In vivo meiotic resumption, fertilization and early embryonic development in the bitch

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Abstract

Early development in canine species follows a very specific pattern. Oocytes are ovulated at the germinal vesicle stage and meiotic resumption occurs in the oviduct. However, because of difficulties in the accurate determination of ovulation time and in the observation of oocyte nuclear stage by light microscopy, these early events have not been fully described. Moreover, the oocyte stage at which sperm penetration occurs is still uncertain since fertilization of immature oocytes has been reported in vivo and in vitro. The aim of this study was to establish the exact timing of in vivo meiotic resumption, fertilization and early embryo development in the bitch with reference to ovulation. Ovulation was first determined by ultrasonography, artificial inseminations were performed daily and oocytes/embryos were collected between 17 and 138 h after ovulation. After fixation and DNA/tubulin staining, the nuclear stage was observed by confocal microscopy. Of the 195 oocytes/embryos collected from 50 bitches, the germinal vesicle stage was the only one present until 44 h post-ovulation, and the first metaphase II stage was observed for the first time at 54 h. Sperm penetration of immature oocytes appeared to be exceptional (three out of 112 immature oocytes). In most cases, fertilization occurred from 90 h post-ovulation in metaphase II oocytes. Embryonic development was observed up to the eight-cell stage. No significant influence of bitch breed and age on ovulation rate, maturation and developmental kinetics was observed. However, some heterogeneity in the maturation/development process was observed within the cohort of oocytes/embryos collected from one bitch. In conclusion, the most peculiar aspect of the canine species remains oocyte meiotic maturation whereas fertilization follows the same pattern as in other mammals.

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Introduction

Among mammals, the canine species is well known for its unusual pattern of oocyte meiosis: at ovulation, an oocyte is released in the germinal vesicle (GV) stage and meiotic resumption occurs about 48 h after ovulation in the oviduct. Moreover, the endocrine environment at ovulation in the bitch is highly different to that observed in other species, since follicles undergo a preovulatory luteinization after the luteinizing hormone (LH) peak and serum progesterone has already reached high levels at ovulation.

The kinetics of meiosis and embryonic development with reference to ovulation has not, however, been fully described. The precise determination of this chronology is very important for canine reproductive biotechnologies, especially to select the optimal moment for artificial insemination with frozen semen or embryo transplantation. In previous studies, the time of ovulation has not been precisely defined and embryonic development was either assessed in respect to the moment of the LH peak or the progesterone level, or was observed by laparoscopy (Tsutsui & Shimizu 1975, Archbald et al. 1980, Bysted et al. 2001). Secondly, determination of the nuclear stage in canine oocytes by stereomicroscopy without DNA staining (Tsutsui & Shimizu 1975) or fluorescent optic microscopy (Saint-Dizier et al. 2004) is quite difficult and uncertain, because of the high lipid content of their cytoplasm. Nowadays, advanced observation techniques such as immunocytochemistry coupled with confocal microscopy are available and have been shown to be especially effective in the observation of the bitch oocyte (Reynaud et al. 2004, Saint-Dizier et al. 2004). Nevertheless, the exact timing of the oocyte maturation step remains to be determined, as well as the exact role of sperm in meiotic resumption. Indeed, sperm heads have already been observed in vivo in immature bitch oocytes.
(Van der Stricht 1923, fox: Farstad et al. 1993). In vitro, this penetration at an immature stage has been shown to occur with a high frequency and to act as an inducer of meiotic resumption (Saint-Dizier et al. 2001).

The aims of this study were thus to evaluate the precise kinetics of oocyte nuclear maturation as well as early embryonic development in the bitch, and to examine whether sperm penetration at immature stages is a physiological feature in vivo. Ovulation time was accurately determined through ovarian ultrasonography, and nuclear stages were defined by confocal microscopy after DNA/tubulin staining.

Materials and Methods

Animals

Fifty bitches, aged 8 months to 9 years (mean 3.80 ± 0.34 years), were included in this study. Twenty-two were Beagles (10–13 kg) from our research kennel and 28 were mongrel or pure-bred bitches from owners and breeders (3–40 kg) attending a consultation for routine ovariectomy. The protocol was approved by the Ethics committee of the National Veterinary College of Alfort.

Oestrus detection and determination of ovulation time

Ovarian cycles of the bitches were followed weekly by performing vaginal smears (for our experimental kennel bitches) and observing vulvar bleeding to detect onset of the heat period. During heat, serum progesterone levels were assayed every day. The progesterone assay was performed using an Elecsys kit (Roche Diagnostics, Meylan, France). Before ovulation, follicles were identified as anechoic structures (starting from 1 mm in diameter and reaching 5–7 mm just before ovulation). At the time of ovulation, the follicular anechoic cavity disappears or its diameter dramatically decreases (2–3 mm) with the inner follicular wall becoming fuzzy. Furthermore, liquid is often visible surrounding the ovary (Hayer et al. 1993). This process lasts for a few hours and therefore the reference to ovulation was set at the mean time between the last ultrasonography with all follicles visible and the one where there was a significant change or complete disappearance of follicles.

Artificial insemination

To allow eventual penetration of spermatozoa in immature oocytes, the permanent presence of sperm in the oviducts was maintained starting before ovulation. Intra-uterine artificial inseminations using a Scandinavian catheter (Andersen 1975) were thus performed once a day from the day before ovulation (progesterone level around 2–3 ng/ml, large follicles >4 mm) to the day of ovariectomy. Semen was collected daily from two Beagle males with known high in vivo fertility, using manual stimulation. After evaluation of the concentration and mobility, the two ejaculates were pooled and fresh semen was used for uterine insemination (spermatic and prostatic fractions).

Collection and fixation of canine oocytes and embryos

Ovariectomies were performed using a conventional surgical procedure from 15 to 136 h after ovulation. Ovaries, oviducts and the tip of the uterine horns were immediately collected and kept at 38 °C. The number of corpora lutea and non-ovulated follicles was evaluated and oviducts were carefully dissected from the fat of the ovarian bursa. A cannula 3.5 French (Tom Cat catheter; Kendall, Coveto, Montaigu, France) was inserted into the distal ostium of the oviduct (when surgery was performed during the first 24 h after ovulation) or in the infundibulum (24 h post-ovulation). Oviducts were flushed with 20 ml warm medium 199 (M199; Sigma, St Quentin-Fallavier, France) supplemented with 20% fetal calf serum (FCS; Invitrogen, Cergy Pontoise, France). Oocytes/embryos were immediately fixed at 38 °C in 2% paraformaldehyde (Merck, Fontenay-sous-Bois, France; w/v in phosphate-buffered saline (PBS) from Sigma) for 30 min, and then in 4% paraformaldehyde (w/v in PBS) for a further 30 min. They were rinsed in PBS +1% fraction V BSA (bovine serum albumin; Sigma). The diameter of the oocyte was measured (zona pellucida excluded; ×400; IX70 inverted microscope; Olympus, Rungis, France) and oocytes were then stored at 4 °C until immunocytochemistry.

Flushing medium was centrifuged (5 min; 200 g) and the presence of spermatozoa was evaluated in the pellet by optical microscopy (×400; microscope BX41; Olympus).

Determination of maturation or developmental stage of oocytes/embryos

When numerous granulosa cells were still present around the oocytes, decoronation was performed by gentle pipeting or incubation in 0.7% pronase for 45 s (Roche; w/v in M199) (Reynaud et al. 2004).

Microtubule visualization was performed by immunocytochemistry according to the technique used in the rabbit (Adenot et al. 1997). Briefly, after fixation and three washes in PBS, oocytes were incubated in a blocking solution (PBS containing 20% FCS and 0.5% Triton X-100) for 30 min at 37 °C, then incubated for 60 min at 37 °C with mouse α-tubulin monoclonal antibody (Sigma) diluted 1:200 in PBS/2% FCS/0.5% Triton. After three washes in PBS/2% FCS/0.5% Triton, oocytes were incubated for 60 min at 37 °C in Alexa fluor-conjugated rabbit anti-mouse antibody + ethidium homodimer-2 (Molecular Probes, Interchim, Asnières, France) diluted 1:500 in PBS/2% FCS/0.5% Triton (final concentration of 4 μg/ml and 2 μM respectively). Stained oocytes were then mounted on slides with Vectashield (Vector Laboratories, USA) and stored at −20 °C.
Results

Collection of canine oocytes/embryos

A mean total of 7.89 ± 0.57 corpora lutea (2–22; n = 46 bitches) was detected in the ovaries. There were no significant influence of bitch age, breed and no interaction between age and breed. The number of corpora lutea was significantly higher on the right compared with the left ovary (4.22 ± 0.40 and 3.15 ± 0.30 respectively; P < 0.05). Non-ovulated follicles, defined as structures full of serous liquid, larger than 5 mm were observed in 12 bitches (32%). For these bitches, between one and four non-ovulated follicles were observed per bitch, representing a mean of 23% (from 10 to 44%) of the follicles present on both ovaries.

A total of 287 oocytes/embryos were collected from the 50 bitches (5.74 ± 0.48 per bitch, from 0 to 19 maximum per bitch). Global recovery rate (number of structures collected/number of corpora lutea) was 72.4 ± 3.9% varying from 0 to 150% according to the bitch. In two cases, the number of oocytes collected was found to be higher than the number of corpora lutea observed (respectively eight and six structures collected for seven and four corpora lutea). No dilatation of the oviduct was observed.

Oocyte/embryo characteristics

Mean oocyte diameter was 82.4 ± 0.6 μm (n = 166; from 58.8 to 100.7 μm) when measured without zona pellucida and 111.3 ± 0.7 μm (n = 162; from 86.2 to 131.5 μm) with the zona, when the limit between zona and granulosa cells was clearly defined. Some intra-bitch variability (maximum 20.4 μm in the cohort of oocytes from the same bitch) was observed.

None of the cumulus–oocyte complexes presented mephication at any stage after ovulation (Fig. 1). Soon after ovulation (Fig. 1a), oocytes were surrounded by two to three layers of dense granulosa cells. Later on, even if the external layer became more loosely attached, the inner ones remained compact (Fig. 1b). Despite the presence of very dense granulosa cells, some immature oocytes were found to be fertilized (Fig. 1c: fertilized MI oocytes). During their stay in the oviduct, oocytes/embryos progressively lost the surrounding cells (Fig. 1d and e) but this denudation was not systematically observed (compare two- and eight-cell embryos in Fig. 1e and f respectively).

Kinetics of meiotic resumption and early embryonic development

Due to the high cytoplasmic lipid content, oocyte and early embryo stages are impossible to determine under light microscopy. Of the 43 bitches, 195 oocytes/embryos were stained and submitted to confocal microscopy. Representative examples of all stages observed are shown in Fig. 2. Maturation and developmental stages observed for each bitch are summarized in Fig. 3.
Until 44 h after ovulation, all oocytes were at the GV stage, with the nucleus peripherally located. Surprisingly, during this post-ovulatory period, the appearance of germinal vesicles was singular. Nuclear membrane, still unfolded and continuous, delimits a space of around 20 μm in diameter but DNA was restricted to much smaller zona of 4–5 μm with chromatin organized around nucleoli (Fig. 2 a–c). Only one GV (out of 82) presented a classical large round-shaped structure (21 μm in diameter), with the DNA dispersed through the whole volume.

After 44 h post-ovulation, some GV oocytes were still present, but later stages began to be observed. No GVBD was seen at any time, MI oocytes were detected starting from 48 h (Fig. 2d) and the first MII oocyte (Fig. 2g) at 54 h. Pronuclear stage embryos appeared in general after 92 h (Fig. 2h and i). Two-cell embryos could be observed from 112 h (Figs 1d and 2j), together with embryos at the four- and eight-cell stage (Figs 1e–f and 2k and l).

It was clear that maturation was very homogenous before 44 h, since all oocytes (51) from 12 bitches were at the GV stage. However, as early as meiosis resumed, cohorts began to diverge. GV, MI, TI and MII stages could be simultaneously observed in the same bitch (Fig. 3, bitch 11). Later, after fertilization, this heterogeneity persisted, as one bitch could have, at the same time, two-, three-, four- and eight-cell embryonic stages (Fig. 3, bitch 30). As a consequence, one maturation stage could be observed over a long period (GV from 17 to 109 h post-ovulation, MI from 48 to 127 h and MII from 54 to 127 h). However, no significant influence of bitch breed and age on maturation and developmental kinetics was observed.

Figure 1 Canine oocytes and embryos observed under light microscopy at different stages after ovulation. Maturation stages were determined by confocal microscopy. (a) Cumulus–oocyte complex at (a) 35 h post-ovulation and (b) 69 h post-ovulation, (c) fertilized MI oocytes at 72 h post-ovulation, (d) two-pronuclei embryo at 110 h post-ovulation and (e and f) two-, four- and eight-cell embryos (112 h and 138 h post-ovulation respectively). Scale bars represent (a, b and d) 50 μm and (c, e and f) 100 μm.

Figure 2 (a) Semi-thin section of a canine oocyte collected 18 h after ovulation. (b) Lamin (green) and DNA (red) staining of the nucleus of an oocyte collected 42 h after ovulation. DNA (red) and α-tubulin (green) staining in canine oocytes/embryos followed by confocal microscopy. (c) The oocyte, 30 h after ovulation, presents a germinal vesicle, peripherally located, containing uncondensed DNA organized around small nucleoli. MI oocytes collected (d) 66 h and (e and f) 72 h after ovulation and (g) an MII oocyte collected 79 h after ovulation showing highly condensed, aligned chromosomes forming the metaphase plate (pb, polar body). Inserts (d, e, g); detail of the gamete DNA. Sperm penetration was observed in the cytoplasm of three immature oocytes: (e and f) slightly decondensed sperm head (sh) was visible at the vicinity of the MI plate. 90 h post-ovulation, embryonic stages were also observed. (h and i) Two-pronuclei stage 110 h after ovulation, (j) two-cell stage 127 h after ovulation and (k and l) four- and eight-cell stage collected 112 h after ovulation. Scale bars represent (f and i) 2.5 μm, (b) 10 μm and (a, c, d, e, g, h and j–l) 20 μm.
Oocyte maturation stage at sperm penetration

Using confocal microscopy, the presence of sperm heads in oocyte cytoplasm could be investigated accurately. Despite the fact that bitches were inseminated during the period ranging from the day before ovulation to surgery, we did not observe any sperm penetration in GV oocytes (n = 82; 21 bitches). Of 30 MI/II oocytes examined, only three were fertilized (one bitch out of 12) (Fig. 2f). Of a total of 112 immature oocytes (30 bitches), sperm penetration in immature oocytes (GV–TI stages) occurred only in one bitch, at the MI stage, at 72 h after ovulation.

Interestingly, despite the presence of spermatozoa in the oviduct, sperm penetration was not observed in 90% MII oocytes present from 54 to 83 h after ovulation (n = 29; nine bitches out of ten). Fertilization occurred generally from 90 h after ovulation.

Polypermy was not observed whatever the oocyte stage.

Discussion

Oocyte maturation and embryonic development in canids follow a number of specific characteristics with respect to timing, site and duration. However, data available in the literature are scarce and still unclear. Most studies, in both the fox and the dog, have been conducted on very few females (from six to 25) over a very large period of time (from 0 to 24 days after ovulation) (Doak et al. 1967, Tsutsui 1975, Tsutsui & Shimizu 1975, Renton et al. 1991, Bysted et al. 2001). Furthermore, the timing of maturation and embryonic development has been described with reference to several events more or less timely related to ovulation: onset of oestrus, acceptance of the male, LH peak, aspect of follicles at laparotomy or serum progesterone levels (see for example Tsutsui 1975, 1989, Archbald et al. 1980, Renton et al. 1991, Bysted et al. 2001). No study has been conducted with real visualization of ovulation, which appears to be the best reference for the description of oocyte maturation and embryo development. In our present work, oocytes/embryos of 50 bitches were collected during a restricted period of time covering 121 h soon after ovulation. Moreover, ovarian ultrasonography was used to assess the occurrence of ovulation precisely. This method has been proven to be accurate for the detection of ovulation (Boyd et al. 1993, Marseloo et al. 2004). Moreover, in contrast to laparotomy (Tsutsui 1975), ultrasonography is a non-invasive technique, respecting the welfare of the animal and inducing less stress which may disturb the LH peak and ovulation. Finally, our observations are physiologically more relevant as oocytes/embryos were collected during spontaneous natural oestrus without any hormonal manipulation such as induction of oestrus or superovulation.

Another bias in most studies focusing on canine oocyte/embryos is the high rate of undetermined nuclear stages which is due to the darkness of the oocyte cytoplasm, rich in lipid droplets. Moreover, the occurrence of sperm penetration is nearly impossible to assess with conventional methods. To overcome this difficulty, we set up and validated an appropriate staining method coupled with confocal laser scanning microscopy. Observation with confocal microscopy made it possible to reduce the rate of oocytes/embryos with non-determined nuclear stage to a minimum (3% versus about 30–40% with optic microscopy; Saint-Dizier et al. 2004).

In our study, the mean number of corpora lutea per bitch was slightly higher than that observed classically (Doak et al. 1967, Tsutsui & Shimizu 1975, Shimizu et al. 1990, Renton et al. 1991, Bysted et al. 2001).

Oocytes/embryos were collected between 17 and 138 h (5.5 days) after ovulation and all of them were found in the oviduct: entry in the proximal part of the uterus has been described to occur between 168 and 240 h after ovulation (16–32-cell, morula or blastocyst stage) (Tsutsui 1975, Harper 1988, Renton et al. 1991). Our recovery rate (73%) was similar to that obtained by others, ranging from 40 to 99% (Tsutsui 1975, Tsutsui & Shimizu 1975, Renton et al. 1991, Bysted et al. 2001). In two cases, we collected respectively one and two structures more than the number of corpora lutea counted at macroscopic examination. Bysted et al. (2001) also reported this observation in one bitch out of nine. This can be explained either by ovulation of polyclavular follicles (Andersen & Simpson 1973, Telfer & Gosden 1987) or by one (or two) corpus luteum being not apparent at the surface of the ovary.

In the dog, oocyte maturation is well known to be delayed 2–3 days after ovulation (Holst & Phemister 1971, Tsutsui 1975) and embryonic development is much slower than in other species (for review see Betteridge 1995). However, the exact timing of meiosis during the first 48 h is not well described. In our study, 56 oocytes from 13 bitches were analyzed between 17 and 48 h after ovulation. The oocytes were found to be at the GV stage up to 44 h, reached MI between 44 and 48 h and the first MII appeared at 54 h. This is in agreement with results obtained by Tsutsui (1975) reporting that MII oocytes can be observed between 48 h and 72 h (also, in this study, the time of ovulation was determined with a precision of only 24 h). No GVBD was observed in our study, or in those of Farstad et al. (1993) and Tsutsui (1975). This is probably because of the short duration of this phase in vivo (1 h in most species; Sirard et al. 1989, Taieb et al. 1997) and, in the case of Tsutsui (1975), the examination technique. On the contrary, the presence of other maturation stages (GV, MI and MII) is spread over 70 to 90 h in our study (Fig. 3). However, it is impossible to discriminate between normal developing oocytes and blocked degenerating oocytes.

Interestingly, the GV aspect that we observed in these in vivo collected canine oocytes was very different; first, from the one observed in other mammalian species and, secondly, from the structure observed in in vitro cultured canine oocytes (Hewitt et al. 1998, Saint-Dizier et al.
Lamin and semi-thin sections showed the persistence of the nuclear membrane and nucleoli were still present, but surrounded by the DNA. Some GV of the *in vivo* collected oocytes drawn by Van der Stricht (1923) also showed this particular aspect. This kind of structure seems similar to the surrounded nucleolus observed in mouse and human oocytes, this configuration being thought to represent a transitional stage of GV towards ovulation (Bouniol-Baly et al. 1999, Miyara et al. 2003).

All the collected oocytes presented two or three layers of dense, compact granulosa cells, without any visible mucification as described by Andersen & Simpson (1973) who described one oocyte in an histological section of an oviduct. No mucified masses were observed in the flushing liquid. However, in preovulatory follicles, after the LH peak, some mucification occurs in peripheral granulosa layers surrounding the three internal dense layers (authors’ unpublished data). Since granulosa cells have been shown to be responsible for the inhibition of meiosis resumption (Whitaker 1996), the persistence of close relations between the oocyte and the corona radiata after the LH peak may contribute to the delay between ovulation and meiotic resumption in the bitch.

Our protocol ensured a constant presence of spermatozoa in the oviduct starting before ovulation and until oocyte/embryo collection. Fertilization was thus possible precociously as this event is physiologically possible *in vivo* in the bitch. However, penetration of spermatozoa in immature oocytes was only exceptionally observed in our study. In the fox, Farstad et al. (1993) reported the opposite for *in vivo* sperm penetration in 46% of immature oocytes. *In vitro*, fertilization of immature oocytes in the bitch is a frequent event (11–46% minimum; Yamada et al. 1992, Nickson et al. 1993, Saint-Dizier et al. 2001), but finally probably artefactual. Sperm penetration of immature oocytes can indeed be achieved *in vitro* in species in which ovulation occurs *in vivo* at the MII stage (mouse: Iwamatsu & Chang 1975, cow: Chian et al. 1992, human: Van Blerkom et al. 1994).

Our study has clearly demonstrated that fertilization in the bitch occurs *in vivo* at the MII stage. However, a striking feature was the observation of a delay in sperm penetration in mature oocytes. Despite the presence of both MII oocytes and spermatozoa for several hours, fertilization was delayed at least up to 83 h after ovulation, suggesting the need for a minimum period in the oviduct before fertilization. The determination of the exact time of fertilization is of clinical interest for reproductive biotechnologies and especially for artificial insemination with frozen semen, because of its short lifespan. Two-pronuclei embryos were first observed in our study 92 h after ovulation. At that time, oocytes are in the distal part of the oviduct (Tsutsui 1975), in contrast to other mammals, in which fertilization generally occurs in the proximal part (Harper 1988). Formation of ampulla was never observed.

![Figure 3 Timing of oocyte meiotic maturation and embryo development in reference to ovulation. Bitches were ovariectomized from 17 to 138 h post-ovulation. Collected oocytes/embryos were stained for observation under confocal microscopy to determine their nuclear stage (GV oocyte to eight-cell embryo). PN, two-pronuclei and + spz, presence of sperm head in oocyte cytoplasm. Heterogeneity of the developmental stages is more visible on bitches (B) 11 and 30 (vertical lines). Beagle bitches (shaded circles) and other breeds and mongrels (open circles).](http://www.reproduction-online.org/articles/130/193-201/f03.jpg)
In vivo fertilization appears to be a very efficient phenomenon, with sperm penetration in 81% of fertilizable oocytes. Polyspermy, observed at a high frequency after in vitro oocyte maturation (6–59%); Yamada et al. 1992, Otoi et al. 2000, Saint-Dizier et al. 2001, did not seem to occur in vivo. Suboptimal oocyte culture conditions before fertilization and excessive sperm concentration in the fertilization medium may be responsible for in vitro polyspermy.

Very few precise data on embryonic development timing are available in the bitch, because most studies refer to an imprecise ‘starting point’ such as the onset of the heat period or the acceptance of the male. The other limiting factors are the restricted number of bitches analysed at each time and the large interval (usually 24 h) between two observations. With reference to ovulation detected by ultrasonography, we observed two-pronuclei stages between 92 and 124 h, in accordance with Bysted et al. 1993 Problems associated with ultrasonography of the canine ovary around the time of ovulation. Journal of Reproduction and Fertility 47 (Suppl) 101–105.

In our study, two-cell embryos were present between 114 and 138 h, and three- to four-cell embryos from 112 h onwards, which agrees with Tsutsui (1975) and Bysted et al. 2001 Embryonic developmental stages in relation to the LH peak in dogs. Journal of Reproduction and Fertility 57 (Suppl) 181–186.


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