Current Relevant Knowledge on Dog Reproductive Physiology – a Review

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Abstract
Successful *in vitro* techniques depend on the possibility to mimic *in vivo* conditions. For this reason, knowledge on reproductive physiology is necessary to define an efficient entirely *in vitro* system to produce embryos. A better understanding of the processes and the factors underlying *in vivo* fertilisation in the dog is necessar. *In vitro* oocyte maturation had been extensively studied, but we could wonder if *in vivo* processes are identical with what was observed during *in vitro* studies. Improving reproductive biotechnologies therefore requires the realization of fundamental studies to better describe and understand phenomena. This review presents a summary of current available knowledge about dog reproductive physiology and biology of the canine oocyte development and maturation, specifically those factors influencing *in vitro* developmental competence of the oocyte. An understanding of the mechanisms controlling oocyte maturation and development is a particularly important target because it would be helpful to developing assisted breeding in this species.

Keywords: Canine, folliculogenesis, *in vivo* oocyte maturation, oogenesis, physiology.

1. Introduction

The oocyte of the domestic dog is unique from that of other mammalian species studied to date. Ovulation occurs either once or twice per year, with the oocyte released at the germinal vesicle stage and then completing nuclear and cytoplasmic maturation within the oviduct under the influence of rising circulating progesterone. *In vivo* meiotic maturation of the bitch oocyte is completed within 48 to 72 h after ovulation, which is longer than 12 to 36 h required for oocytes from most other mammalian species [1].
Formation of oogonia can be recognized in the canine foetus from 42 days post-coitum [2]. During late gestation, the cortical lobules and cords at the cortex of the ovary are composed exclusively of oogonia and pre-granulosa cells. At birth (63 days post-ovulation) oogonia in mitosis are common and degenerating oogonia can be also observed. Folliculogenesis takes place from 2 to 12 weeks after birth and primordial follicles can be seen from about 3 weeks after birth [2]. From 3 to 4 months of age, the oocytes duplicate their size and the zona pellucida (ZP) is formed in oocytes larger than 60 μm in diameter. At the time when the antrum appears, the oocyte has attained almost adult size, approximately 100 μm in diameter. At this stage the oocytes are surrounded by 3–5 layers of granulosa cells [2]. There are few morphological studies of canine oocytes from juvenile bitches. Nickson et al. (1993) investigated gross oocyte morphology in pre-pubertal bitches. Only a small number of oocytes could be collected from ovaries in juvenile bitches and these oocytes had incomplete layers of cumulus cells and were smaller than oocytes collected from adult bitches. A consistent feature of the developing oocyte of the dog is the appearance of lipid yolk bodies [4, 5]. Lipid droplets first appear in the primary oocytes of the pre-antral follicles, and they

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increase in number throughout the entire period of oogenesis [5]. The total number of follicles and total number of oocytes found in monovular follicles per sample area was similar in ovaries of pre- and postpubertal bitches [6]. Likewise the number of polyovular follicles did not change before and after puberty. Despite the popularity of the dog in studies of reproductive physiology and endocrinology there is still very scarce information on the morphology and ultrastructure of canine oocytes [7].

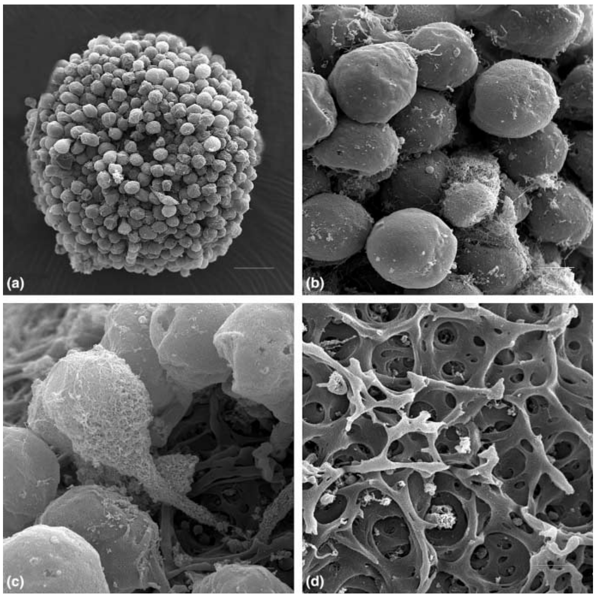

Figure. 1. Scanning electron microscopy of a COC obtained from a pre-pubertal bitch: (a) The round cumulus cells are regularly arranged around the oocyte (scale bar ¼ 17 µm). (b) The cumulus cells are connected with each other by few short processes (scale bar ¼ 3, 4 µm). (c) Near the zona pellucida (ZP) the cumulus cells reveal a bulbous form with one process reaching into a pore of the ZP (scale bar ¼ 2 µm). (d) The ZP reveals numerous spherical or elliptical pores narrowing centripetally (scale bar ¼ 2 µm) [7].

Canine reproductive physiology has unique characteristics. Canines (Canis familiaris) are monoestrous, polyovulatory, with a seasonality nearly lost through domestication in the dog but present in wild species i.e. wolves and foxes [8, 9, 10]. Ovulation occurs 1–2 days after the preovulatory LH peak, at the beginning of the estrus, only once or twice a year at a 5 to 12 month interval [1].

2. The oestrous cycle of the bitch is characterized by four successive periods: a protracted proestrus and estrus (each about 1 week in duration), oestrus, metoestrus (or dioestrus) and anoestrus. Pro-oestrus is defined as the period from onset of vulvar bleeding to the first acceptance of copulation. Its average duration is 9 days, but it displays considerable variation, as it can range from 0 to 27 days [11]. The following phase is oestrus, the period during which the bitch accepts copulation. Here also, the duration of this phase is highly variable: 3 to 21 days [12]. Irrespective of whether pregnancy occurs, estrus is followed by diestrus (metestrus), a luteal phase of elevated circulating progesterone with an average duration of about 2 months [13, 11]. Metestrus (diestrus) begins when the bitch is no longer willing to accept the male. Its average duration is 70 days. It is associated with the presence of active corpora lutea. Toward the end of diestrus, corpus luteum function declines as the bitch enters a prolonged anestrus period of 2 to 10 months duration. Anestrus is the phase between the end of the metestrus and the beginning of next pro-estrus and is characterized by the lack of any sexual behavior or gonadal activity, including nadir circulating progesterone concentrations (P4). Its duration is variable, 2 to 10 months[14]. From mid- to late anestrus, there is an increase in hypothalamic gonadotropin releasing hormone (GnRH) secretion that elicits an increase in follicle stimulating hormone (FSH) and episodic LH release. FSH plays a crucial role in the initiation of folliculogenesis and the onset of proestrus in the bitch [15, 16, 12]. Toward the end of proestrus, the LH surge stimulates a rapid enlargement of mature follicles and preovulatory luteinization that leads to ovulation 40 to 50 h after the LH peak [17, 13]. Behavioral estrus and ovulation occur in the presence of declining circulating estrogen and significantly elevated progesterone [18].

Surprisingly, relatively little is known about the changes in, and temporal relationships between, **reproductive hormones** around the time of ovulation in the domestic bitch [19].

The secretory pattern of **LH** at this period is characterised by frequent increases of short duration [15]. Unlike the other mammalian species, the duration of the LH surge in the bitch is relatively long, ranging from 1 to 5 days [20, 21, 19]. Furthermore, Hase et al. (2000) found that the period in which LH values peaked above 10 ng/mL continued for more than 12 hours. De Gier et al. (2006) found that this LH peak had a bifurcated aspect in 4/6 Beagle bitches. LH is often stated as the ideal technique for **determining with accuracy the ovulation period**, as the LH peak induces ovulation and is, therefore, generally stated as being the "Day zero" of the sexual cycle of the bitch. Assaying LH is in fact not easy as it requires repeated blood samplings and a specific assay technique. Furthermore, De Gier et al. (2006) doubt that assaying LH is a good method to time ovulation, due to the individual variations among bitches.

At the beginning of pro-oestrus, the basal plasma **FSH** concentration is elevated, but becomes relatively low during the progression of the follicular phase [19]. Concurrent or slightly earlier than the LH peak, a pre-ovulatory surge in FSH occurs that lasts about three times longer than the pre-ovulatory LH peak [19].

During pro-oestrus, as tertiary follicles develop within the ovaries, they produce **oestradiol**, leading to peak plasma levels in late proestrus [12]. These oestradiol peak levels differ considerably between oestrous cycles, both within and between individual bitches [23]. De Gier et al. (2006) demonstrated that these high plasma oestradiol concentrations occur concomitantly or just before the LH peak and have a positive feedback effect on LH release, leading to the preovulatory LH surge. However, as soon as the LH surge has occurred, there is a decrease in the plasma oestradiol 17β concentration [24]. Basal values occur around 80 hours after the LH peak [19].

During proestrus, plasma **progesterone** concentrations initially remain low but fluctuate. At the start of the pre-ovulatory LH surge, granulosa cells begin to luteinise and secrete progesterone [19]. The exact temporal relationship between the initial rise in plasma progesterone concentration and the pre-ovulatory LH surge is uncertain. Wildt et al. (1978) found that there was a slight detectable rise in progesterone – 0.5 to 2.5 ng/mL – concomitantly or within 24 hours following the burst of LH. In fact, the initial rise in progesterone concentrations may occur just before, concomitantly or just after the start of the LH surge [19]. After the initial rise, De Gier et al. (2006) also found that the plasma progesterone concentration remained at about the same level for 3 to 4 days before increasing again in 4/6 bitches. According to England and Concannon (2002), 2.0 ng/mL is the progesterone concentration typically observed at the time of the LH surge or on the following day. During metestrus, plasma progesterone concentrations are high. They usually plateau at 10 to 30 days after ovulation. In non-pregnant bitches, the progesterone secretion declines slowly and reaches a basal level at about 75 days after the start of the luteal phase.

3. Folliculogenesis - during anoestrus, follicular growth occurs, but terminal follicular differentiation is absent and maximum follicular diameter is only 0.6-1mm (antral follicles) [2]. Dog follicles can be categorized into five classes depending on morphology, size, type and number of follicle cell layers and the presence of follicular fluid [24]. Data in Table 1 summarizes follicle classification and days in which each class is initially found in the ovaries.

At the onset of proestrus, several follicles measuring 1 to 1.5 mm are already present within the ovary, they grow and reach 1.5 to 5 mm at the end of proestrus, when progesterone concentrations are still basal : < 1 ng/mL [22]. During estrus, follicular diameters increase, reaching the pre-ovulatory stage. Diameters of pre-ovulatory follicles have been reported to range from 3 to 8 mm [17, 25, 22]. Prior to ovulation, the follicles undergo luteinisation. Histologically, the granulosa cells layer takes at this stage a plicated appearance [2].

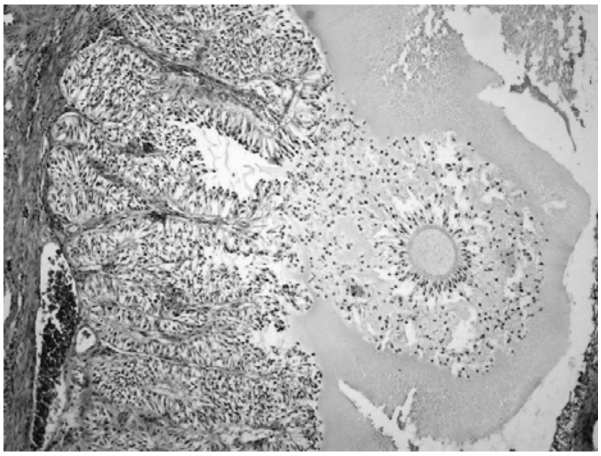

Figure 2. Follicle undergoing preovulatory luteinization [26].

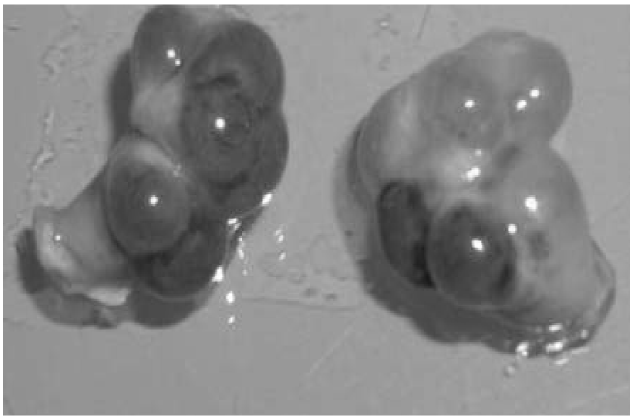

Figure 3. Bitch preovulatory follicles. 100 µm 1 cm [26].

Table 1. Follicle classification, diameter and oocyte type in relation to fetal (or bitch) age during which each class are found within the ovary [24]

Classification	Age (days)	Follicle diameter (mm)	Oocyte	References
Primordial	17-54	0.04-0.05	small oocytes (≈. 25 µm in diameter) with a single granulosa cell layer but no zona pellucida (ZP)	Andersen and Simpson (1973); Tesoriero (1981); Barber et al. (2001); Blackmore et. Al (2004); Durrant et al. (1998)
Primary or preantral	120	0.05-0.08	small, pale oocytes (78 ± 15 µm) with a distinctive ZP	Durrant et al. (1998); Barber et al. (2001)
Secondary or advanced preantral	NA	0.148-0.211	fully grown oocytes (>100 µm) that are comprised of dark cytoplasmic lipid	Durrant et al. (1998); Barber et al. (2001)
Tertiary or early antral	120-160	0.350-0.360	occurs synthesis of follicular fluid	Durrant et al. (1998); Barber et al. (2001)
Advanced antral	240	2-13	as a result of the LH surge, they rapidly enlarge into 4 to 13 mm preovulatory follicles and ovulate approximately 48 h later, but most growing follicles undergo atresia before reaching the preovulatory stage	Wildt et al. (1977, 1979); England and Allen (1989), Concannon et al. (1989)

4. Pre-ovulation oocyte maturation - pre-ovulatory oocytes are difficult to obtain because their collection requires bitches in oestrus and because it must follow a precise monitoring of the heat period to determine the exact time prior to ovulation. In the majority of mammalian species, the pre-ovulatory LH peak represents the stimulatory signal inducing, before ovulation, both the resumption of oocyte meiosis (from prophase I to metaphase II) and the mucification of cumulus cells due to hyaluronic acid accumulation.

Preovulatory luteinization of follicles is exposing oocytes to increasing concentrations of progesterone, as opposed to the situation in many other domestic mammals, where estrogen dominates the preovulatory follicular environment. In most mammals, ovulation of the oocyte occurs when the oocyte has reached the metaphase of the second meiotic division. In canines, the oocyte is ovulated at the beginning of the first meiotic division, and the germinal vesicle is broken down shortly after ovulation.

Subsequent stages of oocyte maturation occur in the oviduct and take 2–3 days to complete [27, 28].

A few hours after the LH peak, mucification is clearly apparent in the granulosa cells of the cumulus [29]. However, the two or three innermost layers of granulosa cells remain unmucified and compact around the oocyte [20]. This mucification depends on the follicular maturity and, in a pre-ovulatory ovary, all the oocytes originating from the antral follicles are not mucified after LH and a minimal follicular diameter (linked to the differentiation, i.e. the receptivity to LH) seems to be required [29]. At the pre-ovulatory stage, oocyte diameters can range from 100 to 120 µm [2].

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5. Ovulation - in the bitch, ovulation is assumed to occur approximately 2 to 3 days after the preovulatory LH surge and sexual receptivity [20, 25]. However, the period at which ovulation occurs ranges from as early as 24 hours until more than 96 hours after the pre-ovulatory LH surge [21].

At ovulation, the rupture site of a follicle can be recognised by a red pin-point area [2]. This point is 0.4 to 0.8 mm in diameter. Follicular rupture does not seem to be associated with extensive haemorrhage [17].

Mean ovulation rate in the canine species can be estimated by several methods (ie: litter size or ultrasonography). However, litter size cannot take into account embryonic or early fetal losses, and it is not clear whether ovarian ultrasonography permits to correctly evaluate the number of pre-ovulatory follicles and if nonovulated follicles remain – and, if so, in which percentage - after ovulation [30]. Some authors evaluated ovulation rate more precisely, after ovariectomy and by counting corpora lutea present on both ovaries, and reported an ovulation rate that ranged from 5.7 ± 0.3 (n=22 bitches – [31]) to 6.0 ± 0.1 (n = 192 bitches – [32]). However, the role of the size or the breed of the bitch on the size of pre-ovulatory follicles and on ovulation rate remains to be further established.

After the LH peak, ovulation occurs but its duration and synchronicity are not well known. Concannon et al. (1986) found that ovulation appeared to occur synchronously in the two ovaries about 36 to 50 hours after the LH peak.

Concerning the duration of the ovulation process, Boyd et al. (1993) suggested that the ovulation process seemed to begin in the right ovary and that the whole process may take as long as 36 hours to be completed.

In the bitch, polyovular follicles are not uncommon [34, 35]. However, it is not known whether these follicles can reach ovulation and release one or more viable oocytes. Follicles containing more than one oocyte are frequently observed in small growing follicles, but only rarely in large pre-antral ones [34]. However, Bysted et al. (2001) reported the collection after flushing of more oocytes or embryos than expected after counting the corpora lutea. We may therefore think that ovulation of more than one oocyte per follicle may occasionally occur.

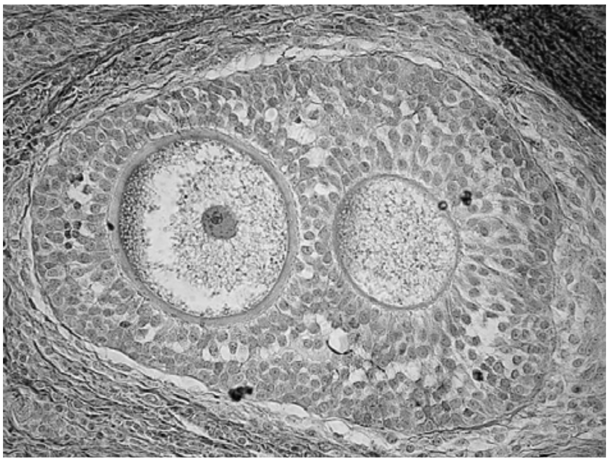

Figure 4. Histological section of a poliovulatory bitch follicle 100 µm 15 [26].

6. Post-ovulation oocyte maturation - This aspect has been poorly studied so far in the bitch. It is now well established that the canine oocyte is ovulated at an immature stage (germinal vesicle, prophase I) and must further undergo meiosis resumption before being fertilised.

A few hours after ovulation, oocytes are found within the uterine tubes [20]. Dense cumulus cell layers are still attached around the oocyte, and will remain until the early embryonic development. This linkage between the cumulus cells and the oocyte may participate in blocking the oocyte meiotic resumption [37].

Twenty-four to forty eight hours after ovulation, the oocytes are found in the proximal and medial parts of the uterine tubes. [20, 38]. *In vivo*, no oocytes at the metaphase I stage were observed until 48 hours post-ovulation, and the metaphase II stage appeared even later [39].

In vivo, Van der Stricht (1923) using optical light microscopy, observed sperm heads inside the cytoplasm of oocytes at the germinal vesicle (GV) or the metaphase I (MI) stage and hypothesised that oocytes may be penetrated by spermatozoa at an immature stage. These findings have also been observed *in vitro* [41, 42], suggesting that sperm penetration may play a role in the resumption of oocyte meiosis. It remains to be demonstrated if this phenomenon may be confirmed *in vivo* using more recent techniques of microscopy [43].

Because mating can occur as early as 3 days before ovulation [44], the immature dog oocyte and spermatozoon meet in the oviduct, and sperm penetration may be involved in inducing nuclear maturation, i.e., resumption of meiosis [41, 42]. However, *in vivo* studies have reported that fertilization does not occur until 44 to 120 h after ovulation when the oocyte has completed nuclear maturation [45, 46, 47].

Thus far, there is no clear explanation for the discrepancy between *in vivo* versus *in vitro* observations. It is known that oviductal cells can maintain longevity of dog sperm by inhibiting calcium flux that, in turn, prevents sperm from undergoing capacitation [48]. Additionally, dog spermatozoa remain viable *in vivo* for as long as 11 days after mating [49]. Based on these observations, it is possible that the discrepancy between *in vivo* and *in vitro* studies is related to delayed sperm capacitation within the oviduct rather than the inability of oocytes to be fertilized [24].

After ovulation, oocytes may also remain fertilisable for a significant time, up to 5 days [31] and even 7 or 8 days [50], and even after the closure of the cervix [51]. However, if the uterine tubes are flushed between 4 to 10 days following ovulation, up to 50% of nonfertilised degenerated oocytes are collected together with normal embryos [36, 52]. Some of these oocytes are still at an immature stage and may have been bad quality oocytes. The end of the oocytes ability to be fertilised may widely be due to changes in the local environment [53].

Moreover, the canine oocyte is very rich in lipids and the cumulus cell mass around the oocyte is tight and multilayered and remains attached to the gamete longer after fertilization [44]. The presence of primary oocytes in the oviducts increases the chance of the oocyte meeting spermatozoa before or during maturation and it has been shown in the fox that primary oocyte can be fertilized and a male pronucleus can be formed irrespective of the stage of oocyte maturation [54]. The canine embryos require long time for the passage of the oviduct and enter the uterus, 7–9 days after ovulation, as an embryo of 16 cells or more [27]. Thus, the oviduct supports long term survival of oocytes that in this tract complete maturation, undergo fertilization, and develop up to the morula-blastocyst stage [55].

7. Conclusions

This summary lays a foundation for identifying the next steps to understanding the mechanisms regulating meiotic maturation and developmental competence of the dog oocyte.

Our inability to regulate or promote reproductive success in the dog is also due to inherent physiological uniqueness of the species and to the lack of research attention (few laboratories study dog reproductive biology).

The immature stage of oocytes at ovulation and the persistence of cumulus cells during the transport and maturation period within the oviduct suggest that the investigation of the relationship between cumulus cells and oocyte could have contributed to clarifying the reasons behind the low efficiency of in vitro maturation of canine oocytes.

All these studies confirm that the morphological appearance and diameter of the oocytes, the developmental stage of the follicles, and the stage of the cycle, coupled with culture conditions are important factors to take into account for the successful IVM of dog oocytes.

Despite the popularity of the dog in studies of reproductive physiology and endocrinology there is still very scarce information on the morphology and ultrastructure of canine oocytes. Overall knowledge in this species compared to others is rudimentary at best, and especially for the oocyte.

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