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Effect of maturation medium on *in vitro* cleavage of canine oocytes fertilized with fresh and cooled homologous semen

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Summary

This study evaluated the effect of three maturation media on the development of *in vitro*-matured and *in vitro*-fertilized dog oocytes. In Experiment 1 (non-comparative experiment) canine cumulus–oocyte complexes (COCs) were matured *in vitro* in TCM199 supplemented with estrous cow serum (10%) + gonadotropins + steroid (treatment A), TCM199 + estrous cow serum (10%) (treatment B), or TCM199 + polyvinylpyrrolidone (PVP) (4%) (treatment C). All maturation media contained a final concentration of 1 μg/ml of human somatotropin (hST). Oocytes were fertilized with fresh ejaculated sperm and development was assessed by cleavage. The objective of Experiment 2 (comparative experiment) was to compare the rates of cleavage and developmental capacity of COCs matured *in vitro* in same medium as in Experiment 1, and fertilized either with fresh ejaculated or with cooled extended homologous spermatozoa. In Experiments 1 and 2, oocytes fertilized with fresh semen were *in vitro*-matured for 48 h, while in Experiment 2 COCs fertilized with cooled semen were matured *in vitro* for 72 h. The results of Experiments 1 and 2 demonstrated that cleavage was not influenced by the oocyte's maturation environment. The results of Experiment 1 showed that pronucleus formation + cleavage (day 7 after IVF) was similar among treatments A, B and C ($p = 0.277$). Also, in Experiment 2, pronucleus formation + cleavage (day 7 after IVF) was not different for oocytes fertilized *in vitro* either with fresh or cooled semen and maturated in media A ($p = 0.190$), B ($p = 0.393$) or C ($p = 0.687$). In both experiments, the numbers of embryos that developed to the 6–8-cell stage were higher for oocytes matured in medium A and fertilized with fresh semen, when compared with numbers of oocytes matured in media B and C. Embryo development to the 6–8-cell stage of oocytes fertilized either with fresh or cooled sperm was observed in treatments A and C in Experiment 2. Cumulus cell expansion was similar among treatments in Experiment 1. In Experiment 2, cumulus cell expansion among treatments A, B and C was similar after 48 h or 72 h of IVM. In both experiments, the greatest expansion category seen was for category 2 (outer cumulus cells slightly expanded). No correlation between cumulus expansion and cleavage were observed. Polyspermy rates in oocytes matured in medium A, and fertilized with fresh sperm were not significantly different from polyspermy rates observed using media B and C, in both experiments. Our findings indicate that treatments A, B and C are similarly effective for the cleavage of dog oocytes. Furthermore, it was demonstrated that canine oocytes matured *in vitro* could be fertilized by homologous cooled spermatozoa and progress to cleavage.

Keywords: Cleavage, Dog, Oocyte, Sperm, Zygote

Introduction

World habitat destruction and pollution have lead to the development of strategies aimed at protecting and

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saving various species of plants and animals, including various canids. Unfortunately, animals continue to be killed by undesirable means, which include hunting, ecotourism, illegal pet trade and road accidents. With only a few inspectors to protect wildlife, biosecurity has become very fragile. Therefore, efforts to safeguard animal species that are living in areas with a severe risk to life preservation are needed.

Studies with *in vitro* fertilization of gametes, using the domestic dog as a model, are useful to expand knowledge in reproductive performance of various canids, as domestic and wild species share a common origin. All dogs are descended from the wolf and their reproductive cycles are nearly identical. In this way, *in vitro* procedures are supposed to serve to rehabilitate wildlife. Methods, such as *in vitro* embryo production, oocyte and embryo cryopreservation of canids, do offer the opportunity to recover infertile or subfertile populations. Other methods, such as short-term storage of sperm, might also be useful for successful *in vitro* procedures under conditions in which the female and male gametes are not synchronous and would be disposable after the claimed period for IVF.

In canine species, the establishment of *in vitro*-maturation protocols, with the aim to promote and maintain competence of the cumulus–oocyte complexes (COCs) for fertilization and cleavage, is still required. Despite the fact that various approaches (Cinone *et al*., 1992; Nickson *et al*., 1993; Srsen *et al*., 1998; Hewitt & England, 1999; Songsasen *et al*., 2002; Willingham-Rocky *et al*., 2002) have been tried in order to attempt *in vitro* meiosis of dog oocytes, acceptable rates of meiotic response for *in vitro*-matured bitch oocytes have not been obtained with most media. Major reasons point to difficulties in identifying which components are lacking in the medium, or which are acting as detrimental or suppressive compounds, or simply because the concentration of a substance needed to induce a response is unknown.

The objective of Experiment 1 (non-comparative experiment) in this study was to evaluate the developmental competence of *in vitro*-matured dog oocytes in three different media for *in vitro* fertilization with fresh ejaculated spermatozoa and cleavage. The objective of Experiment 2 (comparative experiment) was to compare the capability of oocytes, which had been matured *in vitro* in three different media and fertilized either with fresh or cooled extended homologous sperm, for cleavage and embryonic development. To our knowledge, a correlation between *in vitro* expansion of COCs and developmental competence of canine oocytes has not been reported in the literature and was also investigated. The results can serve as a useful guide to *in vitro* embryo production of domestic and wild canids.

Materials and methods

Oocyte collection and maturation

The experiments were performed using oocytes retrieved by slicing the ovaries of bitches at various unknown reproductive stages. Ovaries were collected at two centres for the control of carnivore populations: (1) Centro de Zoonoses – Estrada Bérico José Bernardes, 3489 – (Porto Alegre/RS; Brazil); (2) Associação Leopoldense de Proteção aos Animais (ALPA) – (São Leopoldo/RS, Brazil).

The mean age of 18 bitches used as ovary donors in Experiment 1 was 28.5 months (12–96) and in Experiment 2, the mean age of seven bitches used as ovary donors was 42.2 months (8–96).

Ovaries were transported to the laboratory at ambient temperature either in saline (0.9% NaCl) or phosphate-buffered saline (PBS). Mincing of ovaries was carried out in PBS supplemented with foetal calf serum (FCS) (0.5%). Maximal interval time from ovary collection to placement of oocytes in maturation medium was approximately 2 h. After slicing, the COCs with one or more layers of intact cumulus cells and dark regular and homogeneous cytoplasm (subjectively established as high quality oocytes) – Experiment 1: $n = 253/1155$ (21.9%); Experiment 2: $n = 342/1106$ (30.9%) – were randomly allocated into culture dishes containing four droplets with 100 μl of equilibrated IVM medium each with the following treatments: (A) TCM199 supplemented with 25 mM HEPES (v/v) (Sigma, M2520), 10% heat-inactivated estrous cow serum (ECS), 50 μg/ml gentamicin (Sigma, G1264), 2.2 mg/ml sodium bicarbonate (Sigma, S5761), 22 μg/ml pyruvic acid (Merk, 1.06619.0050), 20 μg/ml estradiol (Sigma, E 8875), 0.5 μg/ml FSH (Follitropin-V, Vetrepharm Inc.), 0.03 IU/ml HCG (Profasi HP) and 1 μg/ml human somatotropin (hST) (Lilly, FF1D44C) (control medium)]; (B) TCM199 supplemented with 25 mM HEPES/l (v/v) (Sigma, M2520), 10% heat-inactivated estrous cow serum (ECS) and 1 μg/ml human somatotropin (hST) (Lilly, FF1D44C); (C) TCM199 supplemented with 25 mM HEPES/l (v/v) (Sigma, M2520), 4 mg/ml polyvinylpyrrolidone (PVP) (Sigma, P0930) and 1 μg/ml human somatotropin (hST) (Lilly, FF1D44C).

Cumulus–oocyte complexes fertilized *in vitro* with fresh semen were previously matured for 48 h at 37 °C in a humidified atmosphere of 5% CO_2 in air, while COCs fertilized *in vitro* with cooled semen were previously matured for 72 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. At the end of maturation period (48 h or 72 h), oocytes were evaluated for expansion on a four-point scale as previously reported (Ball *et al*., 1980). Briefly, complexes were rated as

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follows: 1 (compact, unexpanded complexes), 2 (outer cumulus cells slightly expanded), 3 (outer and inner cumulus cells slightly expanded) and 4 (fully expanded complexes). Oocyte maturation was followed by *in vitro* fertilization either with fresh or cooled extended homologous sperm and presumptive zygotes were cultured for up to 7 days.

Semen collection and spermatozoa assessing viability

A total of 12 ejaculates was used in Experiment 1. Semen was collected from a healthy known and *in vitro* fertility proven Whippet dog (aged 5 years). A total of six ejaculates from the same dog was used in Experiment 2. The samples were collected by digital manipulation in fractions (presecretion, sperm-rich and sperm-free fractions). Only the sperm-rich fraction was used for experiments. Sperm quality was observed by evaluating the sperm motility and the percentage of progressive motility, which were performed in all samples in Experiments 1 and 2 of this study. Sperm quality was assessed at all steps of semen preparation: following collection of spermatozoa, after washing by Percoll gradient and preceding IVF. Motility criteria followed the classification of Mies Filho (1987). The percentage of total motile sperm (number of sperm with progressive motility/the total number of sperm observed) was assessed in 10 μl drops of sperm suspension, using a phase contrast microscope (×200).

Semen preservation

After collection, all ejaculates of sufficient quality were transported diluted (1:1) in Egg Yolk Tris extender (Günzel-Apel, 1994) at 26 °C in water bath to the laboratory.

In Experiment 2, the final volume, which was comprised by the ejaculate and extender, was divided into two samples, from which one half was used fresh to fertilize the oocytes matured for 48 h, while the second half was chilled and used afterwards to fertilize the oocytes matured for 72 h. The second half of the semen sample was placed in a 15 ml conical tube (Falcon #2095), and immersed in a 26 °C water bath inside a closed styrofoam box (17 × 22 × 14.5 cm). Sample was stored overnight at 4 °C in a refrigerator until IVF was carried out.

Sperm preparation

Aliquots of fresh and cooled diluted spermatozoa (∼1 ml) were pipetted into a 15 ml conical tube (Falcon # 2095) onto a two-layer discontinuous gradient formed by layering 1 ml of the 45% Percoll solution on top of 1 ml of 90% and centrifuged at 500 *g* for 20 min at room temperature. The supernatant was removed and the sperm pellet was washed with 5 ml of low bicarbonate–Tyrode's albumin lactate pyruvate (TALP) (Parrish *et al*., 1988) by centrifugation at 500 *g* for an additional 10 min.

The pellet was then recovered after aspiration of the supernatant, and the spermatozoa were resuspended to give a final concentration of 2×10^6 cells/ml in Fert–TALP medium supplemented with 0.56 μg/ml heparin to induce capacitation (Rodrigues *et al*., 2004). The cooled sperm samples were allowed to warm back up to room temperature before washing by Percoll.

In vitro fertilization (IVF) and *in vitro* culture (IVC) of oocytes

In Experiments 1 and 2, oocytes that had been previously matured either for 48 h or 72 h, were co-incubated at 37 °C with fresh or cooled sperm in a 5% CO_2 in air atmosphere. At 24 h after insemination, the presumptive zygotes were partially denuded by repeated aspiration into a pipette and transferred to 70 μ1 drops of Synthetic Oviduct Fluid (SOF) (Tervitt *et al*., 1972) medium containing 4 mg/ml BSA, with Na pyruvate, glutamine, gentamicin and amino acids. Incubation was carried out under mineral oil in the same environmental conditions used for IVF, and COCs were cultured *in vitro* for up to 7 days.

Microscopy of embryos

Presumptive embryos completely dislodged from cumulus cells were evaluated subjectively with stereoscopic microscopy before being fixed and stained as described previously (Rodrigues *et al*., 2004). Oocytes were mounted on slides, covered by coverslips, and afterwards fluorescence microscopy was performed to evaluate polyspermy, pronucleus formation, cleavage and embryonic development. Cleavage percentage was the per cent of total COCs that had more than one cell after fertilization. Polyspermy was the percentage of total COCs containing more than one decondensed sperm head, or one sperm head in the presence of more than one pronucleus, or more than two pronuclei (Kidson, 2004).

Statistical analysis

Data were evaluated by chi-squared analysis. Differences at the $p < 0.05$ level were considered significant. Experiment 1 was carried out using 12 replicates. Experiment 2 was carried out using six replicates.

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Table 1 Effect of different maturation media on IVF and cleavage of canine oocytes

Treatments	Oocytes cultured *n*	Pronucleus + cleavage *n* (%)	Others *n* (%)	Polyspermy *n* (%)
TCM199 + ECS + estradiol + FSH + HCG	86	05 (5.81)	64 (74. 41)	17 (19.76)
TCM199 + ECS	86	03 (3.48)	58 (67.44)	25 (29.06)
TCM199 + PVP	81	01 (1.23)	55 (67.90)	25 (30.86)

ECS, estrous cow serum; PVP, polyvinylpyrrolidone; TCM, tissue cultured medium; others, immature/degenerated/unidentified.
Data from 12 replicates.
$p \geqslant 0.05$.

Table 2 Embryo development of oocytes matured *in vitro* in three media

Treatment	Oocytes cultured *n*	Pronucleus + cleavage *n* (%)	Embryos 2–5 cells *n*	Embryos 6–8 cells *n*
TCM199 + ECS + estradiol + FSH + HCG	86	5 (5.81)	2	2
TCM199 + ECS	86	3 (3.48)	2	0
TCM199 + PVP	81	1 (1.23)	1	0

ECS, estrous cow serum; PVP, polyvinyl pyrrolidone; TCM, tissue culture medium; others, immature/degenerated/unidentified.
Data from 12 replicates.

Results

IVF Experiment 1 (non-comparative)

Final mean sperm motility of the 12 ejaculates was 71.66 ± 13.7% (± SEM) with a range of 50–95%. Sperm progressive velocity was 3.41% with a range of 3–5.

Seven embryos (2–8-cell stage) from a total of 253 fertilized oocytes (2.76%), were produced in this experiment, from which two achieved the 6–8-cell stage. These two embryos were derived from two out of 18 bitches used in the experiment. One 6–8-cell stage embryo was derived from a 2-year-old mixed-breed bitch, whose ovaries at the moment of retrieval showed no presence of follicles or corpora lutea at the surface. The other 6–8-cell embryo was derived from a 1-year-old Dackel bitch, whose ovaries at the moment of retrieval showed the presence of corpora lutea at the surface. The 6–8-cell stage embryos were from oocytes matured in medium A. However, cleavage rate did not differ among treatments ($p = 0.277$). The rates of pronucleus formation + cleavage are presented in Table 1, whereas the distribution of embryo development among treatments is presented in Table 2.

IVF Experiment 2 (comparative)

Fresh spermatozoa exhibited motility and progressive velocity similar to that exhibited by cooled spermatozoa. Percentage sperm motility, in six ejaculates from a donor's dog, was on average 70.8% for fresh samples versus 57.5% for cooled sperm samples. Sperm progressive velocity was 3.6 for fresh samples versus 3.1 for cooled sperm samples.

Ten embryos (2–8-cell stage) from a total of 342 fertilized oocytes (2.92%), were produced in this experiment, from which three achieved the 6–8-cell stage. These embryos were derived from one 5-year-old mixed-breed bitch, whose ovaries at the moment of retrieval showed the presence of corpora lutea at the surface. Embryos developing to the 6–8-cell stage were derived from oocytes matured in medium A and fertilized with fresh semen ($n = 2$), and from one oocyte matured in medium C, previously fertilized with cooled semen ($n = 1$). The distribution of embryo development among treatment groups is presented in Table 4. No significantly differences were observed among treatments A, B and C, when analyzing pronucleus + cleavage rates (Table 3). Similarly to what was observed in Experiment 1, abnormal embryos were not seen in Experiment 2.

Polyspermy in Experiments 1 and 2

Failed membrane block (polyspermic fertilization) consisted mainly as decondensing sperm heads in the oocytes, except for a polyspermic oocyte with pronucleus distribution type II (two pronuclei apposed with one or more supplementary pronucleus or decondensed sperm heads located eccentrically) (Kidson, 2004) (Fig. 1).

In Experiments 1 and 2 lower, but not significant, frequencies of polyspermy were observed in oocytes submitted to treatment A. In Experiment 2, 9.61% (5/52) of oocytes fertilized with fresh ejaculated

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Table 3 Cleavage *in vitro* of canine oocytes matured in three different media and fertilized with fresh and cooled sperm

Sperm/treatment	Oocytes cultured *n*	Pronucleus + cleavage *n* (%)	Others *n* (%)	Polyspermy *n* (%)
Fresh				
TCM199 + ECS + estradiol + FSH + HCG	52	4 (79)	43 (82.69)	5 (9.61)
TCM199 + ECS	54	2 (3.70)	40 (74.07)	12 (22.22)
TCM199 + PVP	63	2 (3.17)	47 (74.60)	14 (22.22)
Cooled				
TCM199 + ECS + estradiol + FSH + HCG	57	2 (3.50)	43 (75.43)	12 (21.05)
TCM199 + ECS	54	1 (1.85)	35 (64.81)	18 (33.33)
TCM199 + PVP	62	2 (3.22)	50 (80.64)	10 (16.12)

ECS, estrous cow serum; PVP, polyvinylpyrrolidone; TCM, tissue culture medium; others, immature/degenerated/unidentified.
Data from six replicates.
$p \geqslant 0.05$.

Table 4 Embryo development of oocytes matured *in vitro* in three media and fertilized with fresh or cooled sperm

Sperm/treatment	Oocytes cultured *n*	Pronucleus + cleavage *n* (%)	Embryos 2–5 cells *n*	Embryos 6–8 cells *n*
Fresh				
TCM199 + ECS + estradiol + FSH + HCG	52	4 (7.69)	1	2
TCM199 + ECS	54	2 (3.70)	2	0
TCM199 + PVP	63	2 (3.17)	2	0
Cooled				
TCM199 + ECS + estradiol + FSH + HCG	57	2 (3.50)	1	0
TCM199 + ECS	54	1 (1.85)	1	0
TCM199 + PVP	62	2 (3.22)	0	1

ECS, estrous cow serum; PVP, polyvinylpyrrolidone; TCM, tissue culture medium; others, immature/degenerated/unidentified.
Data from six replicates.

Figure 1 Canine polyspermic embryo type II stained by Hoechst 33342 (left) and one immature oocyte (right) (×320) (Rodrigues, 2005).

sperm, which were previously matured in medium A, were polyspermic compared with 22.22% oocytes matured in medium B (12/54) and in medium C (14/63), respectively. Nevertheless, oocytes fertilized with cooled sperm, were apparently the least prone to polyspermy in treatments A and C, when this parameter was compared with treatment B. The results for polyspermy in this study are presented in Tables 1 and 3.

Cumulus cell expansion in Experiments 1 and 2

In Experiment 1, expansion of cumulus cells in treatment A was similar to cumulus cells expansion observed in treatment B. In treatment A, 75% (9/12) of COCs and 50% (6/12) in treatment B showed cumulus cell expansion category 2, respectively. In treatment C, approximately 58% (7/12) of COCs showed this category of expansion, while 33% (4/12) of oocytes showed cumulus cell expansion category 1. Category 1 was not observed in COCs matured in media A and B. Frequency of oocytes showing category 3 in Experiment 1 was approximately 25% (3/12), 50% (6/12) and 8% (1/12) for media A, B and C respectively. None of the oocytes matured in the three media showed expansion of cumulus cells of category 4.

In Experiment 2, the most frequently seen cumulus expansion in oocytes matured for 48 h was for category 2. Frequencies were approximately 66% in

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treatments A (4/6) and B (4/6), and 50% (3/6) in treatment C, respectively.

The most frequently seen cumulus cells expansion for oocytes matured for 72 h was category 2 with frequencies of 50% in treatments A (3/6) and B (3/6), respectively. In treatment C, percentage of cumulus cells expansion category 2 was similar to cumulus cells expansion category 3. In treatment B, a single oocyte matured for 72 h was observed with cumulus cells expansion category 4. These data were not subjected to statistical analysis.

Discussion

In the bitch, the establishment of *in vitro* maturation protocols, with the aim to promote and maintain competence of the COCs for fertilization and cleavage is still required. Various protocols have been tried in order to attempt *in vitro* meiosis of dog oocytes (Cinone *et al*., 1992; Nickson *et al*., 1993; Srsen *et al*., 1998; Hewitt & England, 1999; Songsasen *et al*., 2002; Willingham-Rocky *et al*., 2002). Yet, meiotic response of *in vitro*-matured bitch oocytes is very unpredictable, because it is difficult to identify which components are lacking in the medium, or which are acting as detrimental or suppressive compounds, or simply because the concentration of a substance needed to induce a response is unknown. Also, current assays may not encompass all variables influencing cytoplasmic maturation.

It is generally accepted that the process of keeping the medium as simple as possible is the most reliable manner of knowing to what oocytes are being subjected. Despite the complexity, TCM199 has emerged as a commonly used medium for various species, including canids. Apparently, success of nuclear maturation to the metaphase II (MII) stage for canine oocytes matured *in vitro* is independent of gonadotropins, steroids, hormones such as somatotropin and protein supplements in the medium (Songsasen *et al*., 2002; dos Santos *et al*., *Animal Reproduction*, accepted). Nevertheless, it should be highlighted that maturation of COCs is probably the most critical *in vitro* step affecting subsequent embryo development to the blastocyst stage (Massip *et al*., 1995). Many factors may influence the acquisition of oocyte competence during *in vitro* maturation, which can be assessed by the subsequent embryonic development. *In vitro* maturation is associated with migration of the cortical granules, whose contents are released upon sperm penetration and play a role in blocking polyspermy (Zheng & Sirard, 1992).

Polyspermy is a generic term describing the penetration of two or more sperm into the oocyte, and can thus include dispermy, trispermy and tetraspermy or even greater degrees of multiple sperm entry. The pattern of polyspermy can be modulated by the maturational status of the oocyte (IVM conditions), as well as the dose and type of sperm used for IVF (Kidson, 2004).

McAvey *et al*. (2002) reported that perturbation of the oocyte actin cytoskeleton or inadequacy of sperm-induced calcium signalling leads to increased polyspermy during IVF of mouse oocytes. Challenging oocytes with a higher number of sperm, as performed in this study, and/or suboptimal oocyte activation in response to sperm should not be disregarded as possible reasons for polyspermy in the present study. In both experiments, oocytes exposed to treatment A were apparently less prone to polyspermy, which could be the result of substances present in the medium and influenced by the addition of combined hormonal supplements. It might be possible that maturation of cumulus cells was enhanced in treatment A and therefore influenced supernumerary spermatozoa entry into the oocytes to a lesser degree. The rates of polyspermy observed in oocytes submitted to treatment A in this study were lower than the rates of polyspermy observed in a previous experiment, where the same medium was used to mature the oocytes (Rodrigues *et al*., 2004). Polyspermy, and also other factors affecting embryo quality, could be the underlying cause of the low embryonic development we have observed in this study.

The action of endocrine factors that affect oocyte maturation *in vitro* may be quite different from the *in vivo* condition (Chian *et al*., 2004). *In vivo*, there are four factors that are involved with oocyte developmental potential. These are normal growth phase progression, adequate follicle cell support during maturation, completion of intracellular programming before fertilization and the functioning of oocyte checkpoint surveillance mechanisms (Moor & Day, 2001).

Components in medium influence the levels of certain oocyte transcripts. Addition of serum to the maturation medium, such as estrous cow serum (ECS) inactivated by heat treatment, reduces the potentially damaging effects of competent proteins (Glied *et al*., 1996). Nevertheless, it has been suggested that the replacement of serum preparations by large inert molecules may reduce the potential risk of viral or prion contamination and variability associated with the previous sera (Chanson *et al*., 2001). One of these molecules is polyvinyl pyrrolidone (PVP), which has been used to exclude protein influence from culture medium in *in vitro* procedures in the bitch (Songsasen *et al*., 2002; Bolamba *et al*., 2002). Indeed, culture serum-free systems allow researchers to examine more precisely oocyte requirements. However, serum has the effect of masking problems in the culture system. Moreover, it has been reported that serum-free

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medium results in zona pellucida hardening (Kito & Bavister, 1996). Nevertheless, a chemically defined IVM medium, such as TCM199 plus PVP, seems to supply canine oocytes requirements, supports both cleavage and development of normal embryos; this aspect was confirmed by the results in the present study. Regardless of that, it must be emphasized that oocytes were exposed to fetal bovine serum (FBS) during slicing of ovaries in the present study and, therefore, rates of cleavage observed in treatment C might have also resulted from temporary contact between oocytes and FBS, before transfer to culture medium. Rapid steroid actions, which can be stimulated by brief exposure to serum, are involved with a variety of cell signal transduction mechanisms (Chian *et al*., 2003). Assuming that the brief exposure to FBS might have contributed to oocyte priming, embryo development in treatment C was more likely a consequence of a directly or indirectly steroid-induced stimulation.

Information involving IVF of *in vitro*-matured dog oocytes is limited to a few papers (Yamada *et al*., 1992; Metcalf, 1999; Otoi *et al*., 2000; England *et al*., 2001; Songsasen *et al*., 2002, Rodrigues *et al*., 2004). Current canine embryo culture systems are able to support *in vitro*-derived zygotes up to the 8-cell stage (Songsasen *et al*., 2002; Rodrigues *et al*., 2004). In the majority of *in vitro* studies, dog zygotes do not progress to advanced stages (morula or blastocyst), with the exception of one report (Otoi *et al*., 2000).

Although culture medium supplemented with a physiological concentration of FSH or LH stimulates steroid (estradiol and progesterone) secretions from the cultured granulosa and cumulus cells (Chian *et al*., 1999), *in vitro* maturation studies examining the effect of exogenous steroid hormones on developmental competence of dog oocytes have described either negative (Rodrigues & Rodrigues, 2003; Willingham-Rocky *et al*., 2003) or positive effects (Nickson *et al*., 1993; Wilingham & Kraemer, 2001).

It is still not known how supplements added to medium affect dog embryo development *in vitro*. For Experiments 1 and 2 in this study, embryos derived from *in vitro*-matured oocytes showed cleavage blockage around the 6–8-cell stage. Both incomplete maturation of cell cycle regulators and inadequate culture conditions may have been associated with the arrest of embryonic development and, in this study, poor embryonic competence may have originated at a nuclear and/or cytoplasmic level.

Similar to previous reports (Rodrigues *et al*., 2004) and also in this study we observed a clearly variation between females for *in vitro* meiosis, cleavage and embryo development. Immature oocytes from two females in Experiment 1, and immature oocytes from one bitch in Experiment 2 underwent cleavage. Morphological appearance of donor's ovaries suggested that these three females were at a non-proliferative phase of estrous cycle at the time of ovary removal. Recently, it was demonstrated that larger follicles could exist in the dog cycle during diestrus (Songsassen & Wildt, 2005). Cha *et al*. (1992) showed that human immature oocytes from the luteal phase had a significantly higher maturation rate than those obtained during the follicular phase. Interesting, these data support our previous (Rodrigues *et al*., 2004) and present findings, in which *in vitro* cleavage in the dog can be achieved regardless of the female reproductive stage. Nevertheless, the main problem in IVF studies in the bitch remains the limited number of donors, whose oocytes have maturational and developmental potential.

In various species (mouse, rat, cow and pig) oocytes produce an oocyte-secreted factor, termed the cumulus expansion-enabling factor (CEEF), which presumably plays an important role in formation and stabilization of the mucoid matrix molecules (Gilchrist *et al*., 2004). Similarly to COC expansion within the follicle *in vivo*, COCs expand during *in vitro* maturation under the influence of gonadotropins (Yokoo *et al*., 2002). Cumulus cell expansion, a parameter included in our evaluations in this study, requires a cAMP signal and this is readily induced by *in vitro* treatment with FSH. Luteinizing hormone (LH) may act directly upon cumulus cells from isolated COCs to stimulate synthesis of hyaluronan after FSH-mediated upregulation of sufficient numbers of suitable LH receptors on cumulus cells and oocyte membranes (Chen *et al*., 1994). The most vital influences on the oocyte are the cumulus cells, which are thought to be the source of a putative positive meiosis-inducing signal (Eppig, 2003, 2004) under the influence of gonadotropins. Furthermore, developmental competence of *in vitro*-matured oocytes has been reported as questionable when surrounding cumulus cells are removed (Chang *et al*., 2005). Cumulus cell function and viability can be estimated by evaluating the degree of cumulus cell expansion, and this parameter is referred to as a gauge for approximating the COCs developmental potential (Kidson, 2004). Intriguingly, cumulus expansion seems to follow an unusual pattern in canids (Srsen *et al*., 1998). We have previously observed that oocytes from ovaries of bitches at various cycle stages and, with large expanded and/or mucified cumulus cells after *in vitro* maturation, are not necessary the ones achieving meiosis. Furthermore, as it was not possible to establish a correlation between cleavage and COC expansion in this study, this parameter seems negligible and so far has not proved reliable in indicating whether an oocyte is developmental competent and if a successful fertilization and early embryonic development will be established.

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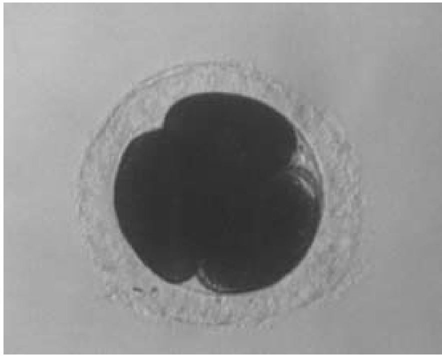

Figure 2 Canine embryo (3–4-cell stage) derived from one oocyte matured in TCM199 supplemented with estrous cow serum (ECS) (×320) (Rodrigues, 2005).

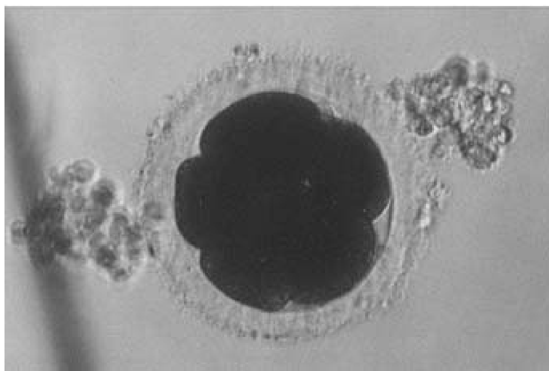

Figure 3 Canine embryo (5–6-cell stage) derived from one oocyte matured in TCM199 supplemented with estrous cow serum (ECS) + estradiol + FSH + HCG (×320) (Rodrigues, 2005).

It is noteworthy that four out of seven zygotes produced in Experiment 1 of this study arose from oocytes matured in medium A, which had FSH and LH added, while the remaining three arose from oocytes matured in medium B ($n = 2$) and medium C ($n = 1$). This observation suggests that gonadotropins and steroids seem to play a role in cytoplasmic maturation of canine oocytes rather than nuclear. Conversely, the addition of gonadotropins and steroids in medium was not sufficient to improve oocyte cleavage ability.

From the few zygotes produced, the highest percentage of embryos was arrested at the 2–5-cell stage (Fig. 2). It was previously hypothesized that arrested embryos enter a senescence like state and that the cell cycle regulatory protein p53 and the stress-related protein $p66^{shc}$ are responsible for this early embryonic arrest (Faveta *et al*., 2004).

It is noteworthy that the majority of embryos progressing to the 6–8-cell stage (Fig. 3) arose from oocytes matured in treatment A, except for one embryo produced in treatment C. Components in treatment A may have acted synergistically or cumulatively and contributed to embryo development.

Mammalian embryos can be cultured successfully using both gas phase-containing atmospheric (20%) or reduced (5%) oxygen (O_2) concentrations (Dumoulin *et al*., 1999), which are about 150 mmHg and 40 mmHg, respectively (Karagenc *et al*., 2004). Dog embryos can develop *in vitro* up to the 8-cell stage under a high oxygen culture environment (Rodrigues *et al*., 2004). However, it is known that the oxygen concentration within the reproductive tract of mammals is lower than in air (Fujitani *et al*., 1997). As the concentration to which embryos are exposed *in vivo* varies between 2 and 8% in the oviduct and uterus, depending on species (Fischer & Bavister, 1993), it is therefore advisable to culture embryos at low oxygen concentrations (5–7%) (Gardner & Lane, 2004). Although the interpretation of these data requires some caution, we associate the possible toxic effects of high oxygen during the time of *in vitro* culture, and mainly represented by reactive oxygen species (ROS), among the variables that could have a negative impact on cleavage and embryo development in this study. Moreover, and as already reported for the mouse, it remains unknown if oxygen concentration is a stage-specific phenomenon on embryo development (Karagenc *et al*., 2004).

Also, the high percentage of immature degenerated or unidentified oocytes found in this study, could be related to the yet inappropriate environment provided by the IVM/IVC systems. However, degeneration of oocytes as detected during IVM might be a normal process, because these are oocytes probably not representative of the selected population destined to ovulation (Villamediana *et al*., 1999). Thus, degenerated or not well matured oocytes are more likely those incapable of successfully accomplishing their reproductive purpose, which is the competence to develop into embryos.

Another issue is the influence of sperm affecting *in vitro* cleavage. Cooling sperm decreases semen quality, and predisposes to altered patterns of sperm fertilization. From various criteria (viability, motility and functional ability of the sperm), the more frequently used for semen evaluation and for comparison between sperm preservation techniques is motility, as this is a clear sign of viability, and spermatozoa need to be motile to reach the oocyte and penetrate the ZP (Rota *et al*., 2004). Besides that, routine semen parameters may not reflect the quality of sperm DNA. Damage to sperm DNA includes ROS, to which spermatozoa are susceptible. This is because their plasma membranes contain large quantities of polyunsaturated fatty acids and their cytoplasm contains low concentrations of scavenging enzymes (Saleh & Agarwal, 2002).

We have previously examined the *in vitro* capacitation status of dog spermatozoa at the time of addition to the fertilization drops. We reported that at the start of IVF the mean number of spermatozoa capacitated

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and acrosome intact (B pattern), and capacitated and acrosome reacted (AR pattern) was measured to be 51.47 ± 6.24, and 20.64 ± 5.02, respectively. At that time, we observed a normal cleavage rate of 10.1% of bitch oocytes in association with these numbers (Rodrigues *et al*., 2004).

In this study, IVF of oocytes with cooled sperm was equally efficient for cleavage, when compared with IVF of oocytes with fresh semen. Therefore, the suboptimal fertilization results obtained in the present study are less likely to be related to the sperm type, i.e., fresh or cooled, and should thus be rather attributed to other cell changes induced during sperm processing, for example inadequate capacitation of spermatozoa, although this statement deserves indepth investigation and remains to be proven.

Sperm susceptibility to cooling was observed in a few samples from the donor dog. This aspect was demonstrated by the reduction in motility after spermatozoa back rewarming. In Experiment 2, the postrewarming score of cooled semen samples showed that cleavage was achieved even when sperm motility was as low as 40%. Despite the fact that higher percentages of sperm motility are an effective predictor of *in vitro* fertilization potential, samples with reduced numbers of motile spermatozoa could still retain most intrinsic characteristics required for fertilize an oocyte. In Experiment 2, cooled sperm was proved to fertilize oocytes, as assayed by cleavage, and therefore this suggests that both cell motility and membrane integrity were preserved in the sperm population. The negative effects of storage have apparently been overcome by maintenance of sperm in Tris–egg yolk diluent, which is known to stabilize the sperm membrane due to the protective effect of egg yolk. *In vitro* preservation of fertility on cooled dog semen in Experiment 2 is encouraging because cooled sperm could be useful in IVF studies in canine species, especially under circumstances where male and female gametes are not synchronous disposable at the moment of insemination.

In conclusion, the results in this study indicate that competence of oocytes matured *in vitro* in TCM199, is independent of medium supplements, as assessed by the subsequent cleavage. The results here suggest that the kind of supplement added to the maturation medium is not involved in the acquisition of competence of *in vitro*-matured canine oocytes. Also, it was demonstrated for the first time that *in vitro*-matured canine oocytes could be fertilized by homologous cooled spermatozoa and progress to cleavage, even when at highly compromised rates. Lastly, it was concluded that canine oocytes cleave *in vitro* regardless the extension of COC expansion.

The presented data reinforces our thoughts that a prepattern of cytoplasmic attributes are responsible for oocyte developmental capacity. Intrafollicular ovarian dynamics coupled to the inherent quality of the oocyte have yet to meet the appropriate *in vitro* culture environment, where the *in vivo* situation could be mimicked. The identification of specific means by which the developmental potential of canine oocytes can be enhanced remains as the great challenge of the *in vitro* programme in the bitch.

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