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Effect of Different Reproductive Stages and Culture Times on Domestic Cat *in Vitro* Oocyte Maturation

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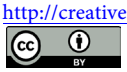

Abstract

The domestic cat has been used as a model to carry out comparative research in assisted reproduction, to be applied in wild cats. The efficiency in domestic cat IVM concerning the reproductive status and/or cultivation times has previously been investigated; however, the studies were carried out separately. The objective of this research was to evaluate the maturation of oocytes of domestic cats of different reproductive stages using two different *in vitro* culture times. The ovaries were obtained by Ooforo-Salpingo-Hysterectomy of cats that were of the following groups: 1) prepubertal, 2) follicular, 3) pregnant or 4) in anestrus. Maturation was carried out with TCM199 medium supplemented with BSA for 24 h and 48 h. On average, 29 ± 25, 20 ± 15, 17 ± 9 and 17 ± 13 oocytes/cat were recovered from the prepubertal follicular, pregnant, and anestrus stages, respectively, but did not show a significant difference ($P > 0.05$). Also, meiotic maturation did not show a significant difference between the different reproductive stages at 24 h and 48 h, respectively ($P > 0.05$). However, in the prepubertal and follicular stages, greater oocyte maturation numbers were observed at 48 h compared to 24 h ($P < 0.05$). In contrast, the aforementioned result was not observed in the pregnant and anestrus stages ($P > 0.05$), indicating that the *in vitro* culture duration is an important factor during *in vitro* maturation of domestic cat oocytes.

Keywords

Domestic Cat, Oocytes, *In Vitro* Maturation, Culture Time, Estrus Cycle Stages

1. Introduction

Most feline species are threatened and endangered due to the destruction of their natural habitat, with the domestic cat being the only species that is not in this situation (*Felis catus*) [1] [2]. The advances in the reproduction of felines are attributed to the comparative investigations of the domestic cat as an experimental model for those felines in danger of extinction [1] [2]. In Mexico, the jaguar (*Panthera onca*), ocelot (*Leopardus pardalis*) and margay or tigrillo (*Leopardus wiedii*) are some of the felines that are in danger of extinction [3], as such the domestic cat can be an alternative to implement reproduction techniques for these threatened species.

Several assisted reproduction techniques have been implemented in companion animals, such as artificial insemination, cryopreservation of germ cells (sperm, oocytes, and embryos), *in vitro* embryo production (IVEP), and embryo transfer [4]. IVEP in wild cats can be an important tool for those oocytes recovered from ovaries obtained by Ooforo-Salpingo-Hysterectomy (OSH), due to medical or post-mortem reasons [5], and help the conservation of threatened and endangered species [6]. It should be noted that the number of oocytes recovered from wild feline ovaries can reach up to 102, depending on several factors such as age, species, state of the estrous cycle, and health status [2].

The domestic cat continues to be essential for IVEP studies, such as increasing the efficiency of the *in vitro* maturation (IVM) of oocytes [7], and these results serve for the implementation of IVEP in wild felines [1] [6]. The domestic cat is a seasonal polyestric species (reproductive season during long days) and requires 12 h of light to maintain normal cycles [4], the best quality oocytes being obtained during the reproductive season [8]. Therefore, the efficiency of the IVEP of the domestic cat is reduced in the non-reproductive stage, affecting IVM and *in vitro* embryonic development (IVD), respectively [9].

Domestic cat IVEP begins with IVM which is expected to result in a large number of oocytes in MII. This first step of IVM, being essential for the following processes such as: *in vitro* fertilization [5] [6], intracytoplasmic sperm injection [10] [11], and *in vitro* embryonic development [5] [11]. However, the efficiency of cat oocyte IVM can be influenced by two factors: the physiological state of the ovary [12] and the culture time [13]. The efficiency in domestic cat IVM concerning reproductive status [12] [14] and/or cultivation times [13] has previously been investigated; however, the studies were carried out separately. The objective of this research was to evaluate the maturation of domestic cat oocytes in different reproductive stages: 1) prepubertal; 2) follicular; 3) pregnant; and 4) anestrus, all cultured for either 24 h or 48 h.

2. Materials and Methods

The present study was carried out in the "Reproduction Management" laboratory of the Universidad Autónoma Metropolitana Unidad Xochimilco, Mexico City. Unless otherwise stated, all reagents used were from Sigma Aldrich (St

Louis, MO, USA).

2.1. Ovary Collection

The domestic cat ovaries were obtained using OSH surgical technique using a mixture of Teletamine and Zolazepan (5 mg/kg) (Zoletil®, Virbac) and acepromazine Maleate (2.5 mg/kg) (Calmivet®, Vetoquinol).

The ovaries were classified according to the following characteristics: 1) prepubertal (female cats under 6 months of age) [15]; 2) follicular (one or more follicles of 2 mm diameter); 3) pregnant (presence of fetuses with one or more corpora lutea (LC); and 4) anestric (ovaries without follicular activity) [14]. After classification, the ovaries were transported in NaCl solution (0.157 M) with Ampicillin (10,000 IU/mL), Streptomycin (10,000 µg/mL), and Amphotericin (25 µg/mL) to the laboratory within 2 h of their recovery.

2.2. Oocyte Collection and *in Vitro* Maturation (IVM)

The cumulus oocyte complexes (COCs) were obtained by microdissection of the ovaries in Petri dishes with modified Tyrode's medium supplemented with sodium lactate (10 mM), HEPES (0.50 mM) and polyvinyl alcohol (0.01%) [16], and using scissors and 18 gauge needles. The search for COCs was carried out with the help of a stereoscopic microscope (Nikon, Japan). Afterwards, only COCs with three or more layers of granulosa cells and homogeneously dark cytoplasm [1] were washed twice with 500 µL of TCM199 medium with Earle's salts supplemented with bovine serum albumin (3 mg/mL) (BSA), cysteine (0.1 mg/mL), HEPES (1.4 mg/mL), sodium pyruvate (0.25 mg/mL), sodium lactate (0.6 mg/mL), L-glutamine (0.15 mg/mL) and gentamicin (0.055 mg/mL) [17]. The wash medium was also used for IVM, but supplemented with human menopausal hormone (4.5 IU/mL) (Merional® IBSA, natural high purified) [18]. The COCs were transferred into 500 µL of maturation medium under mineral oil. The culture of oocytes was done separately according to reproductive state. Also, the culture was carried out in an atmosphere of 38.5˚C, 5% CO_2, 95% air and humidity at saturation for 24 h and 48 h, respectively [13].

2.3. Assessment of Nuclear Maturation

To evaluate IVM, 300 µg/mL of hyaluronidase was used to remove the granulosa cells for 5 minutes at 38˚C. Next, the oocytes were fixed with paraformaldehyde (4%) for 15 min and washed with a mounting solution (Imacel®, *in vitro* Laboratory) and 1.5 µg/mL of 4', 6 Diamidino-2-phenylindole dihydrochloride (DAPI) was added [19]. The stained oocytes were evaluated under a microscope (NIKON, Eclipse E600) equipped with a fluorescence lamp and a UV filter (Ex 330 - 380 nm).

The oocytes in the germinal vesicle (GV) and metaphase I (MI) were considered immature, whereas the mature oocytes were those in metaphase II (MII) with the 1_{st} polar body (PB) (**Figure 1**) [12] [20].

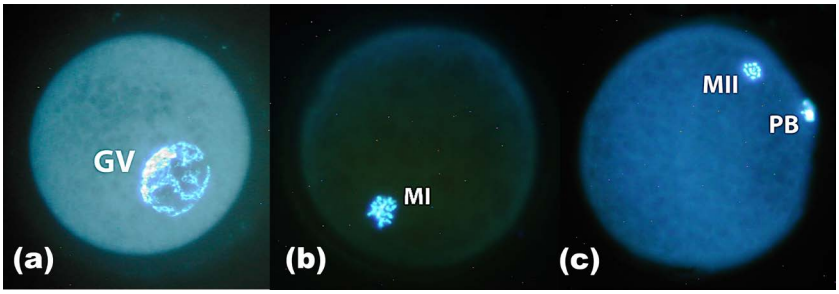

Figure 1. Domestic cat oocytes stained with DAPI. (a) Germinal vesicle (GV); (b) Chromosomes in metaphase I (MI); (c) Chromosomes in metaphase II (MII) with 1st polar body (PB) 400×.

2.4. Experimental Design

The present study was carried out between spring and autumn. The domestic cat ovaries were recovered in a veterinary clinic. Two experiments were performed to determine the maturation of domestic cat oocytes in different reproductive stages (Prepubertal, Follicular, Pregnant and Anestrus) at 24 h and 48 h cultured time.

Experiment 1. Maturation determination of domestic cat oocytes in different reproduction stages at 24 h of culture. A total of 789 oocytes were evaluated with DAPI (8 replicates): 319, 170, 95 and 205 oocytes from prepubertal, follicular, pregnant and anestrus, respectively.

Experiment 2. Maturation determination of domestic cat oocytes in different reproduction stages at 48 h of culture. A total of 616 oocytes were evaluated with DAPI (6 replicates): 267, 113, 108 and 128 oocytes from prepubertal, follicular, pregnant and anestrus, respectively.

2.5. Statistical Analysis

The number of oocytes recovered for each of the reproductive stages was analyzed by one-way ANOVA and followed by a Tukey-Kramer's significant difference test. The percentages of maturation of the different reproductive stages (prepubertal, follicular, pregnant and anestrus) were analyzed with the Chi-square test (X^2). In both tests, a significance value of $P < 0.05$ was utilized [21].

3. Results

A total of 210 ovaries were obtained from 105 female cats of the following reproductive stages: prepubertal (n = 38), follicular (n = 25), pregnant (n = 18), and anestrus (n = 24) (Figure 2). A total of 1405 oocytes were recovered in the present study. The prepubertal, follicular, pregnant and anestrus stages had a similar number of oocytes recovered per cat: 29 ± 25, 20 ± 15, 17 ± 9 and 17 ± 13, respectively ($P > 0.05$) (Table 1).

The meiotic maturation between the different reproductive stages in the *in vitro* oocyte culture times of 24 h (Experiment 1) and 48 h (Experiment 2) did not show a significant difference ($P > 0.05$) (Table 1). But when comparing the

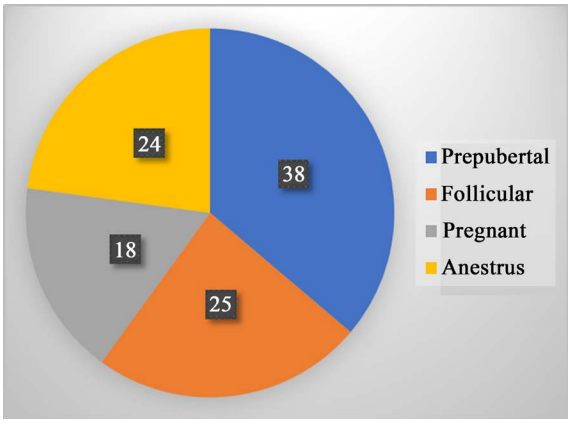

Figure 2. Number of domestic cat with respect to the groups: Prepubertal, Follicular, Pregnant and Anestrus States.

Table 1. Number of oocytes obtained per domestic cat and meiotic maturation of oocytes at 24 and 48 hours of *in vitro* culture for different reproductive stages.

Group	MEAN ± SD*	Total Oocytes	Culture Time			
			24 hours		48 hours	
			n	Mature n (%)	n	Mature n (%)
Prepubertal	29 ± 25[a]	586	319	102 (32)[1a]	267	129 (48)[2a]
Follicular	20 ± 15[a]	283	170	42 (25)[1a]	113	52 (46)[2a]
Pregnant	17 ± 9[b]	203	95	29 (31)[1a]	108	46 (43)[1a]
Anestrus	17 ± 13[b]	333	205	66 (32)[1a]	128	57 (45)[1a]
Total	**13 ± 8**	**1405**	**789**	**239 (30)[a]**	**616**	**284 (46)[a]**

*MEAN ± SD: Average and standard deviation of oocytes per cat. n: Number of oocytes. Mature: Oocytes in metaphase II with polar body. Different letters in columns means a significant difference ($P < 0.05$). Different numbers in rows means a significant difference ($P < 0.05$).

prepubertal and follicular reproductive stages, a greater maturation was observed at 48 h of culture compared to 24 h ($P < 0.05$), however, in the pregnant and anestrus stages, a significant difference was not observed ($P > 0.05$).

4. Discussion

The domestic cat can be used as an experimental model and the basis for the implementation of IVEP programs in wild felines [2] and can contribute to conservation efforts involving endangered feline species in Mexico [3].

The number of oocytes recovered from domestic cats for each reproductive stage was similar. However, a greater trend was observed in the prepubertal females (29 oocytes) compared to females in follicular (20 oocytes), pregnant (17 oocytes) and anestrus (17 oocytes) stages. This is possibly due to the fact that the ovaries of prepubertal cats present up to 102 follicles with diameters between 400 and 1200 μm, compared to follicular-stage ovaries with only 63 follicles [12]. This characteristic becomes more evident when the method commonly used to obtain the cat oocytes is used, making multiple cuts in the ovary "slicing" [22].

The ovary slicing technique releases those oocytes from antral follicles that are not visible, those of approximately 160 to 300 μm [23], and this increases the number of oocytes obtained in the prepubertal stage.

In the present study, 20 oocytes/cat were obtained from the follicular stage, this is similar to that reported by Karja *et al.* [14] with 18.8 oocytes/donor. The activity of the hypothalamic-pituitary axis is influenced by the photoperiod, consequently, it regulates the secretions of FSH, responsible for the development of multiple follicles [14]. The domestic cat ovaries showed 71 follicles of different sizes during the follicular stage, but only 8 ± 5 follicles have diameters between 1200 and 2000 μm [12].

In the pregnant and anestrus stages, 17 oocytes/cat were recovered in the present study, with a lower number of oocytes recovered compared to the previous stages (prepubertal and follicular). The absence of stimuli that promote follicular development, such as FSH [14], can decrease the efficiency in the recovery of oocytes, since during the pregnancy and anestrus stages, other types of hormones predominate, such as progesterone, melatonin and prolactin [24].

However, other studies have recovered similar numbers of luteal and anestric stage oocytes, 18 and 20 oocytes/cat, respectively [25], and 21 oocytes/cat in both stages [14]. Uchikura *et al.* [12] reported a higher number of follicles/ovary in the anestrus stage (92 follicles) compared to the luteal stage (71 follicles), but these results cannot be conclusive, because they used 8 and 7 cats in anestrus and luteal stages, respectively.

The IVM of the oocyte is essential for achieving nuclear maturation [26], an essential process for fertilization and embryonic development [7]. Currently, there is not enough information on the efficiency of IVM using different culture times [13], while considering the reproductive stages of the domestic cat [12]. It has been reported that the meiotic maturation of the domestic cat oocyte begins at 24 h (38%) of culture and reaches its maximum at 45 h (67%) [13].

The meiotic maturation that was obtained from the oocytes of different reproductive stages (prepubertal, follicular, pregnant, and anestrus) were similar at 24 h of culture. This argument has been previously reported by Karja *et al.* [14] where they mentioned that the reproductive stage of the domestic cat (follicular: 52%, luteal: 64%, and anestrus: 60%) did not influence the efficiency of IVM at 24 h of culture. Also, the present study showed that the number of matured oocytes was similar in the prepubertal, follicular, pregnant, and anestrus stages at 48 h of culture.

However, the proportion of MII oocytes increased in the follicular stage from 24 h (25%) to 48 h (46%) of culture, therefore culture time is important in sexually active animals. The oocytes of sexually mature cats (n = 69) began to present MII after 17 h of IVM (36%), at 24 h (38%), and reached up to 67% after 45 h of culture [13]. Subsequently, Nagano *et al.* [27] observed that the percentage of MII oocytes was lower at 24 h (50%), being higher after 30 h (75%), 36 h (67%), and 48 h (69%) of culture, however, both studies did not consider other

reproductive stages.

The MII oocytes of the prepubertal stage increased from 24 h (32%) to 48 h (48%), respectively. This aforementioned result demonstrates that oocyte culture time does not only influence oocyte maturation for mature cats, but also for prepubertal cats.

The expression of LH receptors has been identified in granulosa cells of ≥800 μm and ±300 μm follicles, although the expression is lower in small follicles [28]. Therefore, granulosa cells can respond to exogenous LH, this hormone being essential for follicular development and inducing the final maturation of the oocyte [12] [28]. It is clear that cumulus cells conduct the LH signal to the oocyte to continue meiosis [29] [30]. It has been observed that oocytes from prepubertal and mature domestic cats matured *in vitro* for 12 h present cellular projections of the corona radiata through the zona pellucida, allowing the connection and interaction between the granulosa cells and the oocyte [31].

The prepubertal and follicular stages showed similarity during follicular development, although it is thought that follicular growth stops at diameters of <1200 μm of prepubertal cats, due to insufficient LH secretion [12]. The follicles that stop their development probably could continue until the point of oocyte maturation completion with the appropriate stimuli (LH or HCG) [28]. Notably, it has been reported that oocytes recovered from 100 - 120 days old prepubertal female cats have the ability to mature, fertilize and develop *in vitro* to the blastocyst stage [23].

The proportion of MII oocytes was similar from 24 h to 48 h of culture for the pregnant and anestrus stages. This could be a consequence of the hormones that are predominant in these stages. Progesterone in the case of pregnant cats and melatonin and prolactin in the anestrus stage cats [24].

It has been reported that in bovine species the follicular development can continue in an environment where progesterone predominates [32]. This can also happen in the domestic cat, where follicular development continues during the pregnant stage and the oocytes obtained from these follicles can reach 31% and 43% of MII at 24 h and 48 h of culture, respectively. Importantly, though meiotic maturation is lower in oocytes retrieved from ovaries of pregnant cats or luteal phase compared to the follicular and anesthetic stage [25] as was also observed in the present study.

In the anestrus stage of the domestic cat, ovarian activity stops abruptly when exposed to less than 8 h of daylight, increasing melatonin synthesis [25], but it has been reported that oocytes recovered in this stage can reach 60% of MII in 24 h [14], compared to the present study with 32% and 45% maturation at 24 h and 48 h of culture, respectively.

5. Conclusion

Based on a comparison of matured oocytes from 24 h and 48 h, concerning prepubertal and follicular groups, a greater number of oocytes was observed in MII

when cultured for 48 h. It can be observed that culture time is an important factor in the IVM of domestic cat oocytes.

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Authors' Contributions

José Ernesto Hernández Pichardo contributed to the idea and design of this study, data analysis, interpretation, and drafting of the manuscript. Miguel Ramses Del Moral Reyes contributed to gamete collection and *in vitro* maturation. Michael E. Kjelland contributed to the experimental design, data analysis, and manuscript correction. José Luis Rodríguez Suástegui contributed to data analysis, interpretation and wrote the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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