Sperm distribution in the genital tract of the bitch following artificial insemination in relation to the time of ovulation

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Abstract

In the present study, sperm distribution in the genital tract of the bitch following artificial insemination (AI) in relation to the time of ovulation was investigated by histology, scanning electron microscopy (SEM) and flushing. Ten bitches were inseminated intravaginally with 500×10^6 spermatozoa: three dogs before ovulation, four dogs during ovulation and three dogs after ovulation. Ovariohysterectomy was performed 24 h after AI. Half of the genital tract was divided into nine segments (cervix, corpus uteri, caudal, middle and cranial uterine horn (UTH), utero-tubal junction (UTJ), isthmus, ampulla and infundibulum), which were processed for histology and SEM. The contralateral UTH and uterine tube (UT) were flushed, and several sperm characteristics were assessed. Histology revealed that the spermatozoa were mainly located in the uterine glands and at the UTJ, while very few spermatozoa were detected in the UT. Insemination during ovulation resulted in higher percentages of glands with spermatozoa in the different parts of the uterus ($P < 0.05$). Evaluation by SEM showed higher numbers of spermatozoa in several parts of the uterus for bitches inseminated during ovulation ($P < 0.05$). The mean number of spermatozoa flushed from the UTH and the UT was low. No significant differences in the evaluated sperm quality parameters were found between the flushings of the UTH and the UT. In conclusion, based on our findings, the uterine glands and the UTJ might act as sperm reservoirs in the bitch and sperm transport in the genital tract is affected by the time of AI in relation to ovulation.

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Introduction

The transport and survival of spermatozoa within the female reproductive tract have been studied extensively, mainly in human, cattle and pigs (Drobnis & Overstreet 1992, Hunter & Nichol 1993, Hunter 1995, Mburu *et al.* 1996, Suarez *et al.* 1997). Although the mechanisms involved are complex, sperm transport in the reproductive tract of mammals appears to be a dynamic and highly regulated process, resulting in spermatozoa reaching the site of fertilization simultaneously with the appearance of fertile oocytes (England & Pacey 1998).

In dogs, studies on the distribution and survival of spermatozoa in the female reproductive tract are limited. During natural mating, canine spermatozoa are deposited in the cranial vagina (England & Pacey 1998). Subsequently the spermatozoa are distributed rapidly in the genital tract mainly by vaginal and uterine contractions (England & Burgess 2003). The fertile lifespan of spermatozoa in the reproductive tract of the bitch is considerably longer than in other domestic species since natural

matings as early as 9 days before ovulation may still result in pregnancy and litters (England *et al.* 1989, England & Pacey 1998). In order to remain functionally competent until the time of fertilization, storage of spermatozoa in a sperm reservoir is required (England & Pacey 1998). Canine spermatozoa were reported to be stored at several locations within the reproductive tract of the bitch. In a study by Doak *et al.* (1967), spermatozoa were mainly clustered in the uterine glands, most frequently in the neck of these glands. More recently, other studies associated the sperm reservoir with the utero-tubal junction (UTJ) (England & Burgess 2003). Several authors suggested that sperm survival at these sites was prolonged by the intimate association between the spermatozoa and the epithelium of the uterine tube (UT) or uterine gland (Doak *et al.* 1967, England & Pacey 1998), which was confirmed in several *in vitro* studies using explants from the UT (Ellington *et al.* 1995, Pacey *et al.* 2000, Kawakami *et al.* 2001, Petrunkina *et al.* 2004). However, in most of the *in vivo* studies in dogs, sperm distribution and survival

were investigated following natural mating (Doak *et al.* 1967, England & Pacey 1998, England & Burgess 2003) with little or no information on the number and quality of spermatozoa actually entering the female genital tract. Consequently, part of the variation in the number of recovered spermatozoa in the previous studies may be attributed to variations in mating interactions or even to the use of different male dogs (Doak *et al.* 1967). Furthermore, as far as we know, little information is available on the sperm distribution in the genital tract of the bitch following artificial insemination (AI). Nevertheless, sperm distribution following AI may differ from natural mating as, for example in the golden hamster, it has been shown that the number of spermatozoa entering the UT after AI was considerably lower than in naturally mated animals (Smith *et al.* 1987), which might have been due to the use of lower insemination doses compared to the normal sperm number deposited in the reproductive tract during natural mating.

In several mammalian species, the ovulation event has been demonstrated to influence sperm transport and distribution in the female genital tract, probably due to changes in hormone concentrations which occur around the ovulation period (Hunter 1988, Mburu *et al.* 1996, Kaeoket *et al.* 2002). In pigs, the number and membrane integrity of spermatozoa in the UTJ and isthmus were influenced by ovulation, in that higher numbers of spermatozoa were recovered from the upper isthmus during the peri-ovulatory period than the post-ovulatory period (Mburu *et al.* 1996). Moreover, the transport of spermatozoa towards the UTJ and UT was impaired if sows were inseminated 15–20 h after ovulation (Kaeoket *et al.* 2002). In the hamster, prevention of ovulation yielded fewer spermatozoa in the caudal isthmus and ampulla, whereas super-ovulation resulted in significantly higher numbers of spermatozoa at these sites (Ito *et al.* 1991). In dogs, however, little information is available on the effect of ovulation on sperm transport. Moreover, the dog has an unusual reproductive cycle compared to other domestic animals. Concannon *et al.* (1977) previously showed that ovulation in the dog occurs approximately 38–44 h after the luteinizing hormone (LH) peak and Wildt *et al.* (1978) found that 77.2% of the follicles had ovulated 24–72 h after the LH peak. Just prior to or concomitant with the LH surge, the progesterone secretion starts (i.e. the pre-ovulatory rise), reaching concentrations between 1 and 3 ng/ml (Concannon *et al.* 1977). Canine oocytes are ovulated as primary oocytes, which are not capable of being fertilized (Tsutsui 1989). They first have to undergo the first meiotic division to become secondary oocytes, a process which is completed about 48–72 h after ovulation (Concannon *et al.* 1989, Tsutsui 1989). Fertilization of the secondary oocytes subsequently takes place approximately 60–108 h after ovulation, i.e. 4–7 days after the LH surge (Tsutsui 1989, England & Pacey 1998).

The aim of the present study was to investigate sperm distribution in the genital tract of the bitch following AI in

relation to the time of ovulation. Three techniques (i.e. histology, scanning electron microscopy (SEM) and flushing) were used to quantify the number of spermatozoa in the different segments of the female genital tract. Additionally, several sperm characteristics (i.e. motility, membrane integrity and acrosomal status) of the flushed spermatozoa were determined.

Materials and Methods

All experiments have been approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University, Belgium.

Animals

Ten clinically healthy, female dogs were used for the experiments: nine beagle dogs and one fox hound. The dogs ranged in age between 2.5 and 7.5 years and varied in body weight from 12.5 to 21.0 kg. Three clinically healthy male dogs with normal sperm characteristics were used for semen collection: two sexually mature Anglo-Normands (5 and 7 years old) and one crossbred (9 years). All dogs were obtained from the kennel of the Small Animal Department of Ghent University, Belgium. They were housed with outdoor access and fed twice a day with commercial dog food. Water was freely available.

Determination of the oestrous cycle

All female dogs were examined at least three times per week for the presence of vulvar swelling and serosanguinous vaginal discharge, which were considered to signify the onset of pro-oestrus. When the bitches were in pro-oestrus, the serum progesterone concentration and the cornification of the vaginal cells were determined at least once every 2 days until 1 day after surgery. Blood samples (2 ml) were collected by cephalic venipuncture. The samples were centrifuged for 10 min at 1620 *g* to collect the plasma. The progesterone concentrations were determined by a previously validated RIA (Henry *et al.* 1987). The detection limit for progesterone was 0.05 ng. The inter- and intra-assay variations for progesterone were 7.05 and 8.75% respectively (Henry *et al.* 1987). All hormonal analyses were performed at the Department of Reproduction, Obstetrics and Herd Health of the Faculty of Veterinary Medicine (Ghent University, Belgium).

Semen collection and evaluation

Semen was collected by digital manipulation as described by Linde-Forsberg (1991), if necessary in the presence of a teaser bitch. The second, sperm-rich fraction of the ejaculate was collected into a plastic vial. Immediately after collection, the ejaculates of the three dogs were pooled and the sperm quality was evaluated. The total (velocity average pathway (VAP) > 30 $\mu\text{m/s}$) and the progressive motility (VAP > 50 $\mu\text{m/s}$ and straightness > 70%) was assessed by the Hamilton–Thorne Ceros 12.1 semen

analyser (Rijsselaere *et al.* 2003). Sperm concentration was determined using a Bürker counting chamber (Merck, Leuven, Belgium). Spermatozoal morphology was examined on nigrosin/eosin stained smears. Membrane integrity and acrosomal status of the spermatozoa were evaluated by a fluorescent SYBR14–propidium iodide (PI) (Catalogue No. L-7011; Molecular Probes, Leiden, The Netherlands) and *Pisum sativum* agglutinin (PSA) (Catalogue No. L-0770; Sigma-Aldrich, Bornem, Belgium) staining technique respectively. At least 100 spermatozoa were evaluated using a Leica DMR fluorescence microscope. The procedures of the fluorescent stainings have been described in detail by Rijsselaere *et al.* (2002).

AI

Each bitch was inseminated by AI once deep intravaginally with 500×10^6 spermatozoa using a plastic insemination catheter (Catalogue No. 273404; Kruuse, Manslev, Denmark). Three dogs were inseminated at a progesterone concentration of 1–2 ng/ml (Group 1: pre-ovulatory). Four females were inseminated at a progesterone concentration of 4–8 ng/ml (Group 2: estimated ovulation period) (Johnston *et al.* 2001) and three dogs were inseminated 2–3 days after the presumed ovulation, i.e. at a progesterone concentration of 11–12.5 ng/ml (Group 3: post-ovulatory). After AI, the hindquarters of the dogs were elevated for 10 min while the perineal region was gently stroked to enhance sperm transport (Linde-Forsberg & Forsberg 1989).

Ovariohysterectomy (OVH)

OVH was performed 24 h after AI. The bitches were pre-medicated using 0.1 mg/kg acepromazine (ACP; Eurovet, Bladel, The Netherlands)/0.1 mg/kg methadone (Mephemon; Denolin, Brussels, Belgium) i.v., after which anaesthesia was induced with 10 mg/kg i.v. thiopental (Pentothal; Abbott, Ottignies, Belgium) and maintained using isoflurane (Abbott Laboratories Ltd, Queensborough, Kent, UK) in an O₂/N₂O carrier mix. A routine OVH was performed to remove the ovaries and the reproductive tract with careful manipulation of the tracts. While holding the uterine horn (UTH), special care was taken not to damage the UT. The abdominal incision was closed in a routine manner.

Immediately after OVH, the genital tract was ligated twice at the cervix and the infundibulum, cleaned of surrounding tissues and immersed in physiological saline solution at 37°C. One half of the genital tract was divided into nine segments (i.e. infundibulum, ampulla, isthmus, UTJ, cranial, middle and caudal parts of the UTH, uterine body and cervix), which were used for histological (Experiment 1) and SEM (Experiment 2) examination. The infundibulum was separated at the end of the conical shaped tubal segment and the ampulla was differentiated from the isthmus by its larger diameter. The UTJ consisted of 0.3 cm of the cranial part of the UTH and 0.3 cm of the

caudal isthmus. The contralateral UTH and UT were used for flushing (Experiment 3).

Experiment 1: histology

Directly after excision, two sequential samples of approximately 5 mm thickness were taken from the infundibulum, isthmus, cervix and the different parts of the uterus, whereas only one sample of this size was taken from the UTJ and the ampulla. The samples were fixed for 24 h in a phosphate-buffered 3.5% formaldehyde solution (pH 6.7). After fixation, all samples were embedded in paraffin in an automated system (Shandon Citadel Tissue Processor; Shandon, Cheshire, UK). Subsequently, 8 µm paraffin sections were cut, mounted on uncoated slides and dried overnight at 37°C. The sections were deparaffinized in xylene, rehydrated in descending grades of alcohol, stained with haematoxylin (8 min) and eosin (3 min). For each of the nine localizations, 30 slides were examined using light microscopy ($\times 400$), i.e. 15 from the first paraffin block and 15 from the consecutive block for the infundibulum, isthmus, cervix and the different parts of the uterus, and 15 slides at a superficial level and 15 slides at a deeper level (2 mm) of the same paraffin block for the UTJ and the ampulla.

For the cervix, UTJ, isthmus, ampulla and infundibulum, the total number of spermatozoa counted in 30 histological sections was determined. Since spermatozoa could often not be counted individually in the uterine glands due to clustering (Doak *et al.* 1967), the percentage of uterine glands containing spermatozoa was determined for the different parts of the uterus. Therefore, for each of the 30 sections, 100 uterine glands were evaluated for the presence of spermatozoa and were divided into glands with no spermatozoa, glands with one sperm cell, glands with two to five spermatozoa, and glands with either more than five spermatozoa or in which the spermatozoa were clustered. Additionally, in these uterine segments, the number of intraluminal spermatozoa were determined in 30 histological sections.

Experiment 2: SEM

Immediately after OVH, a sample of each of the segments (except the UTJ which was used for histology in Experiment 1) from one side of the genital tract was fixed in a Hepes-buffered 2% paraformaldehyde–2.5% glutaraldehyde solution (pH 7.2; 1100 mosmol) for 24 h. Subsequently, the samples were postfixated in an unbuffered 1% osmium tetroxide solution for 2 h followed by dehydration in ascending grades of alcohol. Subsequently, they were critically point-dried with CO₂ mounted on a metal stub, platinum coated and examined by a Jeol JSM 5600 LV scanning electron microscope (Jeol Ltd, Tokyo, Japan). For each of the eight localizations, the total number of spermatozoa was determined in 20 randomly placed rectangular areas of 10 000 µm² at a magnification of $\times 900$.

Experiment 3: sperm recovery by flushing

To recover the intraluminal spermatozoa, the contralateral UTH and UT, i.e. isthmus, ampulla and infundibulum, including the UTJ), were flushed with 5 × 3 ml and 5 × 1 ml PBS at 37 °C respectively. To flush the UT, PBS was injected into the ovarian end of the UT with a 26 G needle connected to a syringe and collected on the uterine end of the UT. A clamp was used to prevent back flushing or leakage of collection fluid. The flushings of the UT and the UTH were collected into plastic vials and centrifuged at 720 *g* for 5 min (Rijsselaere *et al.* 2002). The supernatant was removed and the spermatozoa were resuspended in the remaining 1 ml fluid. The total number of spermatozoa in the flushings was calculated by placing 10 µl of the resuspended sample in a Neubauer counting chamber. The motility parameters were assessed subjectively. At least 100 spermatozoa were evaluated individually and classified into one of the following categories: progressively motile, statically motile with vigorous flagellar activity, or immotile. In order to concentrate the spermatozoa in a small pellet, the resuspended semen sample was centrifuged again (720 *g*, 5 min) and, the membrane and acrosomal status of 100 spermatozoa were determined using the fluorescent SYBR14–PI and PSA staining techniques respectively.

Statistical analysis

Throughout the study, results were presented as means and variation was expressed as ranges or s.d. In Experiment 1, the data of all dogs were analysed using ANOVA with reproductive segment and series as fixed factors. Differences between the three groups for each segment were analysed using a general linear model or a Kruskal–Wallis test. In Experiment 2, the data of all dogs were analysed using a general linear model with reproductive segment as fixed factor. Differences between the three groups for each segment were analysed using a general linear model. Possible differences in the evaluated sperm quality parameters between the UTH and UT in Experiment 3 were analysed using a paired *t*-test. Statistical analyses were performed with procedures available in SPSS 11.0 (SPSS Inc. Headquarters, Chicago, IL,

USA). Values were considered to be statistically significant at *P* < 0.05.

Results

Semen evaluation of the pooled ejaculates immediately before AI revealed a mean (±s.d.) sperm concentration of 346.4 ± 107.3 × 10⁶/ml. The percentage of motile and progressively motile spermatozoa was 79.4 ± 10.7 and 63.5 ± 10.0% respectively. The percentage of spermatozoa with a normal morphology and with an intact membrane was 83.7 ± 8.4 and 83.1 ± 3.2% respectively. The percentage of spermatozoa with an intact acrosome was consistently higher than 90%. The mean sperm volume used for AI was 1.61 ± 0.61 ml.

At the time of AI, the mean (±s.d.) progesterone concentrations for the pre-ovulatory, ovulatory and post-ovulatory groups were 1.2 ± 0.1, 6.5 ± 1.0 and 12.0 ± 0.5 ng/ml respectively. The mean (±s.d.) progesterone concentrations at the time of OVH were 1.5 ± 0.2, 9.0 ± 1.0 and 13.7 ± 0.8 ng/ml respectively. No significant differences in body weight, age or inseminated volume of sperm were found among the three groups of female dogs.

Experiment 1: histology

Both within and between groups, large variations in the number of detected spermatozoa were observed. No significant differences were found between the first and the second series of 15 histological sections for each of the nine segments of the reproductive tract. The spermatozoa were mainly located in the corpus uteri, in the different parts of the UTH and at the UTJ, whereas relatively few spermatozoa were found in the cervix, and few if any spermatozoa were found in the different segments of the UT (Tables 1 and 2). In the corpus uteri and the UTH, the majority of the spermatozoa were located in the endometrial glands whereas few spermatozoa (fewer than five per histological section) were found in the lumina (Fig. 1). Irrespective of the time of insemination, there were no significant differences in the mean percentage of glands with spermatozoa among the four different parts of the uterus (Table 2). Insemination during the ovulation period

Table 1 Histological evaluation (×400) of the number of spermatozoa (mean and range) counted in 30 histological sections of the cervix, UTJ and the different segments of the uterine tube of bitches (*n* = 10) 24 h after AI in relation to the time of ovulation.

Location	Total group (<i>n</i> = 10)	Time of insemination		
		Pre-ovulatory (<i>n</i> = 3)	Ovulatory (<i>n</i> = 4)	Post-ovulatory (<i>n</i> = 3)
Cervix	51.8 (0–288) ^A	9.5 (0–19)	122.0 (0–288)	9.7 (0–24)
UTJ	707.6 (3–2922) ^B	567.3 (4–1672) ^{a,b}	1334.8 (119–2922) ^a	10.9 (3–24) ^b
Isthmus	0.4 (0–3) ^C	0.3 (0–1)	1 (0–3)	0 (0–0)
Ampulla	0.2 (0–1) ^C	1 (1–1)	0 (0–0)	0 (0–0)
Infundibulum	0 (0–0) ^C	0.7 (0–1)	0 (0–0)	0 (0–0)

^{A–C} Values with different superscripts in the first column are statistically different (*P* < 0.05).
^{a,b} Values with different superscripts indicate statistical differences between the three groups for a given reproductive segment (*P* < 0.05).

Table 2 Histological evaluation ($\times 400$) of the percentage of endometrial glands containing spermatozoa (mean and range) in the different sections of the uterus of bitches ($n = 10$) 24 h after AI in relation to the time of ovulation.

Location	Total group ($n = 10$)	Time of insemination		
		Pre-ovulatory ($n = 3$)	Ovulatory ($n = 4$)	Post-ovulatory ($n = 3$)
Corpus uteri	17.9 (1.5–37.0)	12.4 (7.4–15.2) ^a	26.5 (18.7–37.0) ^b	12.0 (1.5–19.2) ^a
Caudal UTH	18.2 (0.1–18.5)	14.0 (8.9–18.5) ^a	27.1 (24.4–28.6) ^b	10.5 (0.1–25.0) ^a
Middle UTH	15.3 (0.1–27.5)	10.0 (5.3–14.3) ^a	24.8 (19.4–27.5) ^b	8.0 (0.1–22.6) ^a
Cranial UTH	15.2 (0.0–38.8)	10.2 (0.1–16.6) ^a	27.9 (21.5–38.8) ^b	3.2 (0–8.7) ^a

^{a,b}Values with different superscripts indicate statistical differences between the three groups for a given uterine segment ($P < 0.05$).

resulted in higher ($P < 0.05$) percentages of endometrial glands with spermatozoa in the different parts of the uterus compared to AI carried out before or after ovulation (Table 2). Moreover, for the ovulatory group, 54.7% of the uterine glands with spermatozoa contained more than five spermatozoa (or clusters) compared to 19.9 and 28.2% for the pre- and post-ovulatory group respectively ($P < 0.05$; Table 3). In the pre-ovulatory group, the uterine glands mostly contained one sperm cell, whereas in the post-ovulatory group, two to five spermatozoa were frequently found per gland (Table 3). Furthermore, higher numbers of spermatozoa were found at the UTJ of bitches that had been inseminated before or during the ovulation period (Table 1).

Experiment 2: SEM

As in Experiment 1, high variations in the number of spermatozoa were detected between individual dogs and between the three groups. Regardless of the time of AI,

the spermatozoa were mainly found in the corpus uteri and the different parts of the UTH, whereas few or no spermatozoa were observed in the cervix and the different parts of the UT (Table 4). The heads of the spermatozoa that were detected in the uterus were frequently located in the uterine glands, which made it difficult to visualize the entire sperm cell. Consequently, frequently only the tails of these spermatozoa were clearly visible on SEM (Fig. 2). Although there was a tendency towards higher sperm numbers in the different parts of the UTH compared to the corpus uteri, the differences were not significant. When the total number of spermatozoa in the four segments of the uterus were added, significantly higher numbers of spermatozoa were found in the ovulatory group (mean $\pm$ S.D., 586.5 ± 242.7) compared to the pre- and post-ovulatory group (118.6 ± 100.9 and 38.7 ± 38.6 respectively). Furthermore, significantly higher sperm numbers were found in the caudal and the cranial part of the UTH for the ovulatory group compared to the pre- and the post-ovulatory group ($P < 0.05$; Table 4).

Figure 1 Groups of clustered spermatozoa (white arrows) located in the endometrial glands of the cranial part of the canine UTH and spermatozoa in the uterine lumen (black arrow) 24 h after AI ($\times 400$).

Table 3 Relative percentage (mean and range) of uterine glands with one, two to five and greater than five spermatozoa in bitches ($n = 10$) 24 h after AI in relation to the time of ovulation.

	Time of insemination		
	Pre-ovulatory ($n = 3$)	Ovulatory ($n = 4$)	Post-ovulatory ($n = 3$)
Glands with 1 sperm cell	59.6 (20.4–100.0) ^a	26.2 (16.1–48.5) ^b	34.0 (0–54.5) ^a
Glands with 2–5 spermatozoa	20.5 (0.0–38.3) ^a	19.1 (10.3–30.3) ^a	37.9 (19.9–100.0) ^b
Glands with > 5 spermatozoa	19.9 (0.0–60.8) ^a	54.7 (21.7–71.9) ^b	28.2 (0.0–43.6) ^a

^{a,b}Values with different superscripts indicate statistical differences ($P < 0.05$) between the three groups.

Glands with 1 sperm cell = (% uterine glands with 1 sperm cell/% uterine glands containing spermatozoa) $\times$ 100; glands with 2–5 sperm cells = (% uterine glands with 2–5 spermatozoa/% uterine glands containing spermatozoa) $\times$ 100; glands with > 5 spermatozoa = (% uterine glands with more than 5 spermatozoa/% uterine glands containing spermatozoa) $\times$ 100.

Experiment 3: sperm recovery by flushing

Spermatozoa could be recovered from the flushings of the UTH and UT for seven out of ten and five out of ten dogs respectively, whereas for three out of ten and five out of ten dogs, no spermatozoa could be found after flushing of the UTH and UT respectively (Table 5). The mean ($\pm$ s.d.) number of flushed spermatozoa was low: $16.4 \pm 14.3 \times 10^4$ for the UTH vs $12.1 \pm 26.4 \times 10^4$ for the UT ($P > 0.05$) with large variations between individual dogs (Table 6). Higher numbers of spermatozoa were flushed from the UTH, when the AI was performed during ovulation ($P < 0.05$).

The membrane and acrosome integrity, and the motility characteristics of the spermatozoa flushed from the UTH and UT are summarized in Table 6. Although there was a tendency towards slightly lower sperm quality parameters for the spermatozoa flushed from the UT compared to the UTH, the differences were not significant. In the pre-ovulatory and ovulatory groups, relatively high percentages of membrane- and acrosome-intact spermatozoa were recovered from the flushings of the UTH and UT, whereas in the post-ovulatory group all the spermatozoa flushed from the UTH and UT were membrane- and acrosome-damaged. However, in both the pre- and the post-ovulatory groups, spermatozoa could only be flushed for one out of three dogs in the UT. Therefore, possible differences between the three groups could not be analysed statistically. The percentage of spermatozoa with a progressive

motility was very low ($\leq 5\%$) for all the dogs in the three groups, which may have been due to the lack of protein in the flushing medium. However, in the pre-ovulatory and ovulatory group, relatively high percentages of static spermatozoa with vigorous flagellar beating were recovered in both the UTH and the UT.

Discussion

From our study three conclusions can be made: (i) 24 h after AI, spermatozoa were mainly found in the endometrial glands of the uterus and at the UTJ, (ii) the time of AI in relation to ovulation influenced sperm transport in the female genital tract, and (iii) histology appeared to be the most accurate technique to study sperm distribution in the genital tract of the bitch.

Our findings in Experiment 1 revealed large numbers of spermatozoa in the uterine glands and at the UTJ after AI, indicating that these sites probably act as the major sperm reservoirs in the bitch. The UTJ and the lower part of the isthmus were reported to function as a sperm reservoir in several other species including cow (Hunter *et al.* 1991), sow (Fléchon & Hunter 1981, Mburu *et al.* 1997), mare (Scott 2000), rabbit (Overstreet & Cooper 1978), sheep (Hunter *et al.* 1980), hamster (Smith *et al.* 1987) and bat (Krishna & Dominic 1978). Although storage in the uterus is rather uncommon, survival of spermatozoa in the uterine glands has previously been described in bats (Racey *et al.* 1987). Our findings are in agreement with previous

Table 4 SEM evaluation ($\times 900$) of the total number (mean and range) of spermatozoa counted in 20 rectangular areas of $10\,000\ \mu\text{m}^2$ in the different segments of the genital tract of bitches ($n = 10$) 24 h after AI in relation to the time of ovulation.

Location	Total group ($n = 10$)	Time of insemination		
		Pre-ovulatory ($n = 3$)	Ovulatory ($n = 4$)	Post-ovulatory ($n = 3$)
Cervix	0.1 (0–1) ^A	0 (0–0)	0.3 (0–1)	0 (0–0)
Corpus uteri	35.0 (4–89) ^B	21.3 (10–39) ^{a,b}	60.5 (48–89) ^b	14.7 (4–35) ^a
Caudal UTH	73.2 (1–261) ^B	23.0 (9–30) ^a	157.3 (102–261) ^b	11.3 (1–21) ^a
Middle UTH	81.2 (3–306) ^B	43.3 (6–97) ^{a,b}	165.0 (67–306) ^b	7.3 (3–14) ^a
Cranial UTH	92.4 (0–370) ^B	31.0 (1–63) ^a	203.8 (98–370) ^b	5.3 (0–12) ^a
Isthmus	0.1 (0–1) ^A	0 (0–0)	0 (0–0)	0.3 (0–1)
Ampulla	0 (0–0) ^A	0 (0–0)	0 (0–0)	0 (0–0)
Infundibulum	0 (0–0) ^A	0 (0–0)	0 (0–0)	0 (0–0)

^{A,B}Values with different superscripts in the first column are statistically different ($P < 0.05$).

^{a,b}Values with different superscripts indicate statistical differences ($P < 0.05$) between the three groups for a given reproductive segment.

Figure 2 SEM image ($\times 900$) of spermatozoa located in the cranial part of the canine UTH 24 h after AI. The white arrows show spermatozoa with the sperm head in a uterine gland as a result of which only the sperm tail is visible.

in vivo studies in dogs following natural mating (Doak *et al.* 1967, England & Burgess 2003). However, the percentage of uterine glands with spermatozoa 24 h after mating was markedly higher (i.e. 48.3–54.5%) in the study by Doak *et al.* (1967), which may be due to the fact that the bitches were naturally mated. In the golden hamster, higher numbers of spermatozoa were also found in the UT following natural mating compared to AI (Smith *et al.* 1987), possibly because the insemination doses were lower than the normal sperm number deposited in the reproductive tract during natural mating. However, the lower percentage of uterine glands with spermatozoa found in our study was probably not due to a reduced number of spermatozoa deposited in the vagina, since all the bitches were inseminated with 500×10^6

spermatozoa, which approximates the total sperm number introduced in the vagina of a beagle dog during natural mating. Our findings might therefore indirectly indicate that the uterine and vaginal contractions generated during coitus in the dog (England & Pacey 1998) probably propel the spermatozoa more actively than after AI. In the present study, the spermatozoa were mainly arranged in groups or clustered in the uterine glands and appeared to be bound with their heads to the epithelium of the uterus or the UTJ. Whether these sperm–epithelial interactions are based on carbohydrate recognition, as has been shown in the hamster (sialic acid; DeMott *et al.* 1995), the mare (galactose; Dobrinski *et al.* 1996), the cow (fucose; Lefebvre & Suarez 1997) and the pig (maltose, lactose and mannose; Green *et al.* 2001), needs to be determined in the dog.

Whereas high numbers of spermatozoa were found in the uterine glands and at the UTJ, the number of spermatozoa detected in the UT by histology and SEM was very low. In our opinion, the uterine glands might therefore not only act as a sperm reservoir in the dog, but may also play an important role as an initial selection mechanism for the spermatozoa that will reach the fertilization site. Moreover, based on our findings, the UTJ also appears to form an important barrier to spermatozoal ascent in dogs (England & Pacey 1998), which strongly limits the number of spermatozoa entering the UT (Fléchon & Hunter 1981). Several factors may cause spermatozoa to be retained at the UTJ, such as the narrow lumen (Hunter *et al.* 1991, Suarez 2002), the presence of a thick viscous secretion in this region (Hunter *et al.* 1991, Mburu *et al.* 1996) and the binding of spermatozoa to species-specific receptors

Table 5 Detection of spermatozoa in the UH and UT, including the UTJ, in ten dogs 24 h after AI by means of three different techniques (histology, SEM and flushing).

Location/ Technique	Total (n = 10)	Time of insemination		
		Pre-ovulatory (n = 3)	Ovulatory (n = 4)	Post-ovulatory (n = 3)
UH				
Histology	10/10	3/3	4/4	3/3
SEM	10/10	3/3	4/4	3/3
Flushing	7/10	2/3	4/4	1/3
UT + UTJ				
Histology	10/10	3/3	4/4	3/3
SEM*	1/10	0/3	0/4	1/3
Flushing	5/10	1/3	3/4	1/3

* The UTJ was used for histology; consequently only the different parts of the uterine tube were examined by means of SEM.

Table 6 Total number of flushed spermatozoa (total number), percentage of membrane-intact spermatozoa, percentage of acrosome-intact spermatozoa, spermatozoa with flagellar activity (flagellar-beating), static spermatozoa and progressively motile spermatozoa (progressive) (mean and ranges) flushed from the UTH and UT of bitches ($n = 10$) 24 h after AI in relation to the time of insemination.

Sperm parameter	Location	Total group ($n = 10$)	Time of insemination		
			Pre-ovulatory ($n = 3^*$)	Ovulatory ($n = 4$)	Post-ovulatory ($n = 3^{**}$)
Total number ($\times 10^4$)	UTH	16.4 (0–38.3)	14.8 (0–29.0) ^{a,b}	27.8 (15.3–38.3) ^a	2.6 (0–7.8) ^b
	UT	12.1 (0–83)	0.8 (0–2.3)	29.3 (0–83.0)	0.4 (0–1.3)
Membrane-intact (%)	UTH	52.2 (0–86)	56.5 (45–68)	72.5 (54.5–86.0)	0
	UT	49.3 (0–84.6)	28.0	72.9 (60.0–84.6)	0
Acrosome-intact (%)	UTH	76.7 (70–90)	80.0 (70.0–90.0)	80.8 (70.4–87.0)	0
	UT	75.5 (50–87)	87.0	71.7 (50.0–85.0)	0
Flagellar-beating (%)	UTH	35.3 (0–77)	40.0 (22.0–58.0)	45.7 (15.0–77.0)	0
	UT	26.0 (0–48)	48.0	27.3 (0–44.0)	0
Static (%)	UTH	62.4 (19–100)	57.5 (37.0–78.0)	51.3 (19.0–85.0)	100
	UT	72.6 (47–100)	47.0	72.0 (56.0–100)	100
Progressive (%)	UTH	2.3 (0–5)	2.5 (0–5.0)	3.0 (0–5.0)	0
	UT	1.4 (0–5)	5.0	0.7 (0–2.0)	0

* For the pre-ovulatory group, spermatozoa could only be flushed from the UT for one of three dogs.

** For the post-ovulatory group, spermatozoa could only be flushed from the UTH and UT for one of three dogs.

^{a,b} Values with different superscripts indicate statistical differences ($P < 0.05$) between the three groups for a given sperm parameter.

(England & Pacey 1998, Suarez 2002). Furthermore, the number of spermatozoa penetrating the UTJ is influenced by the total number of spermatozoa present at this site (Settlage *et al.* 1975) and by the type of sperm motility. In the hamster capacitated and hyperactivated spermatozoa were unable to pass through the UTJ (Shalgi *et al.* 1992). A low number of spermatozoa at the site of fertilization (i.e. the UT) is reported in several other studies (Doak *et al.* 1967, Hunter *et al.* 1991, England & Pacey 1998). Doak *et al.* (1967) found, using histology and flushing, considerably lower numbers of spermatozoa in the canine UT than in the uterus. Moreover, frequently no spermatozoa were found in the UT. While several *in vitro* studies showed that the intimate contact between canine spermatozoa and explants from the UT resulted in a prolonged viability and motility (Ellington *et al.* 1995, Pacey *et al.* 2000, Kawakami *et al.* 2001), in the present study, no evidence could be provided that an *in vivo* sperm reservoir is established in the canine isthmus or ampulla. Therefore, it might be interesting to evaluate whether canine sperm interaction with epithelial explants from the uterine body or UTH also leads to a prolonged flagellar activity and viability of spermatozoa. Despite the fact that almost no spermatozoa were detected in the UT by histology and SEM, a mean of $12.1 \pm 26.4 \times 10^4$ spermatozoa could be flushed from the UT in five out of ten dogs. Several explanations are possible for these conflicting findings. First, the UT which was flushed in Experiment 3 also included the UTJ. Consequently, a part of the flushed spermatozoa probably originated from the UTJ and not from the UT. Secondly, the relatively high mean number of spermatozoa was mainly due to one dog in which 83.0×10^4 spermatozoa were flushed from the UT. Indeed, when the median was determined, only 0.63×10^4 spermatozoa could be flushed from the UT. Finally, to prevent polyspermic fertilization, only limited numbers of spermatozoa are released at a time from the reservoirs (Suarez 2002). These

limited numbers of intraluminal spermatozoa in the UT might have been washed off or lost during the staining procedure for histology and SEM. Moreover, even if there were small numbers of intraluminal spermatozoa present in the UT, they appeared not to be bound firmly to the UT epithelium.

In pigs, it has been shown that the boar had a significant influence on the sperm population established at the UTJ and the lower isthmus (Mburu *et al.* 1996). In the present study, the variation caused by the male was minimized since all female dogs were inseminated with an equal number of pooled spermatozoa from the same dogs. Nevertheless, variations in the number of recovered spermatozoa were detected between females with all three evaluated techniques. Consequently, part of this variation may be attributed to differences in sperm-transporting ability between individual females, which has been described in several other species (Overstreet & Katz 1990) or may be due to the different time of insemination in relation to ovulation. Based on the progesterone concentrations, ovulation had not taken place at the time of OVH in the bitches which were inseminated before ovulation (Group 1). Regarding the long maturation period of canine oocytes after ovulation (Concannon *et al.* 1989, Tsutsui 1989), fertilization probably had not occurred at the time of OVH in the ovulatory group (Group 2). In the post-ovulatory group (Group 3), however, fertilization might have taken place. In the present study, there was a clear tendency towards higher percentages of uterine glands containing spermatozoa (Experiment 1) and higher numbers of spermatozoa recovered by SEM (Experiment 2) and flushing (Experiment 3) when the bitches were inseminated during ovulation compared to AI performed before or after ovulation. The influence of the ovulation event on sperm transport has been reported in several species (Ito *et al.* 1991, Mburu *et al.* 1996, Kaeoket *et al.* 2002) and may be due to a number of factors. First, the contractions

of the uterus and the UT, which are thought to propel sperm through the reproductive tract (England & Pacey 1998), may be affected by the ovulation event. Around the ovulation period, the hormone profiles change resulting in increasing progesterone and decreasing oestrogen concentrations (Kaeoket *et al.* 2002). In pigs, the peri-ovulatory contractions of the UT were suggested to result from the local delivery of ovarian steroids and prostaglandins (Hunter *et al.* 1983), whereas after ovulation, the rising plasma progesterone levels were associated with a decline in isthmus frequencies of pressure fluctuations and amplitudes (Mwanza *et al.* 2000). In the ewe, cow and sow, a counter current transfer of ovarian follicular hormones to the utero-tubal artery has been proposed to alter the tubal environment (Hunter 1995). Secondly, after natural mating or AI, spermatozoa are removed from the female genital tract by physical clearance due to myometrial contractions (Hawk & Conley 1971, Overstreet 1983) and phagocytosis, mainly by neutrophils which enter the uterus shortly after AI (Lovell & Getty 1968, Rozeboom *et al.* 1998). The level of sperm phagocytosis may be affected by ovulation, resulting in a more rapid clearance of spermatozoa after fertilization (Hunter *et al.* 1991). In pigs, the oestrous cycle stages and the progesterone levels were reported to be related to the uterine inflammatory response (De Winter *et al.* 1996) probably due to the infiltration of different white blood cells during different stages of the oestrous cycle (Kaeoket *et al.* 2002, 2003). Thirdly, although the initial transport of spermatozoa in the female genital tract is mainly due to vaginal and uterine contractions, the inherent motility of spermatozoa may also be of importance (England & Pacey 1998). In dogs, a cycle-stage-dependent effect of female plasma on sperm motility has been shown *in vitro*, finding higher percentages of hypermotile spermatozoa (VAP > 180 $\mu\text{m/s}$) when plasma was added from a bitch in oestrus compared to plasma from bitches in pro-oestrus, during the LH peak or during metoestrus (Iguer-ouada 2000). Similarly, in the human, sperm motility and velocity were enhanced by follicular fluid released at ovulation, probably due to chemotactic and chemokinetic activities on the spermatozoa (Falcone *et al.* 1991, Ralt *et al.* 1994). These higher sperm velocities might partly explain the higher number of spermatozoa which were able to reach the sperm reservoirs during the ovulation period in the present study. Fourthly, in rabbits (Harper 1973) and hamsters (Ito *et al.* 1991) there is evidence for the stimulatory role of ovulatory products such as oocyte-cumulus complexes on sperm transport, resulting in a facilitated sperm ascent (Van Soom *et al.* 2002). Shortly after ovulation, cumulus cells secrete progesterone and prostaglandins, the latter being a potent stimulator of smooth muscle activity in the UT (Ito *et al.* 1991). Finally, it might be possible that the number of binding sites expressed on the epithelium of the UTJ and uterine glands in dogs is influenced by the ovulation event. In cattle, however, the hormonal state of uterine

tubal epithelium did not appear to affect the number of binding sites (Suarez 2002).

Although flushing is an easy and quick technique for sperm recovery, it appeared less suitable than histology for the determination of the number of spermatozoa present in the genital tract, since most of the spermatozoa resided in the uterine glands presumably by strong sperm-epithelium interactions. Additionally, in several dogs spermatozoa were detected in the uterus by histology but not by flushing, whereas the opposite was never the case (Table 5). Consequently, in our opinion, examination by histology is preferable, although this technique is considerably more time consuming. While flushing allows for the evaluation of several sperm characteristics (i.e. motility, membrane integrity and acrosomal status), this technique can lead to an underestimation of the actual number of spermatozoa present in the genital tract since probably only the intraluminal spermatozoa are recovered (Smith & Yanagimachi 1990, Mburu *et al.* 1996). Although we clearly showed that spermatozoa could be visualized and quantified in the uterus by SEM for all dogs, they were almost never found in the UT. Previously, several authors have reported difficulty in quantifying sperm numbers in the UT using SEM, since spermatozoa frequently reside deep in the folds of the UT or may be obscured by viscous intraluminal fluid (Mburu *et al.* 1996).

We conclude that both the uterine glands and the UTJ might act as the major sperm reservoirs in the bitch following AI. *In vivo*, no spermatozoal storage could be demonstrated in the isthmus or ampulla. The time of AI in relation to ovulation clearly influenced sperm transport as evaluated by three different techniques. Histology appeared to be the most accurate technique to study sperm distribution in the genital tract of the bitch.

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