Ciência Animal, 27 (1): 41-54, 2017.

AVALIAÇÃO DA ATIVIDADE DA PROTEÍNA QUINASE P34^{CDC2} NA MATURAÇÃO *IN VITRO* DE OÓCITOS CANINOS

(Evaluation of p34^{cdc2} kinase activity in the in vitro maturation of canine oocytes)

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ABSTRACT

The control of the cell cycle is regulated by a cascade of coordinated events that can act by influencing the expression or repression of the activity of proteins related to meiosis resumption. Studies have indicated that the activity of these proteins is time-dependent in the *in vitro* maturation process (IVM). This work aimed to evaluate the kinetics of the p34^{cdc2} kinase activity during IVM of canine oocytes. Ovaries were obtained from 40 bitches submitted to elective ovary-salpingo-hysterectomy (OSH). After OSH, ovaries were immediately transported at temperature of 4 °C. In the laboratory, ovaries were sliced for the release of cumulus-oocyte complexes (COCs). Only grade 1 COCs were selected and placed in maturation medium for a period of 24, 48 and 72 h of maturation. After culturing, COCs were plated of 0.2% hyaluronidase solution for complete removal of cumulus cells. The activity of the p34^{cdc2} protein was detected by ELISA. Based on the results, it was observed that the activity of the protein is time-dependent, peaking after 48 hours of IVM (p 0.01). After 72 hours, activity declined. Based on this study, it could be concluded that the p34^{cdc2} protein kinase plays a very important role in the meiosis progression in bitches. Thus, a better understanding of this protein as well as of others that participate in the maturation process may contribute to the establishment of more adequate media to significantly improve maturation rates.

Keywords: Protein kinase, oocytes, p34^{cdc2}, maturation, bitches.

RESUMO

O controle do ciclo celular é regulado por uma cascata de eventos coordenados que podem atuar influenciando na expressão ou repressão da atividade de proteínas relacionadas a retomada da meiose. Estudos indicam que a atividade dessas proteínas mostra-se tempo-dependente no processo de maturação *in vitro* (MIV). Esse trabalho teve o objetivo de avaliar a cinética da atividade quinase p34^{cdc2} durante a MIV de oócitos caninos.

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Os ovários foram obtidos de 40 cadelas submetidas à ovário-salpingo-histerectomia (OSH) eletiva. Após a OSH, os ovários foram imediatamente transportados a uma temperatura de 4 ºC. No laboratório, os ovários foram seccionados em fatias finas ("*slicing*"), para a liberação dos complexos *cumulus*-oócito (COCs). Apenas os COCs grau 1 foram selecionados e colocados em meio de maturação por um periodo de 24, 48 e 72 h de maturação. Após o cultivo, os COCs foram colocados em placas contendo solução de hialuronidase 0.2% para a retirada completa das células do *cumulus*. A atividade da proteina $p34^{cdc2}$ foi detectada por ELISA. Com base nos resultados verifica-se que a atividade da proteina mostra-se tempo-dependente, atingindo o pico após 48h de MIV ($p<0,01$). Após 72h, a atividade demonstrou um decréscimo. Com base neste estudo pode-se concluir que a proteina quinase $p34^{cdc2}$ desempenha uma função de suma importância na progressão da meiose em cadelas. Dessa forma, a compreensão melhor dessa proteina assim como de outras que participam do processo de maturação poderá contribuir para o estabelecimento de meios mais adequados que melhorem significativamente as taxas de maturação.
Palavras chave: Proteina quinase, oócitos, $p34^{cdc2}$, maturação, cadela.

INTRODUCTION

In mammals, when removed from the follicular environment, oocytes no longer receive the signal that maintains meiotic block and resume meiosis. In bitches, although the oocyte may spontaneously resume *in vitro* meiosis, maturation rates are very low and the percentage of oocytes remaining in the germinal vesicle (GV) is high. During ovogenesis, the information required for the initial embryonic development is transcribed and stored as mRNA or translated and stored as proteins (ALLARD *et al*., 2005). Any alteration in the synthesis and consequently in the stock of these proteins can have serious consequences in oocyte development.

The resumption of the meiotic cell cycle is mainly influenced by the meiosis promoter factor (MPF), which is one of the main regulators of morphological changes that occur during oocyte maturation, regulating chromosome condensation, nuclear envelope rupture and the reorganization of microtubules (MOTLIK, 1998).

MPF is a heterodimeric protein belonging to the family of kinases, consisting of a catalytic subunit, the cyclin-dependent kinase called cdk1 or $p34^{cdc2}$, which controls cell division and a regulatory subunit, cyclin B1 (MERMILLOD, 2000). Meiosis progression requires both cyclin B synthesis and its cytoplasmic shift to the

nucleus, along with dephosphorylation of the catalytic subunit.

Kinases and phosphatases are involved in resumption and complete meiotic maturation of the oocyte. In the growth phase, oocytes have very low $p34^{cdc2}$ levels and are not able to progress from the G2 phase (interval between DNA synthesis and cell division) to the M phase (cell division, meiosis). The acquisition of meiotic competence is associated with the activation of $p34^{cdc2}$ at the end of oocyte development and with the presence of adequate amount of cyclin B1 to provide sufficient raw material for pre-MPF activation (DE VANTERY *et al.*, 1996). In its inactive form, pre-MPF, the catalytic subunit is phosphorylated into Thr14 (threonine 14) and Tyr15 (tyrosine 15) residues (GAUTIER *et al.*, 1988). MPF is activated when the two subunits, $p34^{cdc2}$ and cyclin B, are associated and Thr14 and Tyr15 residues are dephosphorylated, whose reaction is catalyzed by cdc 25 phosphatase enzyme. Dephosphorylation of these residues as well as the subsequent conversion of MPF-inactive to MPF-active is the key to G2/M passage (CURCIO *et al.*, 2006). However, it is still unclear which of the heterodimeric subunits of mammalians MPF plays a determinant role in the control of MPF activity (LEDAN *et al.*, 2001).

Kanatsu-Shinohara *et al.* (2000) compared the concentrations of $p34^{cdc2}$ and cyclin B1 in incompetent and competent mouse oocytes to resume meiosis and found that the concentrations of both are approximately three times higher in competent oocytes when compared to oocytes unable to resume meiosis. However, the cyclin B1 concentration was up to seven times higher than that of $p34^{cdc2}$ in both stages. According to these authors, since cyclin B1 is in excess in both immature and mature oocytes, $p34^{cdc2}$ would play a more central role in the regulation of the MPF activation.

The aim of this study was to evaluate the influence of $p34^{cdc2}$ kinase activity at different moments of the *in vitro* maturation of canine oocytes.

MATERIAL AND METHODS

Animals

Ovaries were obtained from 40 healthy adult domestic bitches of different breeds that underwent ovariohysterectomy at the Small Animals Reproduction Service of the Department of Animal Reproduction and Veterinary Radiology, Faculty of Veterinary Medicine and Animal Science, UNESP. The study was accepted by the Ethics Research Committee of FMVZ – UNESP, campus

of Botucatu, under protocol number 176/2011.

Obtaining and classifying oocytes

After OSH, ovaries were aseptically isolated, immersed in physiological solution (0.9% NaCl) and immediately transported at 4 °C to the Laboratory of Reproduction of Small and Wild Animals, where they were processed within 6 hours after removal. In the laboratory, ovaries were transferred to sterile glass Petri dishes containing PBS/PVA solution heated at 37 °C added of 10% fetal bovine serum (FBS) (Fig. 1A). In this solution, they were sliced (Figs. 1B and 1C), along their length and width for the release of cumulus-oocyte complexes (COCs).

COCs were identified and quantified using a stereomicroscopic magnifying glass (Leica® MZ 12.5) and evaluated for homogeneity, cytoplasm staining and number of cumulus cell layers, according to morphological criteria adopted by Hewitt and England (1997) (Fig. 1D). Thus, only grade 1 COCs, that is, those with homogeneous, dark ooplasm and completely surrounded by one or more cumulus cell layers, were selected for this study.

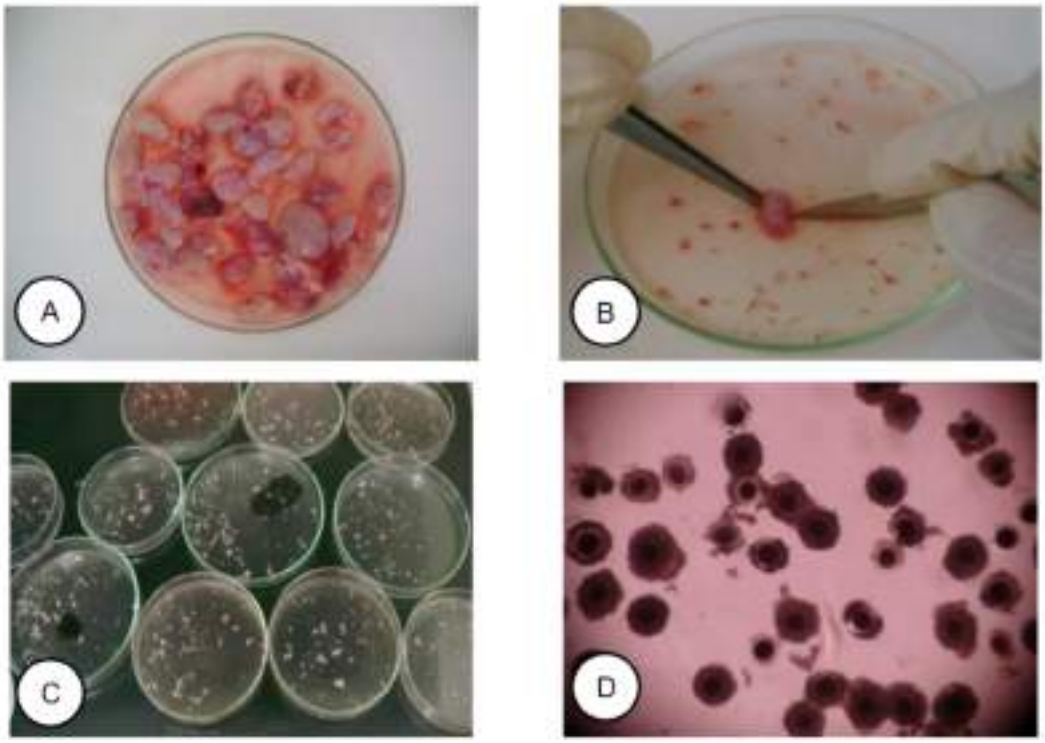

Figura 1: (A) Ovaries of bitches to be processed; (B) Slicing of an ovary; (C) Petri dishes with sliced ovaries; (D) Selection and classification of oocytes. Magnification of 40x. Botucatu, 2014.

During the selection process, COCs were washed three times in TCM-199 washing medium supplemented with 25mM HEPES, 0.2mM sodium pyruvate, 5mM sodium bicarbonate and 75 µg / mL gentamicin.

In vitro maturation (IVM)

After selected and washed, grade 1 COCs were divided into groups of up to 20 oocytes and transferred to 4-well culture plates containing 500 µL of maturation medium. The medium used was TCM 199 supplemented with 25 mM HEPES, 50 µg / ml gentamicin, 26 mM sodium bicarbonate, 1.5 mM sodium pyruvate, 2.9 mM sodium lactate pentahydrate, 0.6 mM cysteine, 0.03 IU / mL hCG, 0.5 µg / ml FSH, 20 µg / mL E_2 and 10 ng / mL epidermal growth factor (EGF) (SUZUKAMO *et al.*, 2009). The cultures were perfomed at 38 °C in a humidified atmosphere of 5% CO_2 for a period of 24, 48 or 72 hours.

At the end of each culture period, COCs were placed in plates containing 200 µL 0.2% hyaluronidase solution for 5 minutes and repeatedly aspirated with the 50 µL pipette for complete removal of cumulus cells. After complete removal, oocytes were then washed in PBS/PVA for hyaluronidase inactivation. Oocytes were then transferred to PBS solution supplemented with 3.7% paraformaldehyde, washed again in PBS and stained with 10 µg / ml Hoechst 33342. Oocytes were placed between slide and coverslip, and evaluated under light and fluorescence microscopy (Leica® DFC 310 FX) for evaluation of nuclear maturation.

Oocyte extract preparation

Oocyte extract was prepared according to methodology described by Suzukamo *et al.* (2009) at times 0, 24, 48 and 72 hours. After completion of each maturation time, oocytes were washed several times in PBS/PVA and transferred to a graduated conical glass tube containing a buffer solution (lysing solution) consisting of TRIS-20 mM, 150 mM NaCl, 1.0 mM EDTA, 1.0 mM EGTA, 1% Triton X-100, 2.5 mM sodium phosphate, 1.0 mM β-glycerophosphate, 1.0 mM Na_3VO_4, 1.0 mg / mL leupeptin and 1.0 mM phenylmethylsulfonyl fluoride (PMSF), to prepare oocyte extract. The proportion of 5µL of buffer solution for five oocytes was used. After this process, the glass tube was placed in styrofoam containing liquid nitrogen for three minutes. Then, this tube was transferred to Sonifier (Branson Digital Sonifier®) for complete oocyte fragmentation and consequent release of

its proteins. The oocyte extract was sonicated five times for 25 seconds, with an interval of one minute. At the end of this procedure, the oocyte extract was transferred to an identified microtube and stored at -80 °C.

Measurement of the $p34^{cdc2}$ kinase activity

The extract of lysed oocytes (5 oocytes / 5 µL buffer solution) was mixed with 45 µL kinase-A buffer solution composed of 25 mM HEPES buffer (pH = 7.5), 10 mM $MgCl_2$ (MBL), 0.1 mM ATP and 10% mouse vimentin peptide solution. This mixture was incubated at 30 °C for 30 minutes. The reaction was terminated with the addition of 200 µL PBS containing 50 mM EGTA (MBL). Subsequently, the extract was centrifuged for 15 seconds and the mouse vimentin peptide phosphorylation was detected by ELISA (MESACUP® cdc2 Kinase Assay Kit).

Statistical analysis

Logistic regression models (SAS Institute, 2011) were constructed in order to estimate the chances of oocytes to be observed at germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (IM), metaphase II and degenerate (DEG) maturation stages in different growing times (24h, 48h and 72h). The ELISA technique was performed three times and each experiment was replicated four times. The measurement of $p34^{cdc2}$ kinase activity during times 0, 24, 48 and 72 hours of canine oocyte was performed by analysis of variance in a completely randomized design, followed by Tukey's test for multiple comparisons between means. The significance level used to reject H_0 (null hypothesis) was 5%, that is, for $p<0.05$ (significance level less than 0.05).

RESULTS AND DISCUSSION

The chances of observing an oocyte at different times of *in vitro* maturation are presented in Tab.1. This tab shows that the chances of finding an oocyte in the GV stage in time of 24 h were 3.15 times greater than in 72h ($p<0.0001$). Similarly, the chances of finding an oocyte at the VG stage were 2.4 times greater at 48 h when compared to 72 h ($p<0.0001$). In the time of 24h, there were 1.26 times more chances of having an oocyte at GVBD than in 48h ($p = 0.0002$) and 1.42 times more chance than in 72 hours ($p = 0.0002$). Thus, these results showed that earlier stagies (GV and GVBD) are most present in the first hours of culture *in vitro* and in the dog, even after 48 hours, there is the presence

of oocytes in the early stages of development.

Tabela 1: Odds ratio of finding an oocyte at different stages of nuclear maturation (GV, GVBD, M-I, M-II and DEG) at 24, 48 and 72h in *in vitro* culture medium. Botucatu, 2014.

Variable response	Culture time	RC	CI (95%)	p-Value
GV				<0.0001
	24h	1.36	0.86-2.16	
	48h	Reference		
	24h	4.27	2.37-7.68	*
	72h	Reference		
	48h	3.14	1.71-5.76	*
	72h	Reference		
GVB				0.0002
	24h	1.65	1.08-2.54	*
	48h	Reference		
	24h	2.56	1.64-4.01	*
	72h	Reference		
	48h	1.55	0.97-2.47	
	72h	Reference		
M-I				< 0.0001
	24h	0.37	0.25-0.56	*
	48h	Reference		
	24h	0.32	0.22-0.48	*
	72h	Reference		
	48h	0.86	0.62-1.20	
	72h	Reference		
M-II				0.0001
	24h	1.25	0.28-5.64	
	48h	Reference		
	24h	0.17	0.06-0.49	*
	72h	Reference		
	48h	0.13	0.04-0.45	*
	72h	Reference		
DEG				0.01
	24h	1.02	0.55-1.88	
	48h	Reference		
	24h	0.53	0.31-0.91	*
	72h	Reference		
	48h	0.52	0.30-0.90	*
	72h	Reference		

In vertebrates, oocytes remain for several weeks, months or years in the prophase of the first meiotic division. During this long period, they gradually accumulate mRNA, protein, lipid and sugar molecules as they grow in size. This essential stock for the supply of energy and information during oocyte growth is paramount for the final stage of oogenesis, i.e., oocyte maturation. Oocytes that do not acquire competence do not complete cytoplasmic maturation (MOUROT *et al.*, 2006).

During meiosis resumption to the maternal-zygotic phase, transcription occurs at a very low level, so, oocytes need to have protein and mRNA supplies to meet demand during maturation, fertilization and early embryonic development (RACEDO *et al.* 2008). If this information is absent or insufficient, there may be failures in nuclear or cytoplasmic maturation, or both, impairing subsequent development. Studies have shown that mRNA levels encoding such proteins are related to maturation time and oocyte quality (CALDER *et al.*, 2005). Thus, it is of paramount importance knowing which proteins are most important in meiosis control; which may be acting in an antagonistic or synergistic way, accelerating or delaying the meiotic process; or even if proteins identified in other species as responsible for acquiring competence are being expressed in bitches.

The culture media used for the maturation of canine oocytes are developed based on adaptations of media used for other animals. In spite of the numerous studies aiming to develop a maturation medium based on the endocrine and metabolic needs of bitches, the oocyte indexes that reach the final stages of maturation are very low, making it impossible to develop reproductive biotechnologies. Thus, the study of proteins, such as $p34^{cdc2}$, that are involved in the meiosis control, can elucidate what maintains the majority of oocytes at the GV stage in this species.

According to Kovo *et al.* (2006), the MPF concentration oscillates among cell divisions. MPF shows a marked activity in oocytes during stages of meiotic division resumption, reaching its highest level at meiosis I. The decrease in the concentration of this factor is observed during the transition from anaphase to telophase, before the first polar corpuscle is released. A study in rats demonstrated that MPF is high in the meiosis resumption before GVBD - nucleolus dissolution, chromosomal condensation, microtubular reorganization and

dissolution of the nuclear envelope, reaching maximum level at M-I (metaphase plate formation), declining before formation of the first polar corpuscle and increasing again before M-II. According to the present study, it was observed that the $p34^{cdc2}$ protein kinase, as well as MPF, oscillates among the different times of *in vitro* maturation, exerting different function at each stage of the cell cycle. Before maturation and in the first 24 hours, an increase is observed, but not significant, which shows that in the early stages of development, this protein is still in the process of being translated. In the first 24 hours, it is possible to observe, according to the results found, the highest oocyte index in GV, since this period showed 3.15 times more chances of having an oocyte at this stage than the time of 72 hours and 2.4 times more chances than the time of 48h. Thus, synthesis of the $p34^{cdc2}$ protein in the first 24 hours of *in vitro* maturation may not be sufficient to promote meiosis resumption in the canine species.

In a study carried out with mice through the treatment of oocytes with protein synthesis inhibitor, cyclohexamide, it was suggested that the increase in cyclin synthesis may be responsible for the regulation of MPF due to the decrease in the activation of MPF and to meiosis resumption (HAMPL and EPPIG, 1995). In this research, oscillation of $p34^{cdc2}$ protein kinase at different maturation times, as well as changes in MPF levels during the different maturation stages have been reported in literature. Thus, as reported by these authors, it could be inferred that this protein may exert influence on the regulation of MPF and consequently on cell cycle control.

According to Josefsberg *et al.* (2003), the decline of MPF activity between meiosis I and II divisions occurs due to the cyclin B degradation process. In this study, there was a significant increase ($p<0.001$) in $p34^{cdc2}$ protein kinase between 24h and 48h, reaching peak in 48h and then there is a decline up to 72h. In view of these data, it may be suggested that this protein may play a fundamental role in different stages of the cell cycle, such as in meiosis resumption, transition from GV to germinal vesicle breakdown (GVBD), or as a determinant for the progression for metaphase I (MI) and metaphase II (M-II) stages. Higher chances of obtaining oocytes at VG and GVBD were observed for time of 24 h. However, the time of 48h showed 2.7 times more chances of obtaining oocyte at M-I stage compared to time of 24h ($p<0.0001$). The chances of obtaining oocytes at M-I and M-II stages increase after 72 h of culture. As after 48h, there is

a decline in the activity of $p34^{cdc2}$ protein kinase, and it can be assumed that it can act as a raw material at crucial moments during cell cycle. The functions of this protein can be observed in different ways: directly controlling meiosis, mutually interacting with cyclin B1 to act in meiosis resumption or even serving as raw material for MPF activation.

A study conducted by Suzukamo *et al.* (2009) demonstrated increase of $p34^{cdc2}$ protein kinase during maturation, observing a peak of its activity at 72 h of culture and a decline after that time. According to these authors, the time of 72h would be ideal for the *in vitro* maturation of canine oocytes. In this present study, changes in the $p34^{cdc2}$ kinase activity during times of 0, 24, 48 and 72 h of canine oocyte IVM are shown in Fig.3. The $p34^{cdc2}$ kinase activity at time 24h when compared to 48h showed a highly significant difference ($p<0.001$). This difference was also observed comparing times of 48h and 72h. It was observed that the $p34^{cdc2}$ kinase activity peaked at 48h and decreased at 72h of maturation time. In this way, the time of 48h could be indicated as recommended for *in vitro* culture. However, according to Tsutsui *et al.* (1989) and Reynaud *et al.* (2005), oocytes require more than 48 hours to complete nuclear maturation within the oviduct. There is controversy over culture time but it is evident that for oocyte competence to occur, the medium must provide nutrients, growth factors and proteins that simulate the *in vivo* environment. Unlike Suzukamo *et al.* (2009), this study used epidermal growth factor (EGF) in the maturation medium. This factor has been associated to cascades of cellular signaling. Studies on porcine oocytes have demonstrated that EGF-induced maturation promotes MAPK phosphorylation, raising the hypothesis that gonadotropin-induced maturation occurs due to the action of EGFR and MAPK. EGF regulates MAPK activity by binding to EGF (EGFR) receptors and EGFR inhibition is responsible for blocking meiosis resumption induced by LH and FSH. Thus, EGF, acting on MAPK phosphorylation, may have exerted influence on the phosphorylation of protein kinases during maturation progression.

Thus, it is suggested that only the evaluation of the $p34^{cdc2}$ protein kinase activity is not enough to understand the modifications that occur during the cell cycle, requiring further studies on other signaling pathways that interfere in meiosis, as well as the evaluation of the phosphorylation of proteins involved in this process.

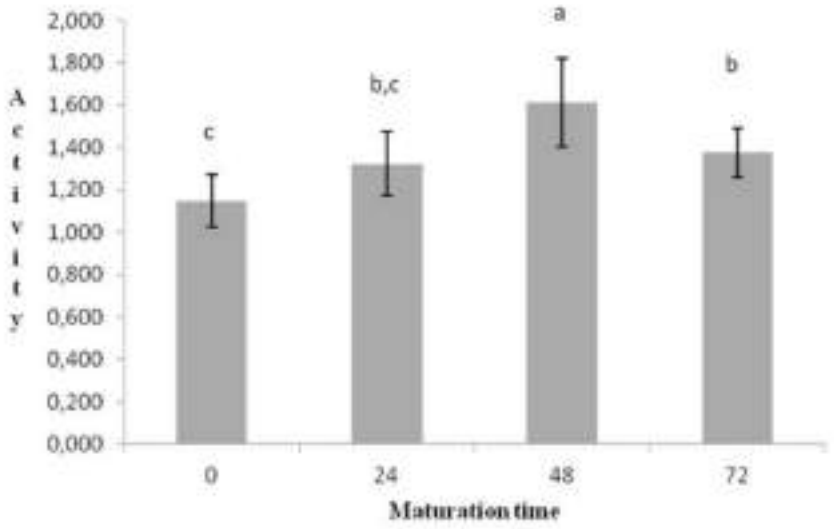

Figura 3: Measurement of the $p34^{cdc2}$ kinase activity in times of 0, 24, 48 and 72 hours during IVM of canine oocytes. Botucatu, 2013.

With this broader understanding, one can evaluate how oocyte competence occurs in this species and determine the most suitable medium and culture time.

According to Tay *et al.* (2000), the administration of cyclin B1 mRNA in oocytes accelerates the maturation rates in mice. The best understanding of which of these proteins are most important in the recovery and control of the cell cycle can influence the development of a culture medium that can more appropriately simulate the *in vivo* environment of female dogs. By identifying what is preventing the progression of meiosis in this species, if they are changes related to gene expression, transcription or translation of proteins, more efficient means of maturation that promote better meiosis resumption and progression to the final stages of nuclear maturation can be developed.

CONCLUSION

Based on the results of this study, it could be concluded that the $p34^{cdc2}$ protein kinase plays a very important role in the progression of meiosis in bitches. Better understanding of this protein as well as of others that participate in the process of cell maturation and signaling may contribute to the establishment of more adequate media that significantly increase maturation rates, allowing the development of reproductive biotechnologies in this species.

FUNDING SOURCES

São Paulo Research Foundation – FAPESP, number 2013/21667-3 and 2014/19776-1.

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