

## Analysis of nuclear maturation in *in vitro* matured oocytes from estrous and anestrous bitches

L.R. Martins<sup>1</sup>, C.B. Ponchirolli<sup>1</sup>, S.L. Beier<sup>2</sup>, F.C. Landim-Alvarenga<sup>1</sup>, M.D. Lopes<sup>1,3</sup>

<sup>1</sup>Department of Animal Reproduction and Veterinary Radiology, FMVZ.

<sup>2</sup>Department of Anesthesiology, FM, São Paulo State University (UNESP), Rubião Junior, Botucatu, SP, 18618-000, Brazil.

### Abstract

The present study evaluated nuclear maturation after *in vitro* culture of oocytes originated from estrous and anestrous bitches. Two culture media were used, TCM 199 (tissue culture medium 199) and synthetic oviduct fluid (SOF). The females were divided in two groups. Group 1 consisted of 11 anestrous bitches, and Group 2 consisted of 6 estrous bitches. In each group, oocytes were harvested from sliced ovaries, and one-half of them were immediately stained with Hoechst 33342. The oocytes were classified as in the GV (germinal vesicle) phase, GVBD (germinal vesicle breakdown) phase, MI (metaphase I), or as D/NI (degenerated/non identifiable). The other half of the oocytes were matured in SOF or TCM media for 24 hours, stained, and evaluated as previously described. There were 511 and 373 Grade I oocytes recovered in Groups 1 and 2, respectively. The mean number of oocytes obtained per anestrous bitch was  $46.5 \pm 25.2$ . For females in Group 2 (estrous),  $62.2 \pm 8.2$  oocytes were obtained per bitch. Oocytes from estrous and anestrous bitches submitted to *in vitro* maturation had similar rates of GVBD (21.7%, TCM 199 and 23.6%, SOF – anestrous; 20%, TCM 199 and 23%, SOF – estrous). However, the MI rate was higher in the estrous group (9.8%, TCM 199 and 9.7%, SOF – anestrous; 25%, TCM 199 and 58%, SOF – estrous). The protocol that utilized estrous donor's oocytes and *in vitro* maturation in SOF was superior to the other protocol tested, demonstrating that the association between estrous versus anestrous and maturation medium is beneficial for oocyte *in vitro* maturation (58% MI). It was demonstrated that 24 hours of *in vitro* maturation was insufficient for obtaining Metaphase II nuclear configurations in oocytes with all of the protocols tested.

**Keywords:** bitches, *in vitro* nuclear maturation, oocytes.

### Introduction

*In vitro* maturation and fertilization still needs to be extensively investigated in the dog before gamete salvage programs can be established in endangered

canine species (Hewitt and England, 1997b). Oocyte maturation and fertilization in these species are unique among mammals. Ovulation occurs anytime between Days 2 and 7 after the initiation of estrous behavior although it most frequently happens between Days 1 and 3 (Yamada *et al.*, 1992). Normally, oocytes are ovulated in the germinal vesicle stage, and the first meiotic division is not completed until at least 48 hours after ovulation into the uterine tubes (Van der Stricht, 1923; Tsutsui and Shimizu, 1975). Recently, Fontbonne *et al.* (2004) observed the presence of oocytes in Metaphase I (MI) in the uterine tube 46 to 84 hours after ovulation and affirmed that the oocyte requires at least 50 hours to reach Metaphase II (MII) in the bitch. However, Nickson *et al.* (1993) observed that the percentage of oocytes that extruded the first polar body did not vary significantly from 24 to 72 hours in culture, indicating that all oocytes capable of maturation had extruded the first polar body after 24 hours of culture.

Canine oocytes may resume meiosis *in vitro* using adaptations of bovine and porcine *in vitro* maturation (IVM) techniques. However, poor results are obtained using these adapted techniques. In canids, *in vitro* maturation has limited success with maturation rates varying from 0 to 58% (Farstad, 2000b). The most common canine IVM culture media is TCM 199 (Hewitt and England, 1997a; Hewitt *et al.*, 1998; Otoi *et al.*, 2000a; b; Rodrigues and Rodrigues, 2002a; b; 2003; Songsasen *et al.*, 2002; Luvoni *et al.*, 2003; Willingham-Rocky *et al.*, 2003) although modified Krebs ringer bicarbonate (Yamada *et al.*, 1992, 1993) and synthetic oviduct fluid (Hewitt and England, 1999a; b; Bolamba *et al.*, 2002) could also be used.

The influence of the *in vivo* preovulatory endocrinological events on the results of oocyte IVM have been studied by several authors (Hewitt and England 1997a; Luvoni, 2000; Otoi *et al.*, 2001; Rodrigues and Rodrigues, 2003; Willingham-Rocky *et al.*, 2003; Songsasen *et al.*, 2004). However, when female donor's reproductive status is considered, the results are still controversial.

Luvoni *et al.* (2001) observed that the functional status of gap junctions between cumulus cells and oocytes is influenced by the phase of the estrous cycle. It has been demonstrated that the presence of cumulus-oocyte communications in the late proestrous

<sup>3</sup>Corresponding author: denise@fmvz.unesp.br

Phone/Fax: +55 14 3811-6249

Received: December 19, 2005

Accepted: May 30, 2006



COC was related to the ability of oocytes to resume meiosis and reach MII at a higher rate than did oocytes collected during anestrus (11.1 % vs. 0%).

The effect of estrous cycle stage has also been documented by Hewitt and England (1997a) and Rodrigues and Rodrigues (2003), who observed no significant difference in IVM rates between proestrous/estrous oocytes compared to anestrus/late luteal phase, and they concluded that the *in vivo* hormonal environment did not inhibit subsequent *in vitro* maturation of high quality oocytes.

The present study was carried out to evaluate whether oocytes from preovulatory follicles obtained from estrous bitches had higher IVM rates compared to anestrus bitches. The efficacy of two culture media, TCM 199 (tissue culture medium 199) and synthetic oviduct fluid (SOF), was also tested for promoting IVM of canine oocytes.

## Materials and Methods

### *Animals and Experimental groups*

This research was carried out at the Animal Reproduction Service (FMVZ, Unesp, Botucatu, São Paulo, Brazil). The animals were divided in two groups: Group 1 – 11 anestrus bitches,  $3.1 \pm 1.2$  years of age, weighing  $8.8 \pm 1.9$  kg, and of various breeds and Group 2 – 6 estrus bitches,  $8.6 \pm 3.2$  years of age, weighing  $8.6 \pm 3.2$  kg, and of various breeds. The ovaries were obtained during routine ovariohysterectomy.

Animals, which had low serum progesterone concentrations ( $<1$  ng/ml) measured by chemiluminescence (Elecsys 2010) and associated with anestrus vaginal cytology, were included in Group 1 (Root-Kustritz, 2001; England and Concannon, 2002). In the females of Group 2, the onset of the follicular phase was identified by behavioral changes, vulvar swelling, serosanguineous discharge, and cytological examination. Progesterone was measured daily from the beginning of the follicular phase until reaching a concentration between 3.0 and 3.9 ng/ml (Root-Kustritz, 2001). Twenty-four hours later, animals were submitted to ovariohysterectomy.

Oocytes harvested from 6 bitches from Group 1 and 3 bitches from Group 2 were immediately stained and evaluated for nuclear configuration. The oocytes harvested from 5 bitches from Group 1 and 3 from Group 2 were submitted to IVM using TCM 199 and SOF.

### *Oocyte recovery*

Ovaries were brought to the laboratory in physiological saline (0.9% w/v) at room temperature (25°C) within 2 hours of removal. The ovaries were sliced repeatedly to release oocytes into PBS. Cumulus-oocyte complexes were classified according to the morphologic criteria of Hewitt and England (1997a). Only Grade I COCs, darkly pigmented and completely

surrounded by one or more layers of cumulus cells, were used. To maximize the number of oocytes utilized in this study, oocytes were not classified by size nor were collected taking into account follicle diameter.

### *Nuclear configuration*

Grade I COCs were washed in hyaluronidase (type V – Sigma<sup>®</sup>, USA – H-6259) 0.4% and 5% of BSA (Sigma<sup>®</sup>, USA - fraction V) solution and then were denuded mechanically. Oocytes were transferred to 10  $\mu$ l of glycerol bisbenzimidazole drops (HOECHST 33342 - Invitrogen<sup>®</sup>, USA) and mounted on slides for fluorescence microscopy (Leica<sup>®</sup> DM IRD). Nuclear maturation was evaluated according to Landim-Alvarenga (2001).

### *In vitro maturation*

*Group 1 – anestrus group.* Grade I COCs were cultured in TCM 199 and SOF, supplemented with FSH (50  $\mu$ g/ml), LH (1  $\mu$ g/ml), BSA (4%), pyruvate stock (11  $\mu$ g/ $\mu$ l), progesterone (1  $\mu$ g/ $\mu$ l), estrogen (1  $\mu$ g/ $\mu$ l), gentamicin (15  $\mu$ g/ $\mu$ l), and 10% estrus bitch serum.

*Group 2 – estrus group.* Grade I COCs were cultured in TCM 199 and SOF, supplemented with BSA (4%), pyruvate stock (11  $\mu$ g/ $\mu$ l), progesterone (1  $\mu$ g/ $\mu$ l), estrogen (1  $\mu$ g/ $\mu$ l), gentamicin (15  $\mu$ g/ $\mu$ l) and 10% estrus bitch serum.

The two groups were matured at 39°C in a humidified environment of 5% CO<sub>2</sub> in air. The oocytes were allocated randomly into groups of 25 to 30 in 400  $\mu$ l of maturation media. At the end of the maturation period, the oocytes were denuded mechanically, mounted on slides, stained with Hoechst 33342, and the meiotic stage of the oocytes was determined.

### *Statistical analysis*

The percentages of different stages of nuclear maturation and degeneration of oocytes between treatments were analyzed using the chi-square test. A confidence level of  $P < 0.05$  was considered statistically significant.

## Results

A total of 981 oocytes were obtained from 11 anestrus bitches and 864 from estrus bitches. Anestrus and estrus bitches provided a total of 511 and 373 healthy-looking COCs, respectively. The mean number of Grade I oocytes per anestrus and estrus donor was  $46.5 \pm 6.1$  and  $62.2 \pm 8.2$ , respectively.

In the anestrus group before maturation, the GV phase was the most prevalent nuclear configuration (42.93%), and no oocytes in the MI/II phase were observed. However, in the group of estrus bitches, 15.34% of the recovered oocytes were already in MI just after slicing of the ovary (Table 1). The number of



oocytes with degenerate/non-identifiable chromatin was significantly higher in oocytes from anestrous than in estrous bitches (36.4% vs. 19.6%, respectively). Considering the results of Tables 2 and 3, we observed that the group of oocytes collected from estrous bitches had a higher percentage of resumption and progression of meiosis when compared to the anestrous group in both SOF and TCM.

No statistical difference was observed

regarding the proportion of TCM 199- to SOF-matured oocytes within each category of development in the group of anestrous bitches (Table 2). When oocytes obtained from bitches in estrous were submitted to *in vitro* maturation, a significant difference in MI rates was observed between TCM 199 (25%) and SOF (58%) groups. Moreover, the number of oocytes with degenerated/non-identifiable chromatin was superior in the TCM 199 matured group (Table 3).

Table 1. Number and percentage of oocytes at various stages of development immediately after collection in anestrous and estrous bitches.

	GV (%)	GVBD (%)	MI (%)	MII (%)	D/NI (%)
Anestrous	85/198 (42.9)	41/198 (20.7)	0/198 (0)	0/198 (0)	72/198 (36.4)
Estrous	35/163 (21.5)	71/163 (43.6)	25/163 (15.3)	0/163 (0)	32/163 (19.6)

Percentages within each column do not differ ( $P > 0.05$ ). GV: germinal vesicle, GVBD: germinal vesicle breakdown, MI: Metaphase I, MII: Metaphase II, and D/NI: degenerated/non identifiable.

Table 2. Number and percentage of oocytes from anestrous bitches at various stages of development after maturation in TCM 199 or SOF.

Treatment	GV (%)	GVBD (%)	MI (%)	MII (%)	D/NI (%)
TCM 199	35/143 (24.5)	31/143 (21.7)	14/143 (9.8)	0/143 (0)	63/143 (44.1)
SOF	36/144 (25)	34/144 (23.6)	14/144 (9.7)	0/144 (0)	60/144 (41.7)

Percentages within each column do not differ ( $P > 0.05$ ). GV: germinal vesicle, GVBD: germinal vesicle breakdown, MI: Metaphase I, MII: Metaphase II, and D/NI: degenerated/non identifiable.

Table 3. Number and percentage of oocytes from estrous bitches at various stages of development after maturation in TCM 199 or SOF.

Treatment	GV (%)	GVBD (%)	MI (%)	MII (%)	D/NI (%)
TCM 199	11/100 (11)	20/100 (20)	25/100 (25) <sup>a</sup>	0/100 (0)	44/100 (44) <sup>a</sup>
SOF	4/100 (4)	23/100 (23)	58/100 (58) <sup>b</sup>	0/100 (0)	15/100 (15) <sup>b</sup>

<sup>a,b</sup>Percentages with different letters within each column differ ( $P < 0.007$ ). GV: germinal vesicle, GVBD: germinal vesicle breakdown, MI: Metaphase I, MII: Metaphase II, and D/NI: degenerated/non identifiable.

### Discussion

The present study is based on protocols that evaluated the effect of the reproductive status of the donor bitch on the oocyte maturation rates obtained using two different culture media, SOF and TCM 199. According to the literature, it is expected that a small percentage of oocytes obtained from fresh ovaries have already resumed meiosis. Mahi and Yanagimachi (1976) and Hewitt *et al.* (1998) reported 1.1 and 5.1% of oocytes from antral follicles at the GVBD stage at 0 hours. In a similar study (Bolamba *et al.*, 2002), 19.4% of the oocytes obtained were in the GVBD stage. The results of the present experiment showed high percentages of GVBD before maturation in both groups

studied (20.7% for anestrous and 43.6% for estrous donors). The break down of the germinal vesicle, characterized by the beginning of condensation of the chromosomes and nuclear membrane dissolution, is promoted by the LH preovulatory peak or by an atresic degeneration. In fact, a high percentage of oocytes in the GVBD stage may be obtained from advanced preantral follicles ( $< 210 \mu\text{m}$ ; Bolamba *et al.*, 2002).

The results referred to in the Table 1 also showed that 15.34% of the oocytes harvested from the estrous group before maturation were in the MI phase. This fact may indicate that some oocytes can be ovulated after the resumption of meiosis, contradicting most of the authors who reported that female dogs ovulate oocytes at the GV stage (Holst and Phemister,



1971; Farstad, 2000a; b; Luvoni, 2000; Otoi *et al.*, 2000a; Songsasen *et al.*, 2004). However, it is important to consider that mammalian oocytes, released from antral follicles, are able to resume meiosis spontaneously.

According to Yamada *et al.* (1993), oocytes from anestrus bitches have no tendency to resume meiosis *in vitro*. A similar result was described by Luvoni *et al.* (2001), indicating that dog COCs isolated from anestrus ovaries are unable to reinitiate meiosis when in culture. Despite these results, the present experiment showed that around 20% of the oocytes from anestrus bitches were capable of resuming meiosis (as evident by GVBD), and 10% achieved the MI stage *in vitro*, independent of the culture media used. However, no MII oocytes were observed after *in vitro* maturation of anestrus bitch oocytes. Although GVBD in anestrus oocytes can be caused by harvesting the oocytes from the inhibitory effect of the follicular fluid, some stimulatory effect of the culture system may be responsible for the organization of the MI phase. This finding is in agreement with the results obtained by Rota and Cabianca (2004) who matured oocytes from anestrus bitches for 72 hours and verified that only a small percentage of oocytes reached MII (1.3% in TCM and 0% in SOF) although a great number were identified as in the GVBD stage (68.6% in TCM and 56.9% in SOF).

Culture conditions have a significant effect on the oocyte's ability to express its potential; however, it is known that the quality of the oocyte is the main factor responsible for controlling its ability to undergo *in vitro* development. Luvoni *et al.* (2001) studied the influence of stage of the estrous cycle on the ability of bitches' oocytes to mature *in vitro* and concluded that the presence of cellular communications between oocytes and granulosa cells observed at the beginning of proestrus is important for oocyte competence. Indeed, the results of this experiment also showed that a high percentage of oocytes obtained from estrous animals were able to resume meiosis. Although the percentage of oocytes in the GVBD stage was similar for anestrus and estrous bitches, there was an increase in the percentage of oocytes in MI in the estrous group (25% for TCM 199 and 58% for SOF) when compared to anestrus animals (9.7% for both media). The higher percentage of meiotic progress observed in the oocytes obtained from estrous ovaries is probably due to the exposure of these oocytes to a follicular environment enriched by estradiol, progesterone, and other unknown factors. In fact, it is known that progesterone exerts a stimulative effect on the synthesis of maturation promoting factor (MPF) in the oocyte; MPF is responsible for GVBD (Salustri *et al.*, 1993).

The collection of oocytes on the day of ovulation was performed to obtain previously primed oocytes. It was expected that these oocytes would have cumulus-oocyte communications, and would be more

capable of finishing meiosis *in vitro*, under the stimulatory effect of estrogen, progesterone, and MPF. In fact these cells, naturally stimulated by the preovulatory LH peak, had already begun to resume meiosis since, just after collection, a higher number of estrous oocytes were in GVBD configurations compared to anestrus oocytes (43.6% and 20.7%, respectively). Moreover 15.3% of the oocytes obtained from estrous bitches were already in the MI stage just after collection, compared to 0% in the anestrus group. However, even taking into account these favorable conditions, no MII was observed in the oocytes obtained from estrous bitches after 24 hours of *in vitro* maturation. Maybe the recovery technique employed to harvest the oocytes resulted in a heterogeneous cell population, which could have been a limiting factor in obtaining better quality cells.

The results of the present experiment do not agree with Songsasen *et al.* (2004) who reported no influence of the different stages of the estrous cycle on oocyte maturation rate (MII) in TCM 199. However, it is important to emphasize that the determination of the stage of the estrous cycle was done by ovarian morphology, differing from the present study where it was identified through behavior, vaginal cytology, and serum progesterone concentration. In the present study, the collection of ovaries was performed 24 hours after serum progesterone concentrations reached 3 to 3.9 ng/ml. According to Root-Kustritz (2001), this corresponds to the ovulation day. This may positively influence the nuclear maturation rates of estrous oocytes.

When the two culture systems were compared in the estrous group, no significant difference was observed in the percentage of oocytes undergoing GVBD (20% for SOF and 23% for TCM 199). However, the proportion of oocytes in MI was significantly greater in SOF than in TCM 199 (58% vs. 25%, respectively). Since canine oocyte maturation occurs in the oviduct, SOF was tested. In addition, Bolamba *et al.* (2002) also evaluated the results of IVM rates in SOF concluding that it is a good medium to support nuclear *in vitro* maturation of canine oocytes.

Bogliolo *et al.* (2002), using TCM 199 supplemented with 10% EBS, FSH, LH, progesterone, estradiol and cisteamine, observed that 40% of oocytes underwent GVBD after 48 hours in culture, and no increase was observed when the oocytes were cultured for 72 hours. Similar results have been described by Otoi *et al.* (1999) and Yamada *et al.* (1993). However, Nickson *et al.* (1993) obtained the highest MII rates after just 24 hours in culture. A balance between the culture system and the incubation period is fundamental for improving the results of IVM. Although long culture periods can lead to higher oocyte degeneration rates during culture, the results of the present work indicate that in all the protocols tested, 24 hours of *in vitro* maturation was insufficient for obtaining a nuclear configuration of metaphase II.



Finally, the results of this experiment, allowed us to conclude that *in vitro* maturation of bitch oocytes is influenced by the interaction between reproductive status of the female and the culture system.

#### Acknowledgment

CAPES for the financial support, and Premier® Pet for the commercial food.

#### References

- Bogliolo L, Zeda MT, Ledda S, Leoni G, Naitana S, Pau S.** 2002. Influence of co-culture with oviductal epithelial cells on *in vitro* maturation of canine oocytes. *Reprod Nutr Dev*, 42:265-273.
- Bolamba D, Russ KD, Olson MA, Sandler JL, Durrant BS.** 2002. *In vitro* maturation of bitch oocytes from advanced preantral follicles in synthetic oviduct fluid medium: serum is not essential. *Theriogenology*, 58:1689-1703.
- England GCW, Concannon PW.** 2002. Determination of the optimal breeding time in the bitch: basic considerations. [database on the Internet]. In: Concannon PW, England G, Verstegen J, Linde-Forsberg C. *Recent advances in small animal reproduction*. Available from: [http://www.ivis.org/advances/Concannon/england2/ chapter\\_fmnr.asp](http://www.ivis.org/advances/Concannon/england2/ chapter_fmnr.asp). Accessed on: Jun 10<sup>th</sup> 2005.
- Farstad W.** 2000a. Assisted reproductive technology in canid species. *Theriogenology*, 53:175-186.
- Farstad W.** 2000b. Current state in biotechnology in canine and feline reproduction. *Anim Reprod Sci*, 60-61:375-387.
- Fontbonne A, Reynaud K, Marseloo N, Dumasy M, Chastant-Mailard S.** 2004. *In vivo* meiotic resumption, fertilization and early embryonic development in the bitch. In: Abstracts of the 5<sup>th</sup> International Symposium on Canine and Feline Reproduction, 2004, São Paulo, SP, Brazil. São Paulo: ISCFR. pp.144-146.
- Hewitt DA, England GCW.** 1997a. Effect of preovulatory endocrine events upon maturation of oocytes of domestic bitches. *J Reprod Fertil*, 51:83-91.
- Hewitt DA, England GCW.** 1997b. The canine oocyte penetration assay, its use as an indicator of dog spermatozoal performance *in vitro*. *Anim Reprod Sci*, 50:123-139.
- Hewitt DA, England GCW.** 1999a. Culture conditions required to induce capacitation and the acrosome reaction of canine sperm *in vitro*. *Vet Rec*, 144:22-23.
- Hewitt DA, England GCW.** 1999b. Synthetic oviductal fluid and oviductal cell coculture for canine oocyte maturation *in vitro*. *Anim Reprod Sci*, 55:63-75.
- Hewitt DA, Watson PF, England GCW.** 1998. Nuclear staining and culture requirements for *in vitro* maturation of domestic bitch oocytes. *Theriogenology*, 49:1083-1101.
- Holst PA, Phemister RD.** 1971. The prenatal development of the dog: preimplantation events. *Biol Reprod*, 5:771-779.
- Landim-Alvarenga FC.** 2001. Maturação *in vitro* de oócitos caninos. *Rev Bras Reprod Anim*, 25:3:477-478.
- Lopes MD.** 2002. *Nuclear maturation, histological and ultrastructural analysis of oocytes in female domestic cats (Felis catus)* [in Portuguese]. São Paulo, Brazil: São Paulo State University. Thesis.
- Luvoni GC.** 2000. Current progress on assisted reproduction in dogs and cats: *in vitro* embryo production. *Reprod Nutr Dev*, 40:502-512.
- Luvoni GC, Chigioni S, Allievi E, Macis D.** 2003. Meiosis resumption of canine oocytes cultured in the isolated oviduct. *Reprod Dom Anim*, 38:5:410-414.
- Luvoni GC, Luciano AM, Modina S, Gandolfi F.** 2001. Influence of different stages of the oestrous cycle on cumulus-oocytes on the efficiency of *in vitro* maturation. *J Reprod Fertil Suppl*, 57:141-146.
- Mahi CA, Yanagimachi R.** 1976. Maturation and sperm penetration of canine ovarian oocyte *in vitro*. *J Exp Zoo*, 196:189-196.
- Nickson DA, Boyd JS, Eckerall PD, Ferguson JM, Harvey MJA, Renton JP.** 1993. Molecular biological methods for monitoring oocyte maturation and *in vitro* fertilization in bitches. *J Reprod Fertil Suppl*, 47:231-240.
- Otoi T, Fujii M, Tanaka M, Ooka A, Suzuki T.** 1999. Effect of serum on the *in vitro* maturation of canine oocytes. *Reprod Fertil Dev*, 11:387-390.
- Otoi T, Fujii M, Tanaka M, Ooka A, Suzuki T.** 2000a. Canine oocyte diameter in relation to meiotic competence and sperm penetration. *Theriogenology*, 54:535-542.
- Otoi T, Ooka A, Murakami M, Karja NWK, Suzuki T.** 2001. Size distribution and meiotic competence of oocytes obtained from bitch ovaries at various stages of the oestrous cycle. *Reprod Fertil Dev*, 13:151-155.
- Otoi T, Murakami M, Fujii M, Tanaka M, Ooka A, Une S, Suzuki T.** 2000b. Development of canine oocytes matured and fertilized *in vitro*. *Vet Rec*, 146:52-53.
- Rodrigues BA, Rodrigues JL.** 2002. Maturation of canine oocytes in TCM-199 supplemented with different proteins. In: Proceedings of the 3<sup>rd</sup> European Veterinary Society of Small Animal Reproduction Congress, 2002, Liège, Belgium. Liège: EVSSAR. pp.94-95.
- Rodrigues BA, Rodrigues JL.** 2002. Meiotic response of *in vitro* matured canine oocytes under heterologous hormone supplementation. In: Proceedings of the 3<sup>rd</sup> European Veterinary Society of Small Animal Reproduction Congress, 2002, Liège, Belgium. Liège: EVSSAR. pp.158-159.
- Rodrigues BA, Rodrigues JL.** 2003. Influence of reproductive status on *in vitro* oocyte maturation in dogs. *Theriogenology*, 60:59-66.
- Root-Kustritz MV.** 2001. Use of commercial luteinizing hormone and progesterone assay kits in canine breeding management. [database on the Internet]. In: Concannon PW, England G, Verstegen J, Linde-Forsberg C. *Recent*



- advances in small animal reproduction*. Available from: [http://www.ivis.org/advances/Concannon/root2/chapter\\_frm.asp](http://www.ivis.org/advances/Concannon/root2/chapter_frm.asp). Accessed on: Aug 20<sup>th</sup> 2005.
- Rota A, Cabianga G.** 2004. *In vitro* maturation rates of canine oocytes from anestrous bitches in simple media. *Reprod Nutr Dev*, 44:105-109.
- Salustri A, Hascall VC, Camaioni A, Yanagishita M.** 1993. Oocyte-granulosa cell interactions. *In*: Adashi EY, Leung PC. (Ed.). *The ovary*. New York, NY: Raven Press. p.209-225.
- Songsasen N, Spindler R, Wildt DE.** 2004. Follicular size, but not stage of reproduction or season, influences meiotic maturation of domestic dog oocytes. *Reprod Fertil Dev*, 16:2:282-283.
- Songsasen N, Yu I, Leibo SP.** 2002. Nuclear maturation of canine oocytes cultured in protein-free media. *Mol Reprod Dev*, 62:407-415.
- Tsutsui T, Shimizu T.** 1975. Studies on the reproduction in the dog. IV. On the fertile period of ovum after ovulation. *Jpn J Anim Reprod*, 21:65-69.
- Van Der Stricht O.** 1923. Étude comparée des ovules des mammifères aux différentes périodes de l'ovogénèse, d'après les travaux du Laboratoire d'Histologie et d'Embriologie de l'Université de Gand. *Arch Biol Paris*, 33:229-300.
- Willingham-Rocky LA, Hinrichs K, Westhusin ME, Kraemer DC.** 2003. Effects of stage of oestrous cycle and progesterone supplementation during culture on maturation of canine oocytes *in vitro*. *Reproduction*, 126:501-508.
- Yamada S, Shimazu Y, Kawaji H, Nakazawa M, Naito K, Toyoda Y.** 1992. Maturation, fertilization, and development of dog oocytes *in vitro*. *Biol Reprod*, 46:853-858.
- Yamada S, Shimazu Y, Kawano Y, Nakazawa M, Naito K, Toyoda Y.** 1993. *In vitro* maturation and fertilization of preovulatory dog oocytes. *J Reprod Fertil Suppl*, 47:227-229.
-