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In vitro nuclear maturation of bitch oocytes in the presence of polyvinyl-pyrrolidone

L.C. Santos[1,3], B.A. Rodrigues[1,2], J.L. Rodrigues[1]

[1]Laboratory of Embryology and Biotechniques of Reproduction, Faculty of Veterinary Medicine, Federal University of Rio Grande do Sul; Porto Alegre, RS, Brazil.
[2]Department of Animal Medicine, Faculty of Veterinary Medicine, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.

Abstract

The main limitation in producing *in vitro* dog embryos succesfully is the low oocyte maturation rate to the Metaphase II stage. The objective of this experiment was to compare the rates of nuclear maturation of dog oocytes cultured in Tissue Culture Medium 199 (TCM 199) supplemented with polyvinyl-pirrolidone (PVP) with oocytes cultured in TCM 199 with estrous cow serum (ECS) or TCM 199 with ECS and hormones. Ovaries were collected from 21 healthy bitches by ovariohysterectomy. Females were at various stages of the estrous cycle at the moment of ovary retrieval. The oocytes were selected and classified subjectively according to their morphology and size and matured for 48 hours at 37°C in an atmosphere of 5% CO_2 in air. Oocytes were randomly allocated to one of three treatment groups: (A) TCM 199 supplemented with 4 mg/ml PVP, (B) TCM 199 with 10% ECS, or (C) TCM 199 supplemented with 10% ECS + 20 µg/ml estradiol + 0.5 µg/ml FSH + 0.03 IU/ml hCG (control). All maturation media contained a final concentration of 1 µg/ml of human somatotropin (hST). There were no significant differences in Metaphase II rates among treatments A (4.7%, 8/170), B (3.52%, 6/183), and C (4.70%, 8/172). It was concluded that *in vitro* nuclear maturation of domestic dog oocytes can be achieved using TCM 199 supplemented with PVP, and this yields results similar to those obtained using media containing serum, gonadotropins, or estradiol.

Keywords: canine, oocyte, maturation, PVP, serum-free media.

Introduction

The development of *in vitro* techniques using the canine oocyte as an experimental model would retain powerful tools for gamete rescue programs, which are important to preserve the existence of various endangered canid species such as the lobo-guará (*Chrysocyon brachyurus*) and the cachorro-vinagre (*Speothos venaticus*) in South America (Brazilian´s list of Ministério do Meio Ambiente, 2004). Moreover, these technologies can be used to understand the basic mechanisms related to gamete development in carnivores and may also be useful in cloning, which requires matured oocytes for use as a cytoplasm receptor for the somatic cell by nuclear transfer.

In vitro maturation (IVM) of oocytes has become a critical step in *in vitro* production (IVP) of canine embryos, and for this reason, several studies have been carried out to test different culture conditions. Nevertheless, the results of nuclear maturation remain low with a percentage of oocytes reaching the Metaphase II stage in the range of 0% to 31.9% (Yamada *et al*., 1993; Bolamba *et al*., 1998; Hewitt and England, 1999; Fujii *et al*., 2000; Saint-Dizier *et al*., 2001; Bogliolo *et al*., 2002; Songsasen *et al*., 2002; Luvoni *et al*., 2003; Rodrigues and Rodrigues, 2003b; Kim *et al*., 2005). At the present time, few researchers have had success with *in vitro* development of canine embryos (Yamada *et al*., 1992; Otoi *et al*., 2000b; England *et al*., 2001; Rodrigues *et al*., 2004).

Many protocols use serum as a source of protein to culture oocytes and embryos in canids (Srsen *et al*., 1998; Bolamba *et al*., 1998; Otoi *et al*., 2000b; Luvoni *et al*., 2003; Rodrigues *et al*., 2004). Two articles reported that serum was not needed as supplement for *in vitro* maturation of dog oocytes (Songsasen *et al*., 2002; Bolamba *et al*., 2002). The disadvantages of utilizing serum or any kind of protein supplementation in the IVP medium are: (1) the inclusion of unkown substances that could be harmful to oocyte maturation and subsequent embryo development; (2) the variability between batches; (3) the difficulties in determining the useful working life of the batch; (4) the need to test a new batch to ensure that the replacement is as close as possible to the previous one; and (5) the risk of contaminating the culture (Freshmy, 2000).

Many studies have been done in order to determine a more appropriate hormonal supplementation in the culture medium that could promote an increase in the results of *in vitro* nuclear maturation of oocytes from canids. Some of these experiments have shown a positive influence of estradiol and progesterone (Kim *et al*., 2005), bovine somatrotopin (Srsen *et al*., 1998), human somatrotopin (Rodrigues and Rodrigues, 2003b), equine chorionic gonadotropin (Hanna *et al*., 2004), and human chorionic gonadotropin (Reyes *et al*., 2005) in the medium. However, two studies performed by Hewitt and England (1997; 1999) did not show a positive effect with *in vitro* nuclear maturation of canine oocytes when the culture medium was supplemented with gonadotropins and steroids hormones. A recent study performed by

[3]Corresponding author: lucilacarb@yahoo.com.br
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Bolamba *et al*. (2006), in which the authors tested the influence of gonadotropin and steroid addition to IVM medium on oocytes collected from preantral and early antral follicles, confirmed these observations.

Polyvinyl-pyrrolidone (PVP) is a synthetic polymer used as a supplement in culture medium that changes the adhesion properties between granulosa cells and oocytes. This positively influences cumulus-oocyte complex (COC) organization and behavior through diffusion of paracrine or autocrine factors that are considered essential to oocyte growth and development and that are produced and secreted by the COCs. These factors are composed of macromolecules, and their movement is affected by interactions with others macromolecules such as PVP. When the culture medium is supplemented with a high concentration of macromolecules, these paracrine and autocrine factors produced by cumulus oophorus cell complexes become less mobile, which in consequence increases the chance for these factors to find their receptors (Hirao *et al.*, 2004).

The present study was performed to compare the nuclear maturation rates of dog oocytes cultured in TCM199 supplemented with PVP to oocytes cultured in TCM 199 with ECS or with ECS and hormones. The main goal of this experiment was to verify the impact of not using animal protein (e.g. ECS) and hormones supplements (e.g. oestradiol, FSH, and hCG) on IVM of dog oocytes by substituting them with TCM199 and PVP.

Materials and Methods

Ovaries and oocyte retrieval

Ovaries were retrieved from 21 healthy pubertal and adult bitches ranging from 11 months to 9 years of age (mean age was 3 years). Purebred (n = 5) and crossbred (n = 16) bitches had their ovaries subjectively categorized according to the stage of the estrous cycle by identification of external ovarian tissue morphology as anoestrus (n = 6; ovaries without follicles or luteal tissue), estrus (n = 4; one or more visible follicles), or diestrus (n = 11; one or more pronounced corpora lutea) according to the procedures of Hewitt *et al*. (1998). Ovaries were obtained by routine and elective ovariohysterectomy (n = 16) or after caesarean section operation (n = 5), following anesthesia for the purpose of neutering at the Veterinary Hospital of Universidade Federal do Rio Grande do Sul (UFRGS), Zoonoses Control Center of Porto Alegre, and at local private clinics. The bitches' ovaries were brought to the laboratory in phosphate-buffered saline (PBS) solution at ambient temperature within one hour after removal. The cumulus-oocyte complexes were recovered by slicing of ovarian tissue at 37ºC in PBS supplemented with 1% fetal calf serum (FCS). Cumulus-oocyte complexes were recovered and morphologically classified as the following grades: (1) darkly pigmented and completely surrounded by one or more layers of cumulus cells or (2) lightly pigmented with incomplete layers of cumulus cells (Hewitt *et al*., 1998). From a total of 2,420 COCs, 623 were morphologically classified as Grade I and were selected for *in vitro* maturation. The medium used to maintain the COCs before maturation was TCM-hepes buffer and was composed of TCM 199 (Sigma, M2520) supplemented with 4.2 mM $NaHCO_3$ (Sigma, S5761), 50 µg/ml gentamicin (Sigma, G1264), 1 mg/ml BSA (Gibco, 11018-017), and 14.3 mM Hepes (Sigma, H3375).

In vitro maturation

The COCs were divided randomly and cultured in 100 µL drops (four drops per dish) under mineral oil (Sigma) at 37°C in a humidified atmosphere with 5 % CO_2 in air for 48 hours. Basal medium was TCM 199 supplemented with 25 mM Hepes/l (v/v; Sigma, M2520, St. Louis, MO), 50 µg/ml gentamicin (Sigma, G1264), 2.2 mg/ml sodium bicarbonate (Sigma, S5761), and 22 µg/ml pyruvic acid (Merk, 1.06619.0050, Darmstadt, Germany). Oocytes were submitted to one of three treatments: (A) TCM 199 supplemented with 25 mM Hepes/l (v/v; Sigma, M2520), 50 µg/ml gentamicin (Sigma, G1264), 2.2 mg/ml sodium bicarbonate (Sigma, S5761), 22 µg/ml pyruvic acid (Merk, 1.06619.0050, Darmstadt, Germany), and 4 mg/ml polyvinyl-pyrrolidone (Sigma P0930); (B) TCM 199 supplemented with 25 mM Hepes/l (v/v; Sigma, M2520), 50 µg/ml gentamicin (Sigma, G1264), 2.2 mg/ml sodium bicarbonate (Sigma, S5761), 22 µg/ml pyruvic acid (Merk, 1.06619.0050, Darmstadt, Germany), and 10% heat inactivated ECS; or (C) TCM 199 supplemented with 25 mM Hepes/l (v/v; Sigma, M2520), 10% heat inactivated ECS, 50 µg/ml gentamicin (Sigma, G1264), 2.2 mg/ml sodium bicarbonate (Sigma, S5761), 22 µg/ml pyruvic acid (Merck, 1.06619.0050, Darmstadt, Germany), 20 µg/ml estradiol (Sigma, E8875), 0.5 µg/ml FSH (Foltropin-V, Vetrepharm, Inc., Ont., Canada), 0.03 IU/ml hCG (Profasi HP, Serono), the control group. All maturation media contained a final concentration of 1 µg/ml human somatotropin (hST; Lilly, FF1D44C, France).

Assessment of meiotic stage

After the *in vitro* culture period, oocytes were transferred into 400 µl of PBS solution and stripped from their cumulus cells by mechanical displacement by gentle mouth pipetting using a small-bore glass pipette. After that, oocytes were made permeable in Triton X solution and fixed in 3.7% paraformaldehyde for 15 minutes. Oocytes were then submitted to an additional 15 minutes in a 2% polyvinyl-pyrrolidone and PBS solution. Groups of 5 oocytes were placed on a slide,

stained with 3 µl of Hoescht 33342 (Sigma St Luis, MO, USA) solution in glycerol (10 µg/ml), and overlaid with a coverslip supported by four small pieces of baton glue. The coverslip was then sealed with nail polish. The bis-benzimide (Hoechst 33342) is a DNA-specific fluorochrome. It allows the evaluation of chromatin configuration in oocytes. Each oocyte was observed using fluorescent microscopy to evaluate the stage of nuclear maturation. Using this method, oocytes were classified as the following: intact germinal vesicle (GV; unidentifiable nucleolus and very fine filaments of chromatin); germinal vesicle breakdown (GVBD; presence of different patterns of chromatin condensation, chromosomes coiled up and no visible individual chromosomes); Metaphase I (MI), Anaphase I (AI), Metaphase II (MII), once the formation of bivalents was completed and appeared one or two sets of chromosomes; and degenerated or unclassified (others; oocytes showed unidentifiable or not visible chromatin).

Statistical analysis

The percentages of various nuclear maturation stages of oocytes among the different treatments groups were analyzed by chi-square analysis. A confidence level of $P < 0.05$ was considered statistically significant. This study was replicated 15 times.

Results

After slicing, 2.420 COCs were obtained from 21 bitches. The mean number of oocytes recovered from individual bitches was 115. A total of 623 (25.7%) healthy–looking cumulus-oocyte complexes were classified as Grade 1 and were used in the experiment (44 COCs per female; Table 1). Ninety-eight oocytes (15.7%) were lost during the processes of removal of *cumulus* cells, fixation, or during preparation for staining (35 oocytes in Treatment A, 29 in Treatment B, and 34 in the control group).

Table 1. General description of the donor oocyte population.

	Mean	Range
Age (months)	38	11 to 108
Number of COCs	115	27 to 266
Number of Grade I COCs	44	6 to 73
Donor weight (Kg)	9.89	4 to 15.7
Ovary weight/donor (g)	1.797	0.617 to 3.513

Nuclear morphology was evaluated in 525 oocytes, which were fixed and stained after 48 hours of culture with their respective treatments. One hundred thirty-two oocytes were classified as degenerated or as possessing unidentifiable nuclear material. Oocyte degeneration was characterized by nuclear pycnosis, unidentifiable intracytoplasmic structures, or loss of the cytoplasmic membrane. As exhibited in Table 2, there were no significant differences among treatment groups at any stage of nuclear maturation (VG, VGBD, MI/AI, or MII). The percentage of oocytes that resumed meiosis was 24.10% (41/170), 25.37% (43/183), and 23.52% (40/172) for Groups A, B, and C, respectively. These percentages were not significantly different. At the end of the *in vitro* maturation period, parthenogenetic activation of the oocytes was not observed.

Table 2. Nuclear maturation status of oocytes after IVM for each treatment group.

Treatment	N	GV (%)	GVBD (%)	MI/AI (%)	MII (%)	Others
Control[1]	172	84 (49.4)	17 (10.0)	15 (8.8)	8 (4.7)	48 (28.3)
TCM 199 + ECS + hST	183	98 (57.6)	23 (13.5)	14 (8.2)	6 (3.5)	42 (24.7)
TCM199 + PVP + hST	170	86 (50.6)	21 (12.4)	12 (7.1)	8 (4.7)	42 (24.7)

[1]Control: TCM 199 + ECS + FSH + hCG + E_2 + hST

GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: Metaphase I; AI: Anaphase I; MII: Metaphase II; Others: degenerated or unclassified.

Chi-square analysis, $P > 0.05$.

Discussion

The results of the present study indicate that supplementation of TCM 199 with PVP provided a similar rate of nuclear maturation to that of oocytes cultured in medium supplemented with gonadotropins, steroids, and serum. In a study by Songsasen *et al*. (2002), canine oocytes were successfully matured *in vitro* in a protein-free medium at comparable rates to those that used medium supplemented with protein. Despite suboptimal rates of maturation, Hewitt *et al*. (1998) previously showed that canine oocyte maturation

was possible in serum-free medium. In other species such as the cat (*Felis catus*), *in vitro* maturation and subsequent fertilization were not affected by the absence of protein supplementation in the medium (Wood *et al*., 1995). Also, Saeky *et al*. (1991) reported that the frequency of *in vitro* nuclear maturation of bovine oocytes did not differ among media supplemented with either fetal calf serum (FCS) or 0.3% PVP exclusively.

In the present experiment, inclusion of 4 mg/ml PVP in maturation medium provided a similar rate of nuclear maturation to the maturation percentage observed in the other treatments when comparison was established among groups. This observation shows that PVP can be used as a substitute for animal surfactant macromolecules present in serum, which are routinely used for *in vitro* maturation systems for dogs. Nevertheless, this statement has to be made cautiously since maturation results cannot be attributed to PVP exclusively, mainly because it is not known in which manner the molecule interacts with the other components in the medium. According to Hirao *et al*. (2004), macromolecules such as PVP and polyvinylalcohol (PVA) provide a colloid osmotic pressure and are useful for preventing loss of oocytes due to adherence of oocytes to the plastic surface of the Petri dish.

In this experiment, meiosis of *in vitro* cultured dog oocytes under the influence of PVP was the result of a variety of cellular processes. Culture conditions are among the major factors interfering with the oocyte´s original pattern of differentiation and are probably responsible for the low developmental competence that was observed in all treatments in this experiment. Without adequate changes at the nuclear and cytoplasmic levels, the number of oocytes that resumed meiosis was low. This fact has been challenging for researchers who are working with *in vitro* maturation in canines.

Otoi *et al*. (1999) observed that the addition of serum to maturation medium contributes to a high number of canine oocytes with unidentified nuclear material after staining. In the present experiment, the percentage of degenerated oocytes or those with unidentified nuclear material was not different among treatments. This indicates that the presence or absence of serum does not influence the incidence of oocytes with those abnormalities.

Some researchers (Leibfried-Rutledge *et al*., 1986, Younis *et al*., 1989, Schellander *et al*., 1990) have shown that the inclusion of serum in IVM medium improves the maturation of bovine oocytes and their subsequent development to embryos, mainly because serum contains a range of substances such as hormones, growth factors, amino acids, and binding proteins all of which are involved with cell maturation. In the mouse, addition of serum to medium has been shown to prevent zona pellucida hardening and enhances fertilization rates as well as embryonic development (Eppig and Schroeder, 1986). However, it is also known that serum has a potential risk to disseminate disease, mainly when it is contaminated by viruses (Gordon, 2003). Another negative point for using serum as a source of protein in IVM medium is the major variability in the results when different batches of serum are used. This variability can be attributed to differences among lots (Bavister, 1995). In the present experiment, the batches of ECS were self prepared. Serum was obtained from blood collected from a cow from the laboratory herd and was immersed in a 56 to 60^{o}C water bath for 30 minutes. This procedure is supposed to deactivate imunnoglobulins and other components such as protein hormones. According to the literature, this process probably reduces the interference of gonadotropins but not the steroids contained in the serum (Isachenko *et al*., 1994).

The majority of reports regarding IVM of canine oocytes refer the use of gonadotropins and steroids hormones as supplements in the medium, which is an adaptation from protocols used in other species. However, there are a few controversial studies indicating that the use of these types of supplementation improves the quality of medium (Hewitt and England, 1997; 1999; Willinghan-Rocky *et al*., 2003; Rodrigues and Rodrigues, 2003b; Kim *et al*., 2005; Reyes *et al*., 2005). Two arguments in favor of supplementation of maturation media with estradiol are that this hormone can act as a co-stimulator for FSH-induced follicular growth by inducing the expression of luteinizing hormone (LH) receptors in granulosa cells, and it promotes the production of insulin-like growth factor I and II (Smitz *et al*., 2001). The addition of gonadotropins in the maturation medium complicates the comparison and the results obtained by different studies testing protein and hormones in medium because there are differences in concentrations and variations in purity among batches (Choi *et al.*, 2001; Reyes *et al*., 2005). However, it is well established that gonadotropins added to maturation medium of bovine oocytes play an important role in triggering the resumption of meiosis and the expansion of the cumulus cells (Gordon, 2003).

In the present study, the rate of nuclear maturation of oocytes in medium with ECS, FSH, human chorionic gonadotropins (hCG), estradiol (E_2), and human somatotropin (hST) was similar to that observed for treatment with ECS and hST or PVP and hST. These findings are in agreement with previous reports (Hewitt and England, 1997; 1999; Bolamba *et al*., 2006) in which the addition of gonadotropins and steroid hormones to the maturation medium was not sufficient to enhance the percentage of canine oocytes that reached MII. Also, Rodrigues and Rodrigues (2003b) tested the addition of FSH and estradiol to the IVM medium without a greater degree of resumption of meiosis in oocytes. In another study with Blue fox oocytes, the addition of FSH to the maturation medium

was not sufficient to improve the rate of nuclear maturation *in vitro* (Srsen *et al*., 1998). However, a recent report by Kim *et al*. (2005) showed that E_2 improved the *in vitro* nuclear maturation rate of canine oocytes only when the ovaries of bitches were retrieved from the follicular phase of the estrous cycle. Reyes *et al*. (2005), testing TCM 199 with different periods of exposure of canine oocytes to hCG for IVM, concluded that the percentage of oocytes that had reached MII was highest in the group matured with hCG for only the first 48 hours after a total period of culture of 96 hours. This study showed that the time of exposure of canine oocytes to this hormone influenced *in vitro* nuclear maturation.

In the present study, a total of 2.420 oocytes were recovered from which 623 oocytes (25%) were classified morphologically as Grade 1 oocytes. This rate of recovery is in agreement with the previous reports from Nickson *et al*. (1993) and Fujji *et al*. (2000) who found an individual rate of 0 to 50% good quality oocytes retrieved from canine ovaries. As previously observed (Rodrigues *et al*., 2004), numbers of COCs and good quality oocytes varied among donors indicating that individual factors influence the rates of recovery but not the MII rates, as verified in this study. Thus, the only factor identified at the moment, influencing oocyte maturation *in vitro* is the inherent quality of the oocyte itself (Hewitt and England, 1998; Otoi *et al*., 2000a). Moreover, stage of the estrous cycle of donors has not affected the achievement of MII in the dog (Rodrigues and Rodrigues, 2003a; Songsasen and Wildt, 2005).

In conclusion, the data presented in this experiment indicate that rate of *in vitro* oocyte nuclear maturation in the dog was similar between modified TCM 199 supplemented with 0,4% PVP, and 10% ECS or 10% ECS and hormones.

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