culture.

Keywords Antral follicles · *Felis catus* · Steroids · Follicle culture · Cumulus-oocyte expansion

Capsule Felis catus ovary as a model to study follicle biology in vitro.

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Introduction

The domestic cat (*Felis catus*) is a species that exhibits a seasonal polyestrous cycle. Cats are classically defined as induced ovulators [1]. Thus, ovulation occurs only if adequate levels of luteinizing hormone (LH), triggered by copulation, are released. Often, more than one copulation is required to achieve ovulation [2–4]. Due to its seasonality, the cycle can be divided into two periods (sexually inactive and sexually active) depending on daylight. The queen is sexually inactive during periods of short daylight (anestrus) and sexually active during long daylight. The first part of the cycle during the sexually active period is known as proestrus. During this stage, which lasts only 1–2 days, follicle stimulating hormone (FSH) induces ovarian antral follicle development [1]. This stage is followed by estrus, characterized as behavioral receptivity to mating and peak levels of estradiol (E2) [5], lasting an average of 7 days. If ovulation occurs, the female enters diestrus, which is characterized by the presence of functional corpora lutea accompanied by high circulating levels of progesterone (P4) [1]. Diestrus lasts about 60 days if the female becomes pregnant, or 40 days if the queen ovulates but fertilization does not occur (pseudopregnancy). However, if the female is not mated or is mated but does not ovulate during estrus, the queen will enter into post estrus (approximately

7 days), instead of diestrus, and then the next cycle starts again returning to proestrus.

The cat is commonly used for the study of oocyte cryopreservation based on its potential to serve as a model species for biomedical research and the conservation of endangered felids. Highly conserved reproductive mechanisms between humans and feline species were recently reported [6]. Cats serve as good models for addressing infertility syndromes in women, such as asynchronous oocyte cytoplasmic and nuclear maturation, ovarian hypersensitivity, and luteal dysfunction after gonadotropin therapy [6]. Interestingly, cat oocytes share several characteristics with human oocytes [7, 8]: (1) the diameter of the oocyte proper and the germinal vesicle is equivalent (110 and 45 μm, respectively) in both species; (2) oocytes reach the metaphase II (MII) stage of meiosis after 24 h in culture; and (3) both species have a similar nuclear configuration with a small nucleolus and a fibrillar chromatin. In contrast, these morphological features are distinct or lacking in the typical laboratory mouse model. Moreover, the cat offers a unique and valuable model to study molecular processes within the preovulatory follicle and cumulus-oocyte complexes (COCs), as each animal provides between 3 and 7 naturally selected preovulatory follicles, in an "ovulation-ready" state waiting for an LH stimulus (triggered by copulation) during estrus. This stage of the cycle, lasting around 7 days, gives a wide window for collecting samples. Plus estrus is easily detected through behavioral changes (crouching, rolling, affection, vocalization, and lordosis) and cornified vaginal cytology.

Follicle culture at different stages of development has been used to study diverse biological processes (folliculogenesis, steroidogenesis, and oocyte maturation) in various species, such as goat [9, 10], mouse [11–13], rat [14], sheep [15, 16], dog [17–19], and monkey [20–23]. Regarding the domestic cat, efforts to date have focused on oocytes [7, 24–30] or preantral follicle cultures [19, 31]. Therefore, studies were designed to characterize and evaluate the response of individual intact antral follicles from adult female domestic cats to a stimulus of recombinant human LH in vitro by assessing cumulus-oocyte expansion (C-OE) and steroid production (E2 and P4).

Materials and methods

Animals

Ovaries at different stages of the natural estrous cycle from adult female *Felis catus* (n=56) were used. The ovaries were donated/obtained from routine spaying procedures conducted at the "Centro de Salud Animal de la Municipalidad de Merlo" (Prov. de Buenos Aires, Argentina). The excised ovaries were immediately transported to the laboratory in physiological solution.

Follicle isolation and culture

Based on preliminary results, antral follicles that measured 0.5–2 mm were used in the present study. Follicle isolation from the ovary was performed under a dissecting microscope using 30-gauge needles as previously described [23]. Isolated antral follicles were individually cultured for 12 and 24 h in the presence or absence of recombinant human LH (rhLH, low or high concentration; Merck Serono) in a 48-well plates containing 300 μl alpha minimum essential medium (αMEM, Sigma) supplemented with 15 ng/ml recombinant human FSH (equivalent to 205 mIU/ml, Merck Serono), 200 μl ITS (Sigma), 200 μl streptavidin/penicillin (Sigma), and 0.30 % BSA (Natocor). An initial set of experiments (n=29 animals, n=188 follicles) was performed using a concentration of 3.4 ng/ml of rhLH. Whereas, a second set of experiments (n=27 animals, n=178 follicles) was conducted using a more physiological dose of rhLH in the feline follicle culture system. This was based on the fact that information about the feline LH surge in vivo was only expressed in ng/ml instead of mIU/ml. The first set of experiments were performed using the human concentration that the IVF clinics normally use for IVM (3.4 ng/ml equivalent to 75 mIU/ml of rhLH, defined as low), whereas during the second set of experiments, they were performed using a bolus equivalent for the reported surge in cats (100 ng/ml of rhLH; equivalent to 2206 mIU/ml and defined as high). At the end of culture, the medium from each well was collected, centrifuged, and stored at −80 °C for subsequent analysis of sex steroids.

Evaluation of LH response: cumulus-oocyte expansion and hormone levels E2 and P4 measurements

At the end of the culture period, COCs were retrieved from each individual follicle. The morphology of the oocyte and its surrounding cumulus cells were observed and analyzed under the dissecting microscope. C-OE is reportedly the most reliable index of oocyte maturation in cats, since polar body extrusion is difficult to identify in feline oocytes due to the dark appearance of their oocytes [32]. Release of the oocyte from the follicle at ovulation must be preceded by detachment of the COC from the inner (granulosa) cell layer of the follicle wall. Detachment involves loss of cell-cell contacts and formation of a hyaluronic acid (HA)-rich extracellular matrix between cumulus cells surrounding the oocyte, resulting in a large increase or "expansion" of the COC. This can be visualized by substantial expansion/enlargement of the adjacent cumulus cells of the COC under the dissecting microscope.

Levels of E2 and P4 in the media were measured from representative samples from each group and time point (n=139) to evaluate their response to LH. The Endocrine Laboratory at CEDIE "Hospital de Niños Ricardo Gutiérrez" analyzed the E2 and P4 levels from the culture media using a

COBAS e411 analyzer, an electrochemiluminescence-based automatic clinical platform (Roche Diagnostics GmbH) [33].

Statistical analyses

Statistical calculations were performed using Sigma Stat software package (Systat Software, Inc., Richmond, CA, USA). A Fisher test was used to analyze differences in proportions in the C-OE rates between control and LH groups. Differences in media hormone content among groups were analyzed using one-way analysis of variance (ANOVA) in each set of experiments (low or high). A *T* test was performed to assess possible differences in hormone levels depending on follicle diameter (divided in two groups: <0.9 vs. ≥0.9 mm) within each group. Differences in hormone levels between low and high concentration within the LH groups were assessed by *T* test. Differences were considered significant at $p<0.05$.

Results

Cumulus-oocyte complex expansion

Even though only healthy antral follicles (devoid of dark follicles/granulosa cells; $n=366$) were isolated and used for this study, at the end of the culture, some (around 10 %) of the retrieved COCs contained naked ($n=24$) or dead ($n=15$) oocytes (see Table 1). And in 9 cases, a COC or oocyte was not retrieved. Thus, these samples were excluded for the C-OE analysis. Most of the naked oocytes ($n=20$) belong to the control groups. But, equal numbers of dead oocytes or absent COC/oocytes were observed in both control and LH groups.

Representative pictures of the COCs retrieved in each group and time points are shown in Fig. 1. The highest percentage of expanded COCs was observed in the LH groups (low and high concentration) at both time points analyzed in comparison to their controls ($p<0.001$). Interestingly, there were significant differences in the proportion of expanded COCs when comparing LH concentrations (high vs. low) at 12 ($p<0.05$) or 24 h ($p<0.0001$). The proportion of expanded COCs retrieved after 12 and 24 h from antral follicles ($n=318$) cultured in the presence or absence of LH at both tested concentrations are summarized in Table 1.

In vitro hormone production

Figure 2 depicts steroid results from the two sets of experiments (low and high; panel a–c and b–d, respectively). Under control conditions, follicles produced greater (around 10-fold) levels of E2 compared to P4 during 12 and 24 h of culture. Higher levels of both steroids (E2 and P4) were observed in the media from antral follicles after 12 and 24 h of culture in the presence of LH (low or high concentration, $p<0.05$).

The obtained results showed a wide range of hormone values within each group at both sets of experiments (low and high concentration). At 12 h of culture, levels of P4 varied from <1 up to 62 pg/ml (control group) and from 208 up to 1354 pg/ml (LH group). In addition, 24 h after culture, P4 levels varied from <1 up to 224 pg/ml in the control group and from 80 up to 4095 pg/ml in the LH group. Also, E2 levels also varied at both time points (between <1 to 1592 pg/ml and 7 to 3589 pg/ml in the control group; between 31 to 7477 pg/ml and 93 to 15,360 pg/ml in the LH group; at 12 or 24 h, respectively). Table 2 summarizes the high variability observed in both hormone produced by antral follicles within each treatment group in both concentration sets.

We also analyzed the hormone levels according to the follicle diameter to evaluate a possible association (Table 2). The isolated antral follicles were measured (before culture) and divided in two groups according to their size (<0.9 and ≥0.9 mm) in both sets of experiments. But, surprisingly, when hormone levels were analyzed according to the follicle diameter within each treatment, this parameter did not correlate with E2 and P4 producing potential ($p>0.05$).

Discussion

The current study indicates that feline antral follicles with different diameters (0.5–2 mm) can be acutely cultured (12 to 24 h) and will respond to an LH stimulus, as evidenced by an increase in C-OE as well as elevated steroid levels in the presence of the gonadotropin. These data are comparable to the results previously observed in rhesus macaques by one of the authors [23]. It is noteworthy that in the LH groups (12 or 24 h), there were decreased numbers of naked oocytes in comparison to the control groups. This may be due to the circumstance that there is greater loss of cell-cell contacts or more cumulus cell death in the control groups during follicle culture and that LH prevents this from occurring. Also, LH exposure (low and high concentration) increased the percentage of expanded COCs in comparison to their controls, as verified by an enlargement of the COC area. There was also a significant increase in the percent of oocytes exhibiting C-OE in the presence of the high dose LH (12 and 24 h) as compared to the low dose of LH. Qualitative as well as quantitative analyses of C-OE has focused on the change in COC area, which is widely considered the main manifestation of this process. However, since C-OE is a complex process involving the formation of a HA-rich extracellular matrix between cumulus cells surrounding the oocyte [34], further studies are warranted to investigate the molecular features of C-OE in felines. Studies are ongoing using feline COCs to study the expression of key genes involved in this process (such as HAS2, TNFAIP6, AREG, and EREG) and to evaluate the formation and deposition of an HA-rich extracellular matrix

Table 1 Proportion of expanded COCs retrieved after 12 and 24 h from antral follicles cultured in the absence (control) or presence of LH (LH)

Treatment group	Low concentration (n=165)	Naked/dead (n=20)	High concentration[#] (n=153)	Naked/dead (n=19)
Control 12 h	2/26 (8)[a]	4	7/34 (21)[A]	7
LH 12 h	27/33 (82)[b]	2	46/47 (98)[B]	1
Control 24 h	7/50 (14)[a]	8	4/29 (14)[A]	9
LH 24 h	41/56 (73)[b]	6	43/43 (100)[B]	2

Two sets of experiments were performed using a low (3.4 ng/ml) or high (100 ng/ml) concentration of LH. Letter "n" represents total number of antral follicles from each set of experiment. Samples containing naked or dead oocytes in each group were excluded for the C-OE analysis and are shown in a separate column. The percentage in each group is expressed in parenthesis. Different letters represent significant differences in proportions within treatment groups in each concentration set of experiments (p<0.01)

[#] There was a significant higher proportion of expanded COCs when comparing high LH concentration vs. low at 12 (p<0.05) or 24 h (p<0.0001)

by a fluorescence-based technique developed for rhesus macaque COCs [35].

Recombinant human gonadotropins used in the present study yielded suitable and similar results with both of the LH concentrations tested. As we mentioned, the two concentrations studied were (1) the concentration normally used for IVM in IVF clinics (low) and (2) a physiological dose (high) equivalent for the reported surge in cats. In literature, however, gonadotropins of porcine or equine origin, plus higher concentrations, were more commonly used for feline studies [7, 19, 24–31]. This was presumably due to experience from in vivo stimulation protocols, where porcine FSH and eCG were used to initiate folliculogenesis in felines (for more information please read review: [36]). No major differences were observed between the low and high concentration of human LH examined, showing in both cases the response of the antral follicle to the LH stimulus. As expected, higher levels of P4 as well as E2 were observed in both LH groups in comparison to their controls. Notably, no significant differences were observed in steroid production when comparing LH concentrations in contrast with the C-OE results. Thus, with the LH lower dose used in the present study, most of the LH receptors were already activated. Surprisingly, when steroid hormone (E2 and P4) levels were analyzed according to the follicle diameter (divided into 2 categories: <0.9 and ≥0.9 mm), this parameter did not correlate with either LH

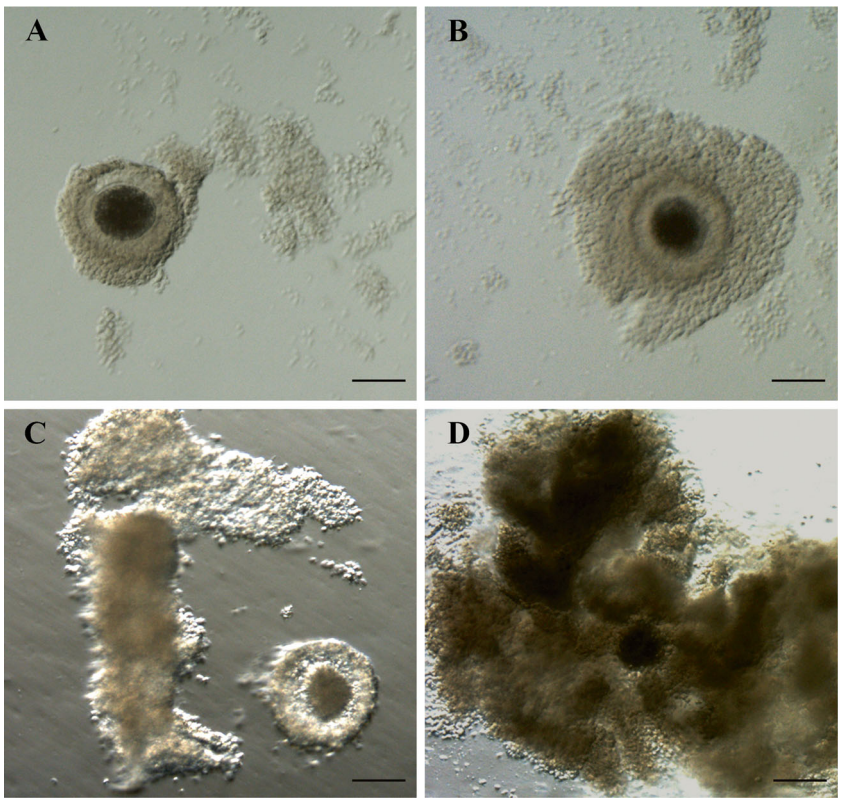

Fig. 1 Representative pictures of feline oocyte-cumulus complexes (COCs) retrieved from individual antral follicles after 12 (**a** and **b**) and 24 (**c** and **d**) h of culture in the absence (control) or presence of LH (LH). **a, c** Compact COCs retrieved from follicles of the control groups (12 and 24 h, respectively). **b, d** Expanded COCs retrieved from follicles of the LH groups (12 and 24 h, respectively). Original magnification 6×. *Bar* represents 100 μm

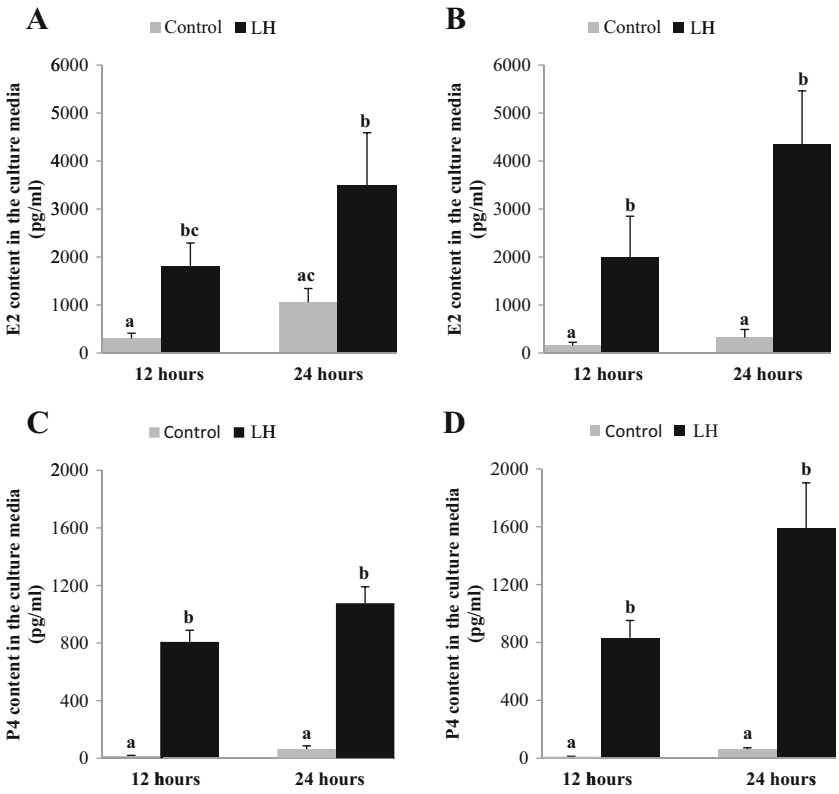

Fig. 2 Steroid **a**, **b** E2 and **c**, **d** P4 levels (pg/ml) in the media of individual feline antral follicles after 12 and 24 h of culture in the absence (control) or presence of LH (LH). **a**, **c** Hormone values from the set of experiments performed with low concentration of LH (3.4 ng/ml). **b**, **d** Hormone values from the set of experiments performed with high concentration of LH (100 ng/ml). Values are the mean ± SEM from representative samples from each treatment group (n=139). Different letters represent significant differences among treatment groups (ANOVA, p<0.05)

concentration. These results differ from those observed in rhesus macaques, where larger follicle diameters correlated with higher levels of E2 in the culture media but follicle size did not affect the levels of P4 [23]. Interestingly, the current results showed a wide range of hormone values within each group of feline follicles. Is it possible that some antral follicles were already being preselected to be dominant follicles at a very early stage? It was reported that dominant follicles have greater concentrations of E2 in their follicular fluid in comparison to the subordinate follicles [37, 38]. And also, a critical decrease in E2, after the LH surge, is necessary to enable normal ovulation in rodent [39]. Gershon et al. demonstrated that estrogen inactivation might allow the LH-induced expression of cyclooxygenase-2 (COX-2) and subsequent C-OE, allowing normal ovulation.

Table 2 Steroid levels according to follicle diameter in the culture media produced by representative antral follicles after 12 and 24 h of culture in the absence (control) or presence of LH (LH)

Treatment group	Low concentration (n=78)			High concentration (n=61)		
	<0.9	≥0.9	Min–max	<0.9	≥0.9	Min–max
E2						
Control 12 h	258±118	529±201	1–1592	206±89	28±27	<1–702
LH 12 h	1482±825	1897±517	96–7477	3002±1994	1253±515	31–2808
Control 24 h	1088±395	1030±430	7–3589	198±92	459±346	11–2533
LH 24 h	5557±2292	2035±930	121–14,658	4017±1562	3852±1285	93–15,360
P4						
Control 12 h	20±10	20±10	1–62	10±5	20±10	<1–48
LH 12 h	800±240	802±110	208–1354	700±180	930±160	300–1175
Control 24 h	50±30	70±30	1–224	70±20	40±10	<1–85
LH 24 h	800±160	1230±140	404–2092	1120±390	1670±390	80–4095

Two sets of experiments were performed using a low or high dose of LH, as explained before. Range of steroids levels are shown by minimum and maximum values in a separate column in each set of experiment. Letter "n" represents total number of representative antral follicles from each set of experiments. Values are the mean ± SEM (pg/ml) of E2 and P4. No significant differences in steroid levels were observed between low and high LH concentration (p>0.05)

In summary, these data indicate, for the first time, that feline antral follicles from different stages of the natural estrous cycle and with different diameters can be cultured and are able to respond to an LH stimulus. LH at both concentration tested elicited similar results with a significant increase in C-OE, as well as E2 and P4 levels, after 12 or 24 h in culture. Thus, the culture of feline antral follicles is a robust and valuable system to study follicular development, steroidogenesis, and periovulatory events as well as follicle biology in general. Moreover, as we mention, the use of a feline model system has the advantage of providing an excellent surrogate for understanding events involving human COCs that are necessary for fertility besides to contribute to the knowledge of feline reproduction. The latter being important for the conservation of threatened felids since most of the species from this family are endangered.

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Conflict of interest The authors declare that they have no conflict of interest.

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