

***In vitro* maturation of domestic cat oocytes subjected to different incubation times**

Maturação *in vitro* de oócitos de gatos domésticos submetidos a diferentes tempos de incubação

Maduración *in vitro* de ovocitos de gato doméstico sometidos a diferentes tiempos de incubación

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Abstract

The aim of this study was to evaluate the effect of three different incubation times on *in vitro* maturation of domestic cat oocytes. Thus, ovaries (n = 42) were submitted to slicing procedure and the oocytes recovered were classified; only good quality oocytes (Grade I and II) underwent *in vitro* maturation for three different periods (24 vs. 30 vs. 36 h) in supplemented TCM-99 medium. After, oocytes were evaluated for *cumulus* cell expansion and presence of the first polar body. After six replicates (7 ± 1,7 ovaries per replicate), a total of 334 viable oocytes were recovered. Differences (p <0.05) were observed regarding the percentage of oocytes presenting expansion of the *cumulus* cells, where higher values were observed in the group of oocytes incubated for 36 h (84.3%), when compared to 30 (73.4%) and 24 h (71.0%). Moreover, differences were also observed regarding the presence of the first polar body (24 h: 29.7%; 30 h: 58.2%; 36 h: 69.8%). We conclude that the incubation period influenced the maturation rates, indicating 36 h as the ideal period for the *in vitro* maturation of domestic cat oocytes in supplemented TCM-199 medium.

Keywords: Domestic feline; First polar body; Maturation; Meiotic competence.

Resumo

O objetivo deste estudo foi avaliar o efeito de três diferentes tempos de incubação na maturação *in vitro* de oócitos de gatos domésticos. Assim, os ovários (n = 42) foram submetidos ao procedimento de *slicing* e os ovócitos recuperados foram classificados; apenas oócitos de boa qualidade (grau I e II) foram submetidos à maturação *in vitro* por três períodos diferentes (24 vs. 30 vs. 36 h) em meio TCM-199 suplementado. Em seguida, os oócitos foram avaliados quanto à expansão celular do *cumulus* e a presença do primeiro corpo polar. Após seis replicatas (7 ± 1,7 por replicata), um total de 334 oócitos viáveis foram recuperados. Diferenças (p <0,05) foram observadas em relação à porcentagem de oócitos que apresentam expansão das células do *cumulus*, onde maiores valores foram

observados no grupo de oócitos incubados por 36 h (84,3%), quando comparados a 30 (73,4%) e 24 h (71,0%). Também foram observadas diferenças em relação à presença do primeiro corpo polar (24 h: 29,7%; 30 h: 58,2%; 36 h: 69,8%). Concluimos que o período de incubação influenciou as taxas de maturação, indicando 36 h como o período ideal para a maturação *in vitro* de oócitos de gatos domésticos em meio TCM-199 suplementado.

Palavras-chave: Felino doméstico; Primeiro corpúsculo polar; Maturação; Competência meiótica.

Resumen

El objetivo de este estudio fue evaluar el efecto de tres tiempos de incubación diferentes sobre la maduración *in vitro* de ovocitos de gatos domésticos. Así, los ovarios (n = 42) se sometieron al procedimiento de corte y se clasificaron los ovocitos recuperados; solo ovocitos de buena calidad (grados I y II) se sometieron a maduración *in vitro* durante tres períodos diferentes (24 frente a 30 frente a 36 h) en medio TCM-99 suplementado. Luego, se evaluaron los ovocitos para determinar la expansión de las células del cúmulo y la presencia del primer cuerpo polar. Después de seis repeticiones ($7 \pm 1,7$ por repetición), se recuperó un total de 334 ovocitos viables. Se observaron diferencias ($p < 0,05$) en relación al porcentaje de ovocitos que presentan expansión celular de cúmulos, donde se observaron valores más altos en el grupo de ovocitos incubados por 36 h (84,3%), comparado con 30 (73,4 %) y 24h (71,0%). También se observaron diferencias en relación a la presencia del primer cuerpo polar (24 h: 29,7%; 30 h: 58,2%; 36 h: 69,8%). Concluimos que el período de incubación influyó en las tasas de maduración, indicando 36 h como el período ideal para la maduración *in vitro* de ovocitos de gatos domésticos en medio TCM-199 suplementado.

Palabras clave: Felino domestico; Primer corpúsculo polar; Maduración; Competencia meiótica.

1. Introduction

In vitro maturation (IVM) of oocytes is a primordial and essential step of *in vitro* embryo production (IVEP), providing the necessary conditions for *in vitro* fertilization (IVF) and subsequent *in vitro* embryonic development. Under optimal culture conditions, approximately 50-79% of immature oocytes from domestic cats reach nuclear maturation (Snoeck *et al.*, 2016; Sowińska *et al.*, 2016; 2017; Prochowska *et al.*, 2019). However, these IVM rates are highly variable according to the individuality of domestic cats (Snoeck *et al.*, 2016), and the *in vitro* development performance tends to decrease as the selected mature oocytes follow the IVEP process (Hribal *et al.*, 2013; Sowińska *et al.*, 2016). Several factors can influence IVM success, ranging from donor reproductive status to *in vitro* conditions, including incubation time (Nagano *et al.*, 2008; Włodarczyk *et al.*, 2009; Zhong *et al.*, 2015).

For domestic cat oocytes, the period proposed by some authors to perform IVM ranges between 24 to 44 h (Comizzoli *et al.*, 2003; Demir *et al.*, 2014; Snoeck *et al.*, 2016; Evecen *et al.*, 2016; Veraguas *et al.*, 2017; Brusentsev *et al.* 2018). Among these, the 24 h interval has been preferentially employed due to laboratory logistics (Moulavi *et al.*, 2017; Ochota & Nizański, 2017; Kochan *et al.*, 2018). However, the maturation rate can gradually increase over a period of 24 h (Zhong *et al.*, 2015), and the pool of immature oocytes at different development stages may require different maturation periods (Gómez *et al.*, 2006). In this context, it was reported that domestic cat oocyte populations undergo two “waves” of nuclear maturation, the first occurring in 26 h of culture and the second after 28-30 h, mimicking the *in vivo* copulation and ovulation given in response to induction of sexual stimulus (Johnston *et al.*, 1989; Katska-Ksiazkiewicz *et al.*, 2003; Gómez *et al.*, 2006). In its turn, it has been reported that incubation for times longer than 36 h makes oocytes very aged, making sperm penetration difficult/unviable (Nagano *et al.*, 2008). Therefore, a study is needed that seeks consensus on the ideal incubation period to promote oocyte maturation in the feline species.

In this context, the domestic cat is valuable as an experimental model for other felines threatened with extinction, given their phylogenetic proximity (Thongphakdee *et al.*, 2020). Thus, IVM results data can be used to potentially preserve endangered wild felids in the era of assisted reproductive technologies. The increasing creation in commercial breeding domestic cat also supports research in the field of reproductive biology and biotechnology in this species (Pieri *et al.*, 2015).

The investigation of intermediate IVM times, between 24 and 36 h, is important to the optimization of results and standardization of protocol between laboratories that perform IVEP in domestic cats. This would facilitate the sharing and

comparisons of data, and consequently, the development of biotechniques in feline species. In this perspective, the objective was to evaluate the effect of three different incubation times (24, 30, and 36 h) on the IVM of domestic cat oocytes.

• 2. Methodology

• 2.1 ethics committee

This study was conducted according to the recommendations of the Brazilian Code of Animal Experimentation (COBEA) (1988) and was approved by the Ethics Committee on Animal Use (CEUA) of the Federal University of Semi-Arid (UFERSA) under protocol 23091.001388/2018-65.

2.2 Experimental animals and ovaries collection

Twenty-one pairs of healthy ovaries (total number = 42) from adult (1-3 years old) cats submitted to elective ovariectomy in local veterinary clinics were used. The ovaries were transported at 4°C in 0.9% NaCl with 0.05 mg/mL penicillin to the laboratory. The time interval between ovaries collection and processing did not exceed 5 h (Martins & Lopes, 2008). Follicular, luteal, intermediate and inactive ovaries were used, and ovaries with ovarian cysts were discarded (Karja *et al.*, 2002).

For each maturation time evaluated, six replicates were performed, and an average of seven ovaries were processed per replicate.

2.3 Immature Oocyte Collection And Selection

The recovery of *cumulus-oophorus* complexes (COCs) was performed according to the protocol described by Kunkitti *et al.* (2016). Briefly, the ovaries were submitted to the slicing technique, with the aid of a sterile scalpel slide, in petri dishes containing oocyte collection medium, consisting of TCM-199 supplemented with sodium bicarbonate (2.2 g/L), bovine serum albumin (BSA; 3 mg/mL), sodium pyruvate (0.25 mg/mL), L-glutamine (0.15 mg/mL), sodium lactate (0.6 mg/mL) and gentamicin (0.055 mg/mL).

The recovered COCs were evaluated according to the morphological classification described by Wood and Wildt (1997), and only oocytes of Grade I and II were used, namely: Grade I COCs- were oocytes with homogeneous and heavily pigmented ooplasm, surrounded by five or more layers of compacted *cumulus* cells; Grade II COCs- had homogeneous, heavily pigmented ooplasm, with some or a few heterogeneous granulations, encased in less than five layers of compacted *cumulus* cells.

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2.4 In Vitro Maturation

After selection, the COCs were quantified and washed three times in IVM medium (oocyte collection medium supplemented with FSH/LH (0.02 IU/mL) and cysteamine (100 µM). The COCs were placed in sterile Petri dishes containing drop of 100 µL IVM medium covered with mineral oil at a rate of 15-20 oocytes/drop. Maturation proceeded at 38.5°C with saturated humidity and atmosphere with 5% CO₂, and the COCs were divided into three groups according to the maturation time: 24, 30 and 36 h.

2.5 Assessment of *in vitro* maturation

The COCs submitted to IVM were observed under a stereomicroscope (20-40x magnification) and initially evaluated for *cumulus* cell expansion. This assessment was based on the appearance of the *cumulus* cells and the enlargement of *cumulus*

cell mass away from the zona pellucida. The percentage of COCs presenting total *cumulus* cells expansion was calculated. Then, the oocytes were mechanically denuded by successive pipetting until the complete removal of the *cumulus* cells adhered to their zona pellucida; and visualized in the inverted microscope (400x magnification), for quantification/classification as to the presence or absence of the first polar body (1st PB). Oocytes that presented extrusion of the 1st PB were considered matured (meiotic maturation). In addition, the percentage of oocytes activated spontaneously (with one or more cell divisions) during IVM was recorded.

2.6 Data analysis

The rates of IVM assessments were calculated by considering the number of oocytes that showed expansion of their *cumulus* cells or extrusion of the 1st PB over the total number of oocytes submitted to IVM multiplied by 100. The spontaneously activated oocytes rate was calculated by considering the number of oocytes that presented one or more cell divisions over the total number of oocytes submitted to a given IVM period multiplied by 100.

The data were expressed as mean in percentage \pm standard deviation (SD) for each evaluated IVM time (24 vs 30 vs 36 h) and compared by the Chi-square test ($P < 0.05$) using the statistical program Bioestat version 5.3.

3. Results

Of the total number of domestic cat ovaries evaluated, approximately 334 Grade I and II oocytes were recovered, of which 41.7% were Grade I and 58.3% Grade II. On average, 3.3 ± 1.3 Grade I oocytes and 4.7 ± 2.1 Grade II oocytes were recovered by ovary. Grade III or IV oocytes were not counted in the study.

Table 1 presents the data regarding the IVM assessment rates for the different incubation times (24, 30 and 36 h). Differences among the three IVM times were observed for both *cumulus* cell expansion and 1st PB extrusion ($P < 0.05$).

Table 1: *In vitro* maturation of domestic cat oocytes after different incubation times.

Incubation times (h)	No. of oocytes (replicate)	Evaluation of IVM (%)			
		Expansion of <i>cumulus</i> cells		Presence of the 1 st PB	
		Mean \pm SD	Min- Max	Mean \pm SD	Min- Max
24	117 (6)	71.0 ± 7.5^c	65.0-85.7	$29.7 \pm 4,3^c$	25.0-33.3
30	111 (6)	73.4 ± 17.4^b	53.3-92.8	58.2 ± 12.2^b	46.2-70.6
36	106 (6)	84.3 ± 7.5^a	73.3-93.8	69.8 ± 18.6^a	43.7-86.4

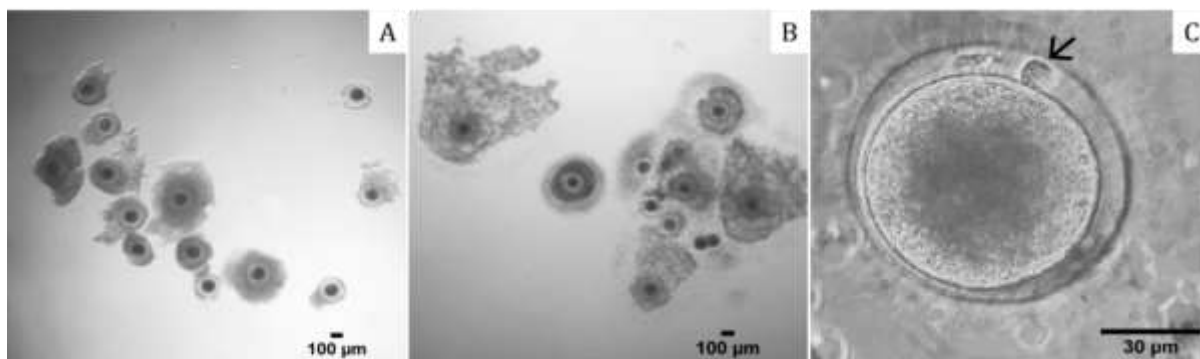
IVM: *in vitro* maturation; 1st PB: first polar body; SD: standard deviation of the mean; Min: minimum percentage; Max: maximum percentage. a, b, c: different letters in the same column indicate different values between compared groups (24, 30 and 36 h; $P < 0.05$). Source: Authors (2019).

The 36 h of oocyte maturation reached the highest mean rate of the study for *cumulus* cell expansion (84.3%) and for the presence of the 1st PB (69.8%), compared to the other incubation times ($P < 0.05$), while the incubation for 24h showed the lowest rates in the evaluation of oocyte maturation ($P < 0.05$; Table 1).

Cleaved oocytes and morula structures obtained by spontaneous parthenogenesis were observed for all evaluated IVM times (24, 30 and 36 h). Considering all replicate, an average of 1.70%, 2.70% and 1.88% of spontaneous parthenogenesis-activated oocytes were observed to 24, 30 and 36 h of IVM, respectively.

The structural characteristics of the oocytes before and after *in vitro* maturation, taking into account *cumulus* cell expansion and the presence of the 1st PB, are shown in Figure 1.

Figure 1. Structural characteristic of domestic cat oocytes before and after *in vitro* maturation.



A. Oocytes before *in vitro* maturation, unstained, visualized in stereomicroscopy. B. Oocytes showing *cumulus* cell expansion after *in vitro* maturation, unstained, in stereomicroscopy C. Denuded oocyte showing extrusion of first polar corpuscle after *in vitro* maturation, unstained, visualized in an inverted microscope; Arrowhead indicates first polar body. Source: Authors (2019).

4. Discussion

In many species, including domestic cats (Kunkitti *et al.*, 2016; Jewgenow *et al.*, 2019), goats (Dos Santos *et al.*, 2013) and cows (Otero *et al.*, 2017), the time taken for IVM is around 24 h, and much of this is due to the adequacy to the laboratory routine (Lonergan & Fair, 2016). We observe a growing increase in maturation rate in relation to the 1st PB extrusion as IVM time increased ($29.7 \pm 4.3\%$, $58.2 \pm 12.2\%$ and $69.8 \pm 18.6\%$ for 24, 30 and 36 h, respectively), even over a longer period than the 30-34 h indicated by Fernandez-Gonzalez *et al.* (2015) in wild felid (*Panthera leo*).

In the present study, IVM for 36 h was ideal for domestic cat oocytes to acquire nuclear maturation, showing superiority for 1st PB extrusion rates and *cumulus* cell expansion over other times evaluated. Oocytes recovered from postmortem ovaries of the Indian leopard (*Panthera pardus fusca*), tiger (*Panthera tigris tigris*) and lion (*Panthera leo persica*) were also successfully matured to MII stage after 36 h of incubation (Rao *et al.*, 2015). It was stated that some meiosis arrest factors, such as type 2 natriuretic peptide receptor (NPR-2) have a relatively longer half-life in feline oocytes (Zhong *et al.*, 2015). Another hypothesis is that prolonged incubation time reduces the concentration of some medium constituents that may have paracrine action on factors promoting meiotic arrest, such as estradiol; it acts by activating the enzyme adenylate cyclase (AC) which in its turn promotes synthesis of cyclic adenosine 3', 5'-monophosphate (cAMP), keeping oocytes in the germinal vesicle stage (Das *et al.*, 2017; Pan & Li, 2019).

The 30-h period of IVM showed intermediate results, with an average nuclear maturation above 50% of cultured oocytes. Similar IVM rate ($51.8 \pm 10\%$) was observed by Veraguas *et al.* (2017) after 30 h of incubation. Possibly, this time promotes the maturation of a small portion of the oocytes that would be activated after 28 h of culture, while the others do not complete nuclear kinetics. It is reported that recovered feline oocytes may have varying degrees of immaturity, so some are in very early stages for gene activation and protein coding for meiosis resumption, such as mitogen-activated protein kinase (MAPK) and MAPK activating protein kinase (MEK), requiring longer *in vitro* incubation times (Johnston *et al.*, 1989; Oliveira *et al.*, 2009).

All IVM periods evaluated were able to trigger oocyte maturation, considering *cumulus* cell expansion at rates above 70%. This is due to the rigorous selection and use of only Grade I and II oocytes, as it is evident that both oocytes are more likely to mature *in vitro* (Katska-Ksiazkiewicz *et al.*, 2003; Włodarczyk *et al.*, 2009; Kunkitti *et al.*, 2016). The protocol

adopted allowed to reach the maximum IVM rate of 93.8% by the *cumulus* cell expansion in the 36 h period, using 6 Grade I oocytes and 10 Grade II oocytes. The results were similar to those observed by Veraguas et al. (2017), suggesting that despite the time influencing IVM rates of domestic cat oocytes, the protocol adopted in the study proves to be satisfactory in the oocyte maturation process.

Additionally, a good oocyte recovery rate was obtained for both Grade I oocytes ($3.3 \pm 1.3/\text{ovaries}$) and Grade II oocytes ($4.7 \pm 2.1/\text{ovaries}$), being this amount sufficient for the distribution between the three IVM times evaluated and considering that the proportions found in the literature are similar to the study (Wood & Wildt, 1997; Baudi *et al.*, 2006). Thus, it is believed that this selection played an important role in obtaining good IVM rates in this study.

Oocytes with one or more cell divisions were observed at all evaluated IVM periods. This process, called spontaneous parthenogenetic oocyte activation, can occur in appropriate media under *in vitro* conditions, associated with oocyte “aging”, supposedly triggered by the reduction of maturation promoting factor (MPF) and MAPK activity (Jiang *et al.*, 2015). Moreover, parthenogenetic activation in domestic cat oocytes may occur more frequently when they are matured for long periods, with a significant increase in this rate after 40 h of incubation (Bogliolo *et al.*, 2004), thus, suggesting the effect of maturation time on this process. In this study, however, the spontaneously activated oocytes rate did not differ between the evaluated times. It is assumed that the occurrence of spontaneously oocyte activation may be associated with extracellular calcium concentrations present in the TCM-199 medium composition used in culture (Sun *et al.*, 2002; Eftekhar *et al.*, 2012). The storage time and temperature may also promote spontaneous activation of oocytes within the ovary (Kochan *et al.*, 2018). Additionally, the spontaneously activated oocytes rates observed in this study were similar to those previously reported during IVM of oocytes after 30 and 32 h of incubation (2.8% and 3.7%, respectively) (Wolfe & Wildt, 1996).

5. Final Considerations

Finally, the efficiency of IVM in domestic cats is influenced by the incubation time. The 36 h incubation period in TCM-199 medium provided higher nuclear maturation rates. Thus, this IVM time can be adopted in other laboratories, aiming at standardizing protocols and the improvement of oocyte maturation rate in the species. Additionally, this study can lead to future investigations regarding the evaluation of ideal IVM media and temperatures for felids.

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