

## In vitro maturation rates of canine oocytes from anoestrous bitches in simple media

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**Abstract** – The meiotic competence of canine oocytes collected from anoestrous bitch ovaries and cultured for 72 h in different media was studied. The base culture medium was TCM 199 enriched with 10% fetal bovine serum (TCM); the effect of supplementation with EGF (50 ng·mL<sup>-1</sup>) or ITS (insulin: 10 µg·mL<sup>-1</sup>; transferrin: 5.5 µg·mL<sup>-1</sup>; selenium: 5 µg·mL<sup>-1</sup>) was also studied. TCM was also compared to a Synthetic Oviductal Fluid (SOF). All the media contained FSH (0.1 UI·mL<sup>-1</sup>), LH (10 UI·mL<sup>-1</sup>), 17β-oestradiol (4 µg·mL<sup>-1</sup>) and kanamycin. Despite the anoestrous stage of the donor bitches, resumption of meiosis occurred in a high proportion of the oocytes, (mean value 77.3%). The number of oocytes showing the ‘germinal vesicle breakdown’ nuclear stage was not influenced by the type of the culture medium used. ITS had a positive effect on nuclear progression to later stages (from metaphase I to metaphase II); however, this effect was not statistically significant.

**bitch / anoestrous / oocytes / maturation / in vitro**

### 1. INTRODUCTION

In vitro maturation of canine oocytes yields rather poor results if compared to that of other species. Canine oocytes have the peculiarity of maturing in the oviducts, after having been released from ovulatory follicles at the germinal vesicle stage, and of requiring 48–72 h to reach the suitable stage for fertilisation [1–3]. If oocytes are collected from preovulatory follicles, they show higher meiotic competence, a fact that suggests that maturative events (although not meiosis resumption) take place inside the follicle [2]. The influence of various factors on the in vitro maturation of canine oocytes has been evaluated, but the results

are not univocal and maturation rates are hardly ever satisfactory. The stage of the oestrous cycle does not seem to affect in vitro maturation [4, 5] although oocytes of the highest quality were collected from the ovaries of proestrous bitches [6] and oocytes with larger diameters (> 120 µm) (which showed a higher ability to undergo meiotic maturation and to reach metaphase II) were recovered in higher numbers from bitches in the follicular phase of the oestrous cycle [3]. Serum supplementation gives contrasting results: canine oestrous serum improved the maturation rate in some experiments [7], but had no effect in others [8]; 0.3% BSA supplementation generally gives better results than fetal calf

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serum, but 20% serum is optimal for maturation when oocytes are cultured for 96 h [9], and the presence of serum may be necessary to prevent zona pellucida hardening. Addition of steroid hormones has been found to be ineffective [4]. Gonadotropins are sometimes a component of maturation media and a recent study showed the beneficial effect of a short exposure of canine oocytes to eCG [10].

The aim of this work was to study the maturation rates of oocytes collected from anoestrous bitches and to test the effect of supplementing a basic medium with Epidermal Growth Factor (EGF) or 'Insulin, Transferrin, Selenium' (ITS); the basic medium was also compared to a Synthetic Oviductal Fluid (SOF).

## 2. MATERIALS AND METHODS

All the products in this study were purchased from Sigma-Aldrich (Milan, Italy).

Ovaries were harvested from 12 bitches of various breeds, after routine ovariectomy. The bitches (1–8 years) were all healthy at the time of surgery, and in anoestrous, as gathered from history, clinical examination and macroscopic appearance of the ovaries. To confirm the stage of the cycle, blood for plasma progesterone and oestradiol determination was collected by puncture of the cephalic vein. Plasma was separated by centrifugation at  $3500 \times g$  for 6 min and stored at  $-25^\circ\text{C}$  until analysis.

Extracted plasma was assayed by microtitre RIA, with polyclonal anti-progesterone and anti-oestradiol antibodies raised in the rabbit. The assays had been previously validated for canine plasma [11].

Within 1 h after surgical excision, the ovaries were placed in PBS at  $30^\circ\text{C}$  and sliced to release cumulus oocyte complexes (COCs).

Grade 1 COCs, i.e. darkly pigmented oocytes, completely surrounded by at least one layer of cumulus cells [4], and  $>100\ \mu\text{m}$  diameter, were washed three times in a

modified HEPES 199 medium supplemented with  $1\ \text{mg}\cdot\text{mL}^{-1}$  polyvinyl alcohol and  $0.075\ \text{mg}\cdot\text{mL}^{-1}$  kanamycin [12], and then placed, in groups of 25–30, in  $500\ \mu\text{L}$  maturation medium, in four well dishes, covered by  $300\ \mu\text{L}$  of mineral oil, at  $38.5^\circ\text{C}$ , in a humidified atmosphere with 5%  $\text{CO}_2$  for 72 h.

Base culture medium was a modified TCM 199 enriched with 10% Fetal bovine serum (TCM). The effect of supplementing EGF ( $50\ \text{ng}\cdot\text{mL}^{-1}$ ) or ITS (insulin:  $10\ \mu\text{g}\cdot\text{mL}^{-1}$ ; transferrin:  $5.5\ \mu\text{g}\cdot\text{mL}^{-1}$ ; selenium:  $5\ \mu\text{g}\cdot\text{mL}^{-1}$ ) was studied. Base culture medium was also compared to SOF (Tab. I). All media contained FSH ( $0.1\ \text{UI}\cdot\text{mL}^{-1}$ ), LH ( $10\ \text{UI}\cdot\text{mL}^{-1}$ ),  $17\beta$ -oestradiol ( $4\ \mu\text{g}\cdot\text{mL}^{-1}$ ) and kanamycin ( $0.075\ \text{mg}\cdot\text{mL}^{-1}$ ). Each maturation treatment was tested in four replicates against the basic medium; for each replicate the oocytes of one bitch were randomly divided into two groups.

After 72 h of culture, cumulus cells were removed by placing the oocytes in a hyaluronidase solution ( $9\ \text{mg}\cdot\text{mL}^{-1}$ ) and then by repeated passages through small-bore glass pipettes. Denuded oocytes were positioned on a slide, fixed with a small drop of buffered 4% formalin solution and

**Table I.** Components of synthetic oviductal fluid (modified from [12]).

SOF (100 mL)	(g)
NaCl	0.63
KCl	0,053
$\text{NaHCO}_3$	0.21
Na lactate	$370\ \mu\text{L}$
$\text{MgCl}_2 \times 6\text{H}_2\text{O}$	0.0098
$\text{CaCl}_2 \times 2\text{H}_2\text{O}$	0.025
Phenol Red	0.001
Glutamine	0.015
$\text{KH}_2\text{PO}_4$	0.016
Glucose	0.027
MEM amino acids solution	1 mL
Bovine serum albumin (fraction V)	1

then stained with 10  $\mu\text{L}$  of a solution 3:1 glycerol/PBS, containing 2.5  $\text{mg}\cdot\text{mL}^{-1}$  sodium azide and 2.5  $\mu\text{g}\cdot\text{mL}^{-1}$  bis-benzimide (Hoechst 33258) [13].

The nuclear stages were classified under a fluorescent microscope, according to what was proposed by Hewitt et al. [9], as the "Germinal vesicle" (GV), "Germinal vesicle breakdown" (GVBD), "Metaphase I" (MI), "Anaphase I" (AI), "Metaphase II" (MII), unidentified or degenerated.

Data were analysed using the Logistic procedure of SAS [14]; the effect of the treatments on GVBD, MI/AI/MII, and on identifiable nuclear stages compared to the basic medium was studied with the "Analysis of maximum likelihood estimates".

### 3. RESULTS

The number of Grade 1 in vitro cultured COCs was 589; each bitch yielded a mean of  $36.8 \pm 8.1$  ( $\pm$  SD) Grade I COCs, ranging from a minimum of 10 to a maximum of 41. Plasma concentrations of progesterone and estradiol confirmed the anoestrous stage of the cycle for all the animals, since they were  $1.3 \pm 0.5$   $\text{ng}\cdot\text{mL}^{-1}$  and  $11.51 \pm 4.6$   $\text{pg}\cdot\text{mL}^{-1}$ , respectively [15].

After maturation, 129 oocytes were damaged during the procedures of decoronisation, fixation and staining; the nuclei of 287 oocytes, that is 62.4 % of the remaining ones ( $n = 460$ ), could be identified and classified.

The overall percentages of matured oocytes in the different media are reported

in Table II. GVBD occurred in quite a high proportion of oocytes (mean value 62.7%), without significant differences between TCM and the other media.

ITS supplementation increased the number of oocytes progressing to MI/AI/MII, while SOF reduced the number of oocytes maturing beyond GVBD; however the differences were not statistically significant. EGF supplementation significantly reduced the percentage of oocytes with identifiable nuclear stages.

### 4. DISCUSSION

Meiotic resumption occurred in quite a high percentage of oocytes, but for the majority of them, the nuclear stage that was identified was diakinesis. A much lower percentage of oocytes reached the MI/AI/MII stages, with a positive effect caused by ITS supplementation, though not statistically significant. EGF had already been experimented on canine oocyte maturation and our results confirm that it is ineffective in promoting maturation to MII [16]. In a previous study [17] ITS was present in the composition of a maturation medium for canine oocytes, but EGF was also added, so that those results are not comparable with ours; the effect of ITS alone was not investigated and our data suggest that it could be worth testing it at different concentrations. The ITS positive effect could be due at least partially to its antioxidative properties, that could be particularly beneficial during the long period of culture in vitro.

**Table II.** Oocyte maturation after 72 h of culture.

Maturation medium	No. oocytes examined	No. (%) identified nuclear stages	No. (%) GVBD	No. (%) MI/AI	No. (%) MII	Total No. (%) of oocytes that resumed meiosis
TCM	229	153 (66.8)	105 (68.6)	21 (13.7)	2 (1.3)	128 (83.6)
TCM+EGF	74	37 (50.0)	20 (54.0)	5 (13.5)	0	25 (67.5)
TCM+ITS	83	46 (55.4)	26 (56.5)	11 (23.9)	1 (2.3)	38 (82.6)
SOF	74	51 (68.9)	29 (56.9)	2 (3.9)	0	31 (60.8)

Culturing canine oocytes has been unrewarding till now, and even complex techniques, such as co-culture with oviductal and infundibulum cells [17, 18] or inside ligated bitch oviducts [19] have not univocally and significantly improved results, as compared to simpler procedures. In our study, in which we used a simple technique, meiotic resumption occurred in a high percentage of cultured oocytes; the low number of MII we registered may be due to the anoestrous stage of the donor bitches. Previous experiments showed that oocytes from anoestrous bitches have a low tendency to resume meiosis as compared to preovulatory oocytes [2]; cumulus-oocyte communications are not open in COCs collected during anoestrous [20], and this reduces their meiotic competence and their ability to reach MII in culture.

In our experiment, as in a large number of previous works, a high proportion (37.6%) of cultured oocytes could not be classified since they were degenerated or their nuclear configuration was not identifiable. Various authors [9, 21] signalled a high percentage of canine oocyte degeneration, already at collection, and also after culture. Hewitt et al. [9] reported a negative effect of fetal bovine serum on oocyte identification, but in our experiment also SOF, containing BSA and not serum, led to more than 30% unidentifiable nuclear stages (31.1 in SOF vs. 33.2 in TCM). It has been demonstrated that the percentage of non determined nuclear stages could be highly reduced by using confocal microscopy [22].

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## REFERENCES

- [1] Hewitt DA, England GCW. The effect of oocyte size and bitch age upon oocyte nuclear maturation in vitro. *Theriogenology* 1998, 49: 957–966.
- [2] Yamada S, Shimazu Y, Kawano Y, Nakazawa M, Naito K, Toyoda Y. In vitro maturation and fertilization of preovulatory dog oocytes. *J Reprod Fertil* 1993, Suppl 47: 227–229.
- [3] Otoi T, Ooka A, Murakami M, Karja NW, Suzuki T. Size distribution and meiotic competence of oocytes obtained from bitch ovaries at various stages of the oestrous cycle. *Reprod Fertil Dev* 2001, 13: 151–155.
- [4] Hewitt DA, England GCW. Effect of preovulatory endocrine events upon maturation of oocytes of domestic bitches. *J Reprod Fertil* 1997, Suppl 51: 83–91.
- [5] Rodrigues BA, Rodrigues JL. Influence of reproductive status on in vitro oocyte maturation in dogs. *Theriogenology* 2003, 60: 59–66.
- [6] Cinone M, Ghein A, Cairra M, Dell'Aquila ME, Minoia P. Collection and maturation of oocytes in the bitch. *Proceedings XII International Congress on Animal Reproduction* 1992, 4: 1767–1769.
- [7] Otoi T, Fujii M, Tanaka M, Ooka A, Suzuki T. Effect of serum on the in vitro maturation of canine oocytes. *Reprod Fertil Dev* 1999, 11: 387–390.
- [8] Rodrigues BA, Rodrigues JL. Meiotic response of in vitro matured canine oocytes under different proteins and heterologous hormone supplementation. *Reprod Domest Anim* 2003, 38: 58–62.
- [9] Hewitt DA, Watson PF, England GCW. Nuclear staining and culture requirements for in vitro maturation of domestic bitch oocytes. *Theriogenology* 1998, 49: 1083–1101.
- [10] Songsasen N, Yu I, Gomez M, Leibo SP. Effects of meiosis-inhibiting agents and equine chorionic gonadotropin on nuclear maturation of canine oocytes. *Mol Reprod Dev* 2003, 65: 435–445.
- [11] Rota A, Mollo A, Marinelli L, Gabai G, Vincenti L. Evaluation of cabergoline and busarelin efficacy for oestrous induction in the bitch. *Reprod Domest Anim* 2003, 38: 440–443.
- [12] Boni R, Tosti E, Roviello S, Dale B. Intercellular communications in in vivo- and in vitro-produced bovine embryos. *Biol Reprod* 1999, 61: 1050–1055.
- [13] Hinrichs K, Schmidt AL, Friedman PP, Selgrath JP, Martin MG. In vitro maturation of horse oocytes: characterization of chromosome configuration using fluorescence microscopy. *Biol Reprod* 1993, 48: 363–370.

- [14] SAS/STAT. User's Guide, version 6, fourth edition, Vol 2, SAS Inst., Inc., Cary, NC, 1990.
- [15] Olson PN, Bowen RA, Behrendt MD, Olson JD, Nett M. Concentration of reproductive hormones in canine serum through late anoestrous, proestrous and oestrous. *Biol Reprod* 1982, 27: 1196–1206.
- [16] Willingham-Rocky LA, Hanna CBM, Westhusin ME, Kraemer DC. Effects of porcine somatotropin and epidermal growth factor on the resumption of meiosis of canine oocytes matured in vitro. *Theriogenology* 2002, 57: 742.
- [17] Hewitt DA, England GCW. Synthetic oviductal fluid and oviductal cell coculture for canine oocyte maturation in vitro. *Anim Reprod Sci* 1999, 55: 63–75.
- [18] Bogliolo L, Zedda MT, Ledda S, Leoni G, Naitana S, Pau S. Influence of co-culture with oviductal epithelial cells on in vitro maturation of canine oocytes. *Reprod Nutr Dev* 2002, 42: 265–273.
- [19] Luvoni GC, Chigioni S, Allievi E, Macis D. Meiosis resumption of canine oocytes cultured in the isolated oviduct. *Reprod Domest Anim* 2003, 38: 410–414.
- [20] Luvoni GC, Luciano AM, Modina S, Gandolfi F. Influence of different stages of the oestrous cycle on cumulus-oocyte communications in canine oocytes: effects on the efficiency of in vitro maturation. *J Reprod Fertil* 2001, Suppl 57: 141–146.
- [21] Theiss T. Investigation on the collection, in vitro maturation and fertilization of dog oocytes. Thesis, Tierärztliche Fakultät der Ludwig-Maximilian Universität Munich, 1997.
- [22] Saint-Dizier M, Renard JP, Chastant-Maillard S. Induction of final maturation by sperm penetration in canine oocytes. *Reproduction* 2001, 121: 97–105.