The seasonal and ovarian status effects on *in vitro* production of domestic cat embryos between Equator and Tropic of Capricorn[1]

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ABSTRACT.- Martins L.R., Fernandes C.B., Villaverde A.I.S.B., Landim-Alvarenga F.C. & Lopes M.D. 2014. **The seasonal and ovarian status effects on *in vitro* production of domestic cat embryos in a region between Equator and Tropic of Capricorn.** *Pesquisa Veterinária Brasileira 34(3):277-280*. Laboratório de Reprodução Animal, Curso de Medicina Veterinária, Universidade Federal de Mato Grosso, Av. Alexandre Ferronato 1200, Sinop, MT 78557-267, Brazil. E-mail: lirigatto@yahoo.com.br

From the Tropic of Capricorn to Equator, the seasonality of domestic cat is known to be absent, i.e., these animals are considered non-seasonal breeders at these regions. We hypothesized that this particularity might have some influence on in vitro embryo production. The aim of this experiment was to determine the percentage of cleavage and morulae and blastocyst formation produced from oocytes recovered from queen ovaries of three distinct status - follicular, luteal or inactive - during two different reproductive seasons experienced by cats in southeast of Brazil (22°53'09" S and 48°26'42" W) – non breeding season (NBS), comprehending January to March; and breeding season (BS), August to October. Thirty queens were neutered. Ovaries were classified according to their status and were sliced in PBS for cumulus oocyte complex (COC) releasing. Grade I COC were washed three times in H-MEM supplemented with BSA, glutamine, sodium pyruvate, cysteine, streptomycin and penicillin. Oocytes were incubated in groups of 20-30 in 400μL of DMEM supplemented with FSH, LH, estradiol, IGF-I and basic fibroblast growth factor under mineral oil for 30 or 36 hours at 38°C in humidified environment of 5% de O2, 5% CO2 and 90% N2. COC were fertilized in Ham's F-10 medium supplemented with BSA, cysteine, pyruvate and streptomycin/penicillin (culture medium) with fresh semen selected through swim up technique. Eighteen hours later, the presumptive zygotes were denuded, the percentage of cleavage was determined and every 10 zygotes were transferred to 100μL drops of culture medium for culture during three days. After 72 hours of culture the percentage of morulae formation was evaluated and these structures were transferred to drops of the same culture medium. At the eighth day of culture blastocyst formation was analyzed. During NBS, from a total of 272 (inactive), 162 (luteal) and 134 (follicular) fertilized oocytes, the percentage of cleaved zygotes, morulae and blastocysts derived from inactive ovaries were 24.63, 16.54 and 8.09 respectively; for those derived from luteal ovaries, the percentage was 21.6, 12.96 and 8.64, and for those from follicular ovaries, they were 24.62, 16.41 and 8.21. Considering BS, from a total of 102 (inactive), 198 (luteal) and 86 (follicular) fertilized oocytes, the relative frequency (%) of cleaved zygotes, morulae and blastocysts derived from inactive ovaries were 64.7, 41.17 and 23.53 respectively; for those derived from luteal ovaries, the percentage was 64.14, 40.41 and 23.73, and for those from follicular ovaries,

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they were 63.95, 39.54 and 24.41. The results of this experiment demonstrate that no statistically significant difference (P<0.05) was verified in the frequency of cleaved embryos and morulae and blastocyst formation when comparing the three ovarian conditions in the same season. However the breeding season presented better results considering cleavage and morulae and blastocyst formation.

INDEX TERMS: Seasonality, felids, oocyte, *in vitro*-maturation.

RESUMO.- [**Efeitos da sazonalidade e da condição ovariana sobre a produção in vitro de embriões de gatos domésticos em uma região entre o Equador e o Trópico de Capricórnio.**] Do Trópico de Capricórnio ao Equador, sabe-se que a sazonalidade no gato domestico é ausente, i.e., estes animais são considerados reprodutores não sazonais nestas regiões. Nós hipotetizamos que esta particularidade possa ter alguma influência sobre a produção embrionária *in vitro*. O objetivo deste experimento foi determinar a porcentagem de clivagem e formação de mórulas e blastocistos produzidos a partir de oócitos recuperados de ovários de gatas em três condições - folicular, lútea ou inativa – durante duas estações reprodutivas pelas quais gatas passam na região sudeste do Brasil (22°53'09" S e 48°26'42" O) - estação não reprodutiva (ENR), que compreende os meses de janeiro a março; e estação reprodutiva (ER), agosto à outubro. Trinta gatas foram castradas. Os ovários foram classificados de acordo com sua condição e foram fatiados em PBS para liberação dos complexos *cumulus oophorus* (COC). COC grau I foram lavados três vezes em H-MEM suplementado com BSA, glutamina, piruvato sódico, cisteína, estreptomicina e penicilina. Os oócitos foram incubados em grupos de 20-30 em 400 μL de DMEM suplementado com FSH, LH, estradiol, IGF-I e fator de crescimento fibroblástico básico sob óleo mineral por 30 ou 36 horas em atmosfera úmida de 5% de O_2, 5% CO_2 e 90% N_2 a 38°C. Os COC foram fertilizados em meio Ham's F-10 suplementado com BSA, cisteína, piruvato e estreptomicina/penicilina (meio de cultura) com sêmen fresco selecionado através da técnica de *swim up*. Dezoito horas depois, os presumíveis zigotos foram denudados, a porcentagem de clivagem foi determinada e cada 10 zigotos foram transferidos para gotas de 100 μL de meio de cultura para cultivo durante 3 dias. Após 72 horas de cultivo, a porcentagem de formação de mórulas foi avaliada e estas estruturas foram transferidas para gotas do mesmo meio de cultivo. No oitavo dia de cultivo a formação de blastocisto foi avaliada. Durante a ENR, de um total de 272 (inativo), 162 (lútea) e 134 (folicular) oócitos fertilizados, a porcentagem de clivagem de zigotos, formação de mórulas e de blastocistos derivados de ovários inativos foi 24,63, 16,54 e 8,09 respectivamente; para aqueles oriundos de ovários na condição lútea, a porcentagem foi de 21,6, 12,96 e 8,64, e para aqueles provenientes de ovários na fase folicular, foi de 24,62, 16,41 e 8,21. Considerando a ER, de um total de 102 (inativo), 198 (lútea) e 86 (folicular) oócitos fertilizados, a frequência relativa (%) de zigotos clivados, mórulas e blastocistos derivados de ovários na condição inativa foi de 64,7, 41,17 e 23,53 respectivamente; para aqueles oriundos de ovários na condição lútea, a porcentagem foi de 64,14, 40,41 e 23,73, e para aqueles provenientes de ovários na fase folicular, foi de 63,95, 39,54 e 24,41. Os resultados deste experimento demonstraram que não houve diferença estatística significante (P < 0.05) na frequência de embriões clivados e na formação de mórulas e blastocistos quando comparadas as três condições ovarianas dentro da mesma estação. Entretanto, a ER apresentou resultados melhores considerando as taxas de clivagem e formação de mórula e de blastocisto se comparada à ENR.

TERMOS DE INDEXAÇÃO: Sazonalidade, felídeos, oócito, maturação *in vitro.*

INTRODUCTION

All the 37 wild felid species are classified as threatened with extinction, except the domestic that may act as a valuable model for human biomedical research. Propagating some wild felids as well as domestic cat populations serving as human models is a major challenge primarily due to difficulties in transporting animals between facilities to ensure the pairing of genetically matched individuals, behavioral incompatibility between pairs and low fertility. *In vitro* fertilization is a powerful tool for helping manage rare populations (Pelican 2006)

In vitro maturation of cat oocytes depends on different factors, such as the stage of the estrous cycle (Spindler & Wildt 1999). Additionally, seasonality ranges from being nonexistent with year-round gonadal activity as cheetah, *Acinonyx jubatus* (Brown et al. 1996) to highly restrictive, with cyclicity occurring during an extremely narrow time window of a few weeks, as Pallas' cat, *Otocolobus manul* (Brown et al. 2002). However, despite higher oocyte *in vitro* maturation (IVM) rates in cats, compared with dogs, they are lower than other species.

The aim of the experiment was to determine the *in vitro* maturation rate and percentage of cleavage and morulae and blastocyst formation derived from oocytes recovered from queen ovaries of three distinct status – follicular, luteal and inactive – during two different reproductive seasons experienced by cats in southeast of Brazil - non breeding season (NBS), comprehending the months of January to March; and breeding season (BS), from August to October.

MATERIALS AND METHODS

All chemicals were purchased from Sigma (St Louis, MO, USA), unless otherwise mentioned. Procedures were in accordance with the ethical standards of the committee on animal experimentation of Unesp.

Oocyte collection and selection. Ovaries were recovered after ovariohysterectomy performed at a local veterinary clinic and transported in Dulbecco phosphate buffered saline (DPBS; Nutricell®, SP, Brazil) containing 1% antibiotic-antimicotic solution at 4°C using ice packs in a thermal box. Ovaries were sliced within 4h of gonadectomy into a plastic Petri dish containing 5ml

of DPBS at 38°C using a scalpel blade to release cumulus oophorus complexes (COC). Under stereomicroscope (MZ 125; Leica®, Wetzlar, Germany), COC were selected and classified in grades I, II and III. Only COC grade I, defined by a uniform, dark cytoplasm surrounded by at least five layers of cumulus was selected. Grade I COC were washed three times in Hepes-buffered Minimum Essential Medium (H-MEM; Gibco®, NY, USA) supplemented with 3mg/ml bovine serum albumin (BSA), 2.0mM glutamine, 1.0mM pyruvate, 1.2 mM cysteine, 100mg/ml streptomycin and 100 UI/ml penicillin (Gibco®, NY, USA).

Oocyte *in-vitro* maturation. Oocytes recovered from follicular, inactive and luteal ovaries were *in vitro* matured. Those selected for maturation were incubated (20 to 30/400µl) in a four-well dish (Nunc®, Denmark or Ingámed®, PR, Brazil), containing 400µl DMEM (Dulbecco's Modified Essential Medium; Gibco®, NY, USA) supplemented with 3mg/ml BSA, 2.0mM of glutamine, 1.0mM pyruvate, 1.2mM cysteine, 100mg/ml of streptomycin and 100 UI/ml of penicillin (Gibco®, NY, USA), 10µg/ml bovine FSH (Folltropin® V, Bioniche® Animal Health, Canada), 1µg/ml LH (Lutropin® V, Bioniche®Animal Health), 1µg/ml oestradiol, 20ng/ml insulin-like growth factor I (IGF-I) and 10 ng/ml basic fibroblast growth factor (bFGF) under mineral oil for 30 to 36h at 38°C in a humidified environment of 5% O_2, 5% CO_2 and 90% N_2.

Oocyte *in-vitro* fertilization and culture. After 30 to 36 hours, oocytes were fertilized with fresh semen in Ham's F-10 medium supplemented with 3mg/ml BSA, 0.13 mM cystein, 1 mM pyruvate and 100 mg/ml streptomycin and 100IU/ml penicillin (culture medium). Eighteen hours later, the presumptive zygotes were denuded, the percentage of cleavage was determined and every 10 zygotes were transferred to 100 µL drops of culture medium for culture during three days. After 72 hours of culture the percentage of morulae formation was evaluated and these structures were transferred to drops of the same culture medium. At the eighth day of culture blastocyst formation was analyzed. Statistics differences between groups were analyzed through ANOVA.

RESULTS

The oocyte mean number/donor during NBS was 23.65±0.8 while this number increased during BS to 48.20±1.2.

Total number of fertilized oocytes, cleaved zygotes, morulae and blastocysts were analyzed by Chi-square test with a significance level of 0.05 using Statistical Analysis Package (SAS).

Ovarian status did not affect the total number of fertilized oocytes, cleaved zygotes, morulae and blastocysts during both studied seasons. However, when breeding season (BS) and non breeding season (NBS) were compared, values obtained during breeding season were higher.

Considering blastocyst production as the most important parameter in *in-vitro* procedures,, breeding season significantly influenced this parameter compared to non breeding season (30.66±14.22 x 15.67±5.68, respectively).

These results are summarized on Table 1 and Table 2.

Table 1. Absolute and relative frequency of cleavage, morulae and blastocyst formation derived from oocytes recovered from queen ovaries of three distinct status during NBS

	Inactive (%)	Luteal (%)	Follicular (%)
Total of fertilized	272	162	134
Cleaved zygotes	67 (24.63)[a]	35 (21.6)[a]	33 (24.62)[a]
Morulae	45 (16.54)[b]	21 (12.96)[b]	22 (16.41)[b]
Blastocyst	22 (8.09)[c]	14 (8.64)[c]	11 (8.21)[c]

Different letters in the same column indicate statistically significant difference (Chi-square test, P<0.05).

Table 2. Absolute and relative frequency of cleavage, morulae and blastocyst formation derived from oocytes recovered from queen ovaries of three distinct status during BS

	Inactive (%)	Luteal (%)	Follicular (%)
Total of fertilized	102	198	86
Cleaved	66 (64.7)[a]	127 (64.14)[a]	55 (63.95)[a]
Morulae	42 (41.17)[b]	80 (40.41)[b]	34 (39.54)[b]
Blastocyst	24 (23.53)[c]	47 (23.73)[c]	21 (24.41)[c]

Different letters in the same column indicate statistically significant difference (Chi-square test, P<0.05).

DISCUSSION

It has been shown by Freisted et al. (2001) that oocyte nuclear maturation *in vitro* is depressed during the months in which day length decreases. The ability to form cleaved embryos, morulae and blastocysts remains low due to this poor oocyte IVM. This information corroborates with our results. Those authors evaluated the effects of season (January-March (I) and, October-December (IV) corresponded to the non-breeding season -; April-June (II) and July-September (III) corresponded to the breeding season) and ovarian status (freshly ovulated, follicular, luteal, intermediate, or inactive) on the efficiency of the *in vitro* production of domestic cat embryos. Our Grade 1 COC recovery rate/donor during non-breeding season was similar to that obtained in their study (23.65±0.8 *vs*. 18.8±0.4, respectively, *P*<0.05). However the rates obtained by Freisted et al. (2001) were significantly lower when compared to the results obtained in our study when breeding season was considered singly (48.20±1.2 *vs*. 18.8±0.4, respectively, *P*<0.05). Those authors also assumed that the average number of COC recovered per donor was not influenced by season, contradicting our results.

This difference may be explained by the period and by the temperature of the storage of those ovaries. In their study, ovaries were stored at room temperature if oocytes were recovered within 8h or at 4°C if stored overnight. We decided to transport Grade I COC at 4°C using ice packs in a thermal box and harvest them within 4h after gonadectomy. According to Wolfe et al. (1996) when ovaries were stored for 24 h, fertilization success was higher (*P*<0.05) than in the 48 and 72h groups, and, although 9.1% of inseminated oocytes from the 24 h storage group developed to blastocysts, none (P<0.05) achieved this stage after 48 or 72 h of storage. They demonstrated that COC recovered from ovaries stored at 4 degrees C for up to 72 h are capable of reaching telophase I or metaphase II *in vitro*. However, only oocytes stored within the ovary for 24 h produced blastocysts, indicating that the ability to achieve nuclear maturation is an inadequate indicator of fertilization and developmental competence.

Data obtained by Wlodarczyk et al. (2009) indicated that storage of domestic cat ovaries at room temperature, even for a short time, can negatively influence the compe-

tence of oocytes to undergo nuclear maturation.

Freisted et al. observed that after IVM/IVF, cleavage rates were significantly higher (P<0.05) during breeding season than during non-breeding season. However blastocyst rates on Day 6 were higher during in seasons I , II, and III but were significantly lower (P<0.01) in season IV The corresponding blastocyst rates on Day 8 were similar between seasons I, II and III but varied significantly from season IV (28.9% ±1.3%, 33.7% ±1.6%, 37.9% ±2.3%, and 23.6% ±2.6%). Authors were not able to explain which factors caused the impaired developmental competence of COC recovered in season IV.

The breeding season in our study presented superior frequencies of cleavage and morulae and blastocyst formation as observed in another study (Comizzoli et al. 2003) where it was suggested that appropriate mechanisms, perhaps seasonal variation in FSH receptors or lack of antioxidant capacity of the cumulus-oocyte complex are inadequate during non-breeding season.

Karja et al. (2002) observed that the number of oocytes reaching cleavage stage and development to the morula and blastocyst stages from follicular stage ovaries were significantly lower (P<0.05) than those obtained from inactive and luteal stage ovaries. According to these authors, these results indicate that the donor's reproductive cycle had no apparent effects on the frequencies of maturation and fertilization of cat oocytes, although it did influence developmental competence of the oocytes following IVM and IVF. In contrast, we found no significant effect (P<0.05) of ovarian status; luteal, follicular, and inactive ovaries on the frequency of cleaved embryos and morulae and blastocyst formation (P<0.05). It is difficult to compare our results to those obtained by Karja et al. and by Johnston et al. (1989) because the difference in the frequencies of development of the blastocyst stage may be attributable to different culture systems or different genetic background of donor cats

Although embryos could be produced throughout the year, the efficiency was significantly affected by season but not by the ovary type.

In the conditions of our study, ovarian condition did not influence the frequency of cleaved embryos and morulae and blastocyst formation (P<0.05). However, there were significantly higher during breeding season compared to non-breeding season.

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