

Effect of estrous cycle stage on oocyte *in vitro* maturation of domestic cats reared under tropical conditions

Efecto de la fase del ciclo estral en la maduración *in vitro* de ovocitos de gatas domésticas criadas en condiciones tropicales

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ABSTRACT

Oocyte maturation is a critical step for *in vitro* embryo production. In female cats, findings on the influence of the estrous cycle stage on oocyte quality and maturation are contradictory. Little is known about this phenomenon in female cats in the tropics. This study aimed to assess the effect of the estrous cycle stage on oocyte quality and subsequent capacity to complete nuclear maturation in cats in a tropical environment. Ovaries from 18 sexually matured cats were collected during ovariectomy. Cumulus-oocyte complexes (COCs) were released from follicles by slicing and fragmentation of the ovarian cortex. According to morphological characteristics, COCs were classified into grades I-II (suitable) and III-IV (no suitable). Only suitable COCs from each cat were cultured for *in vitro* maturation. Nuclear oocyte maturation was assessed by the presence of a telophase I or metaphase II plate with extrusion of the first polar corpuscle. A significantly greater number of oocytes per ovary were collected from queens in inactive than in follicular or luteal phase. Proportions of suitable COCs were similar among groups. Rate of oocyte maturation did not differ among stages of the estrous cycle, nor did the proportion of non-matured or degenerated oocytes. The age of the queens did not affect the percentage of oocyte maturation. In conclusion, the quality and rate of oocytes maturation were similar in the three stages of estrous cycle examined.

Key words: Cat, reproduction; biotechnology; *in vitro* system; tropics

RESUMEN

La maduración de los ovocitos es un paso crítico para la producción de embriones *in vitro*. En las gatas, los hallazgos sobre la influencia de la fase del ciclo estral en la calidad y maduración de los ovocitos son contradictorios. Se sabe poco sobre este fenómeno en las gatas en los trópicos. El objetivo de este estudio fue evaluar el efecto de la fase del ciclo estral sobre la calidad de los ovocitos y la posterior capacidad de completar la maduración nuclear en gatas de un ambiente tropical. Se recogieron ovarios de 18 gatas sexualmente maduras durante la ovariectomía. Los complejos cúmulo-ovocito (COCs) fueron obtenidos de los folículos mediante el corte y la fragmentación de la corteza ovárica. Según sus características morfológicas, los COCs se clasificaron en grados I-II (aptos) y III-IV (no aptos). Sólo los COCs de buena calidad de cada gata se cultivaron para su maduración *in vitro*. La maduración nuclear de los ovocitos se evaluó por la presencia de una placa en telofase I o metafase II con extrusión del primer corpúsculo polar. Se obtuvo un número significativamente mayor de ovocitos por ovario en las gatas en fase inactiva que en fase folicular o lútea. Las proporciones de COCs fueron similares entre los grupos. La tasa de maduración de los ovocitos no difirió entre las fases del ciclo estral, ni tampoco la proporción de ovocitos no maduros o degenerados. La edad de las gatas no afectó al porcentaje de maduración de los ovocitos. En conclusión, la calidad y la tasa de maduración de los ovocitos fueron similares en las tres etapas del ciclo estral examinadas.

Palabras clave: Gata; reproducción; biotecnología; sistema *in vitro*; trópico

INTRODUCTION

Domestic cats (*Felis catus*) are very prolific and fertile animals, so the main efforts to develop an efficient *in vitro* system for embryo production have been the multiplication and preservation of wild species of felines threatened by extinction [1]. Thus, domestic cats have been used as an experimental model [2, 3] for the development of an *in vitro* embryo production system to generate information applicable to the reproduction of wild cats [4, 5].

In vitro studies in small animals are scarce, particularly in domestic cats. In felines, as in other domestic species, *in vitro* oocyte maturation has been considered a critical step for the advancement of this biotechnology in terms of production of transferable embryos [6]. Different methodologies and media for *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) in cat oocytes have been described [3, 7, 8, 9, 10, 11, 12, 13].

The goal of an optimal *in vitro* maturation media is to allow the developmental competence of the oocytes to be fully expressed. Oocyte competence is crucial for the success of *in vitro* maturation and subsequent embryo development. This capacity is influenced by several factors such as the presence of cumulus cells around the oocyte [14], reproductive season [15], the diameter of the follicle from which the oocyte is derived [16] and stage of the estrous cycle at the time of oocyte retrieval [13].

In cats, the estrous cycle progresses throughout different phases: proestrus, estrus, interestrus, diestrus (if copulation and induced ovulation occur), and anestrus [17]. In each phase, there is absence (anestrus), low (interestrus) or high concentration of estrogens (proestrus, estrus), and high (diestrus) or low (proestrus, estrus) concentration of Progesterone. These gonadal hormones modulate the physiological characteristics of the ovaries, oviduct and uterus, and determine changes in the cellular morphology of the vagina [17].

The variation in the developmental capacity of cat oocytes by the influence of the estrous cycle stage has been poorly studied and controversial. Progesterone and or other substances produced by the corpus luteum (CL) seem to affect the ability of oocytes to complete nuclear maturation. Oocytes retrieved during the follicular phase completed metaphase II in a greater proportion, to those recovered from ovaries with a luteal structure [13, 18]. However, other studies found no effects of the stage of the estrous cycle on the maturation rate of cat oocytes, or the cleavage rate and blastocyst development [19, 20].

The studies mentioned above were conducted in regions where cats exhibit seasonal polyestrous reproductive conduct [21]. In the tropics, daylight hours do not vary greatly throughout the year, and cat reproduction is continuously polyestrous [22]. There is no published information about the influence of estrous cycle stages on oocyte quality and nuclear maturation in tropical regions in cats. Therefore, this study aimed to assess the effect of the estrous cycle stage on oocyte quality and subsequent capacity to complete nuclear maturation in cats under a tropical environment.

MATERIAL AND METHODS

All chemicals were purchased from Sigma (St Louis, MO, USA), unless otherwise mentioned.

Animals and surgery

It was studied 18 domestic cat females, sexually matured, aged between 8 and 30 months, of different breeds and crossbreeds. Cats (2.4 kg weight) were in satisfactory physical and health condition before being included in the study. The surgical procedure was performed at the Veterinary Polyclinic of the University of Zulia, Maracaibo, Venezuela. For surgery was used the Hedlund's surgical technique [23]. The study was conducted between March and May 2017. Vaginal smears were taken before surgery to corroborate the stage of the estrous cycle. Ovaries were collected from each female during ovariectomy. Cats were allocated to one of three stages of the estrus cycle, according to the structures found in the ovaries: 1) follicular stage: one or more follicles greater than or equal to 2 mm in diameter in one or both ovaries; 2) luteal stage: presence of one or more CL in one or both ovaries; 3) inactive stage: ovaries without CL and with no follicles greater than or equal to 2 mm in diameter [20].

Ovary collection and oocyte recovery were transported to the *in vitro* fertilization (IVF) laboratory in sterile saline (0.9% NaCl) at 38°C within one h after surgery. Immediately after arriving at the laboratory, ovaries were rinsed twice in a sterile warmed washing medium (NaHCO₃ 0.55 g; Heparin 0.00277 g; TCM-199 3.9 g; Gentamicin sulphate 50 mg·mL⁻¹, 0.4 % Bovine Serum Albumin (BSA), Sodium Pyruvate 20 µL; embryo tested ultra-pure water 250 mL). Surrounding tissues were removed from the ovaries. Ovaries were placed in a sterile 100 mm petri dish containing washing medium, and cumulus oocytes complexes (COCs) were released from follicles by slicing and fragmentation of the ovarian cortex.

COCs were classified according to morphological features under stereoscopic magnification 20X (Nikon, SMZ-2B, Tokyo, Japan) into four categories [24]: 1) Grade I: oocytes with uniform, dark cytoplasm, eccentric spherical nuclei, and five or more compact layers of cumulus cells; 2) Grade II: oocytes with uniform, dark cytoplasm, less than five compact layers of cumulus cells; 3) Grade III: oocytes with inhomogeneous cytoplasm, partially surrounded by not so compact cumulus cells; 4) Grade IV: oocytes with heterogeneous or fragmented cytoplasm, with few or no cumulus cells around them. Grade I and II oocytes were considered suitable and grades III and IV were unsuitable. Only the former group of oocytes (grades I and II) was submitted to Maturation *in vitro* (IVM).

In vitro maturation

Cumulus oocytes complexes from each cat were cultured separately for IVM in groups no greater than 20 structures in 90-µL droplets, covered with mineral oil. IVM medium was composed of TCM-199 supplemented with 1 µg·mL⁻¹ of estradiol 17-β; 0.02 UI·mL⁻¹ of FSH; 0.02 UI·mL⁻¹ of LH 50 µL; 0.3 mM sodium pyruvate; 4 mg·mL⁻¹ BSA; 5% fetal bovine serum; and 50 µg·mL⁻¹ of Gentamicin. Incubation (Thermo Scientific, modelo 3010, Waltham, MA, USA) was performed for 30 h at 38.5 °C in a humidified atmosphere of 5% CO₂.

Oocyte nuclear maturation

After maturation, COCs from each cat were denuded from cumulus cells by gentle pipetting in the maturation medium. Denuded oocytes were fixed in a solution of acetic acid-ethanol (1:3) for 24-48 h at 4°C and then placed on a sterile slide covered with a cover slide setting. Oocytes stained with 1% aceto-orcein solution for 30 min, were rinsed in acetic acid and glycerol solution and left to dry. Nuclear oocyte maturation was assessed by the presence of

a telophase I or metaphase II plate and the extrusion of the first polar corpuscle. Oocytes without the above characteristics were considered immature. Fragmented or irregularly shaped oocytes were considered degenerated [25].

Statistical analysis

The number of oocytes recovered per ovary was analyzed by the general linear model of SAS (SAS®; Version 9.3; SAS Institute, Inc., Cary, NC, USA). Means differences were compared by Tukey's multiple comparison test. Proportions of suitable, matured or immature/degenerated oocytes were analyzed by Chi-square of SAS.

RESULTS AND DISCUSSION

In general, 601 COCs were recovered from female cats in follicular (n=7), luteal (n=9) or inactive (n=2) stage. A significantly greater ($P<0.05$) number of oocytes per ovary were obtained from queens in inactive than in the follicular or luteal phases. This difference was because one queen in the inactive stage yielded 100 COCs and the other 21. Similar proportions of suitable COCs were quantified among groups of ovaries (TABLE I).

TABLE I
Effect of stage of estrous cycle on the quantity and quality of oocytes recovered from cat ovaries

Estrous cycle stage	No. oocytes recovered	Oocytes/ovary (mean ± EE)	Suitable oocytes n (%)
Follicular	228	16.3 ± 6.7 ^a	173 (75.9) ^a
Luteal	252	14.0 ± 6.2 ^a	188 (74.6) ^a
Inactive	121	30.2 ± 27.9 ^b	90 (74.4) ^a
Total	601	16.7 ± 10.2	

^{a, b}: Values with different letters in the same column differ. $P<0.05$

Maturation rate was greater ($P>0.05$) in oocytes from cats in luteal than in follicular or inactive stage (TABLE II). A similar proportion of immature/degenerated oocyte was observed among groups. The odds ratios for oocyte maturation between two groups in different stages of the estrous cycle showed a greater probability of oocyte maturation in luteal than in follicular phase, and in follicular or in luteal than in inactive stage (TABLE III). According to the cat's age, categorized as ≤ 12 (10.1 ± 1.7; n=8) and >12 (20.4 ± 4.2; n=10) months, the rate of maturation did not differ among stages of estrous cycle (data not shown).

TABLE II
Effect of stage of estrous cycle on nuclear maturation of oocytes recovered from cat ovaries

Estrous cycle stage	No. oocytes examined	Matured oocytes n (%)	Immature/degenerated oocytes n (%)
Follicular	89	11 (12.4)	78 (87.6) ^a
Luteal	144	25 (17.4)	119 (82.6) ^a
Inactive	65	5 (7.7)	60 (92.3) ^a
Total	298	41 (13.7)	257 (86.2)

TABLE III
Chi-square value and odds ratios for oocyte maturation among stages of estrous cycle

Estrous cycle stage	χ^2	P-value	Odds ratio (95% CI)
Luteal × Follicular	1.05	0.305	1.49 (0.69–3.19)
Luteal × Inactive	0.87	0.349	1.69 (0.56–5.13)
Luteal × Inactive	3.40	0.065	2.52 (0.91–6.91)

Proportionally more COCs per ovary were retrieved from ovaries in the inactive than in the luteal or follicular stages. Although only two queens with inactive phase ovaries were part of the current study, and the ovaries from one of them produced 5 fold more COCs than the second, the average number of oocytes recovered was close to doubling the average number of oocytes recovered from the other groups. In relation to this issue, there are opposite findings. Naoi *et al.* [20] reported obtaining a greater number of COCs from inactive than from follicular or luteal ovaries, which agrees with the outcomes of Freistedt *et al.* [26] and the present study in South America. However, no difference was found in the number of COC retrieved in different stages of the reproductive cycle [19]. It was found similar proportions of suitable (grade I and II) COCs among groups of ovaries; however, significant differences in the proportions of grade I oocytes was observed between the follicular and luteal stages [20].

In this study conducted in a tropical region, where cats exhibit reproductive cyclicity throughout the entire year, maturation rates were statistically similar among stages of the estrous cycle. However, it is important to note that the maturation rate was 5 and 9.7 percentage points greater ($P>0.05$) in oocytes obtained in luteal than in follicular or inactive phase, respectively. The rate of maturation in this study was considerably lower than that found in other studies [13, 19]. There is no precise explanation for this finding; however, the donor cats included in this study came from widely varied households with varying levels of medical care, husbandry, and nutrition. In general, cats in tropical environments are freer to be outside the house and, therefore, are more exposed to stressful situations and environmental cues that may affect the oocyte developmental competence [27, 28].

The outcomes of previously published studies in felines about oocyte competence and how it is affected by the stages of the reproductive cycle have been variables, but not enough evidence has been gathered. For instance, noticeable differences in oocyte maturation between follicular and luteal stages were found, with maturation rates of 50 percentage points greater in the former than in the later stage [13]. This difference did not change by adding IGF-I, EGF, or both growth factors, in the maturation media [13].

Supporting the previous finding, substances produced by luteal tissue, likely Progesterone, seem to have adverse effects on oocyte competence, because maturation rate was lower when the oocytes were collected during the luteal stage or from ovaries of pregnant cats than in the follicular or inactive stage [18]. However, in ruminants, the CL had favorable effects on oocyte competence and embryonic development [29, 30].

On the other hand, other studies found no difference in the maturation rate [19] or blastocyst development [20] from cat oocytes collected at different stages of the estrous cycle. However, proportionally more oocytes obtained from the luteal or inactive or

intermediate phase cleaved and became blastocysts after IVF than those recovered in the follicular stage [19, 28].

At different stages of the estrous cycle, oocytes are exposed to varying concentrations of gonadal steroids and other substances produced inside or outside the ovary [31, 32, 33, 34]. Therefore, it is plausible that the developmental competence of the oocytes changes throughout the reproductive cycle. Progesterone is essential in determining uterine receptivity in mammals but also plays a relevant role in oocyte maturation and subsequent embryo development [29, 35, 36]. Follicular size, which is related to the stage of the estrous cycle, may affect maturation and blastocyst rates [37, 38]. Even varying rates of oocyte maturation, cleavage, and embryo production in cats in the same stages of the estrous cycle may be supported by numerous factors modifying the oocyte developmental capacity [26, 27, 39, 40, 41, 42, 43].

CONCLUSION

The quality and rate of oocyte maturation were not affected by the estrous cycle stage. Nonetheless there was a greater probability for oocytes obtained from queens in the luteal phase to reach maturation after IVM. Eventhough, there is some indication that the number of recovered COCs increased in the inactive than in the follicular or luteal stage, it cannot at this time conclude this due to the small number of cats in this group and further research should be conducted using cats between 12–24 months of known parity.

Conflicts of Interest

The authors have no potential conflicts of interest with respect to the research, authorship or publication of this article.

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