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DISTRIBUTION OF SPERMATOZOA IN THE GENITAL TRACT OF ARTIFICIALLY INSEMINATED HEIFERS

By

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LARSSON, B. and K. LARSSON: *Distribution of spermatozoa in the genital tract of artificially inseminated heifers.* Acta vet. scand. 1985, *26*, 385—395. — Eight heifers were artificially inseminated in the uterine body with 160×10^6 spermatozoa frozen in French mini-straws. The heifers were slaughtered 2 (n = 4) or 12 (n = 4) h after insemination and spermatozoa were recovered by flushing defined segments of the reproductive tract. The efficiency of the method was checked in different ways. There was a slight underestimation of the number of recovered spermatozoa. This underestimation was randomly distributed among heifers and genital tract segments.

The total number of spermatozoa recovered was higher at 2 than at 12 h (14.6 vs 0.6 % of the total number inseminated). Most spermatozoa were found in the vagina both at 2 and 12 h after insemination and in greater number at 2 h. In uterus there was a slight decline in the number of spermatozoa recovered at 2 versus 12 h after insemination. The number of spermatozoa recovered from the oviducts were similar at 2 (89.6×10^3) and 12 h (71.5×10^3) after insemination. At 2 h spermatozoa were found in all parts of the oviduct with the majority located in the utero tubal junction, whereas at 12 h the most were recovered from isthmus.

More spermatozoa were recovered from the left than from the right side of the tract in 6 of the 8 heifers. Only in 1 heifer were the majority of spermatozoa found in the oviduct ipsilateral to the follicle bearing ovary.

sperm-transport; bovine; frozen semen.

Over the years several investigators have studied transport of spermatozoa in the female genital tract and the distribution of spermatozoa at different time intervals after semen deposition (c.f. *Einarsson* 1980, and *Hawk* 1983). These studies mostly concern natural mating or conditions similar to natural mating. *El-Banna & Hafez* (1970) inseminated heifers in estrus at the ex-

ternal os of the cervix with fresh semen and studied the distribution of spermatozoa 16 or 40 h later. They recovered more spermatozoa at 16 h than at 40 h (1.61 % vs 0.03 %) and the highest number of spermatozoa were found in the anterior vagina. In the oviducts the recovery was 4 times higher at 16 h (i.e. at the time of ovulation) than at 40 h after insemination. When comparing sperm recovery 1, 8 or 24 h after insemination with fresh semen at the external os of the cervix in heifers *Dobrowolski & Hafez* (1970) had the highest recovery rate (13.4 %) at 1 h after insemination and the greatest number of spermatozoa were always recovered from the vagina regardless of time after insemination. In the uterotubular junctions and the oviducts, the greatest number of spermatozoa were recovered 8 h after insemination when the heifers had ovulated, and approximately equal numbers were found at both sites.

Today artificial insemination with frozen semen is used in cattle around the world. This implies a reduction both in the number of spermatozoa and the volume of the inseminate compared to natural mating. The site of semen deposition is also different and it is believed that the site of deposition as well as the timing of artificial insemination are critical for fertilization. There is, however, a lack of information concerning the distribution of spermatozoa in the female genital tract under the conditions prevailing at artificial insemination with frozen semen.

The aim of this study was to investigate the distribution of spermatozoa in the female genital tract of heifers 2 and 12 h after artificial insemination of frozen semen.

MATERIALS AND METHODS

Animals

Eight virgin heifers (A-H), 7 of the Swedish Red and White breed and 1 of the Swedish Friesian breed, were used. The heifers were 15 to 23 months of age. Before being used in the experiment the animals were observed during at least 2 estrous cycles.

Insemination and semen

The heifers were checked for external signs of estrus 3 times daily and were considered to be in standing heat when allowing mounting-attempts from a bull. They were inseminated late in standing, unregulated heat.

Semen used in the experiment originated from 1 ejaculate from a bull with proven good fertility. After collection, the semen was diluted at +35 °C with a diluent containing 73 % Tris buffer-citric acid-fructose, 20 % egg yolk and 7 % glycerol (v/v). After cooling to +5 °C during 1½ h, the semen was placed in French mini-straws which were frozen in liquid nitrogen vapor for 7 min 1½ h later. Immediately before insemination the straws were thawed in +35 °C water for 12 s. Each straw (i.e. insemination dose) contained 160×10^6 spermatozoa and the progressive motility after thawing was 40 %. At insemination the semen was deposited in the posterior part of the uterine body.

Sperm recovery and sperm count

The heifers were slaughtered at 2 h (A-D) or 12 h (E-H) after insemination. The reproductive organs were recovered immediately after stunning and bleeding. After trimming off the connective tissue the organs were divided into different regions by clamping (Fig. 1). The utero tubular junction (UTJ) included 1.5 cm of the uterine horn and 1 cm of isthmus. The junction between isthmus and ampulla was estimated after careful palpation. Each region was flushed with a solution containing 97 % sodium chloride (0.9 % sol.), 2 % formaline (35 % sol.) and 1 % Liqui-Nox (v/v). This was a modification of the solution used by *El-Banna & Hafez* (1970). Each segment of the oviduct was flushed with 3 ml and the other segments with 10 ml. The infundibuli of the oviducts were placed in jars containing flushing solution and were washed. Mucus found in the cervix and vagina was aspirated in syringes. A known volume of 0.5 N NaOH was added to dissolve the mucus. During manipulation of the organ, urine leaked from the bladder and thus flushed the posterior part of the vagina. This urine was collected for sperm counting as was the mucus that drained off the vagina.

Concentrations of spermatozoa in the flushing solution, mucus and urine were determined in a haemocytometer (Bürker chamber, 0.1 mm depth). Two samples per segment were drawn from the flushing solution, mucus and urine. Two independent observers each examined 1 sample. Altogether 288 squares from the 2 samples were examined. If spermatozoa were only seen outside the squares, the result was recorded as zero but the presence of spermatozoa was noted. Intact spermatozoa and detached heads were counted but not loose sperm tails.

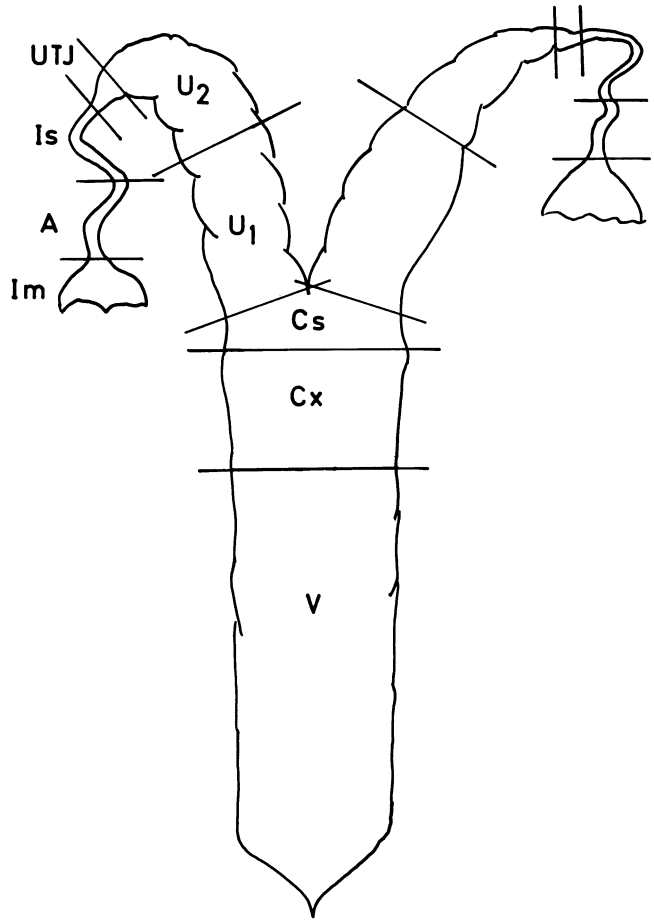

Figure 1. Schematic drawing of the reproductive organ of the cow showing how clamps were placed to divide the organ into different regions.

V = vagina, Cx = cervix, Cs = corpus uteri, U_1 = posterior half of uterus, U_2 = anterior half of uterus, UTJ = uterotubular junction, Is = isthmus, A = ampulla, Im = infundibulum.

Control of the efficiency of the flushings and sperm counts

Small pieces of tissue were taken from each oviduct (the ampullary and isthmic parts and the UTJ), each uterine horn and cervix. The specimens were fixed in Bouin's fixative (24 h), cut to a thickness of 4 μ, stained with hematoxylin and eosin and examined under a light microscope. The occurrence of spermatozoa in the sections was recorded.

If no spermatozoa were found in the Bürker chamber the flushing solution was centrifuged and a smear was prepared from the sediment. The smear was stained according to Williams (*Lagerlöf* 1934) and examined under a light microscope for presence of spermatozoa.

Analysis of data

Numbers of spermatozoa recovered from the different segments were transformed to $\log_{10}$. If no spermatozoa were found in a section the log of half of the lowest number of cells that would have been detected was used.

RESULTS

The efficiency of the method

The efficiency of the method for quantitation of spermatozoa was checked in 3 ways (Table 1). No spermatozoa were found in 60 of the 71 (85 %) sections that were taken from the reproductive organs. Eight of the sections where spermatozoa were found originated from uterus and cervix and 3 from UTJ where the recovery of spermatozoa in the flushing solution was good. No spermatozoa were seen in the sections from other parts of the oviducts indicating that the flushing had efficiently removed spermatozoa from the oviducts.

Table 1. Results of different methods of checking the accuracy of flushings and countings.

Heifer	Sections with spermatozoa	Samples in which no spermatozoa were found inside the squares of the Bürker chamber	
		Samples with spermatozoa only outside the squares of the Bürker chamber	Smears with spermatozoa
A	3/10	1/4	0/3
B	0/9	0/9	0/9
C	1/9	1/2	1/1
D	1/9	1/7	3/6
E	1/7	4/7	0/3
F	1/9	2/16	0/14
G	4/9	0/5	0/5
H	0/9	1/11	2/10
Total	11/71	10/61	6/51

Totally 120 flushings and 25 mucus specimens were examined. Spermatozoa were found inside the squares in the Bürker chamber in 84 of these (58 %). Of the remaining 61 samples, 39 were from heifers slaughtered 12 h after insemination. There was very good conformity between the results obtained by the 2 persons who made the countings. In 10 cases spermatozoa were seen only outside the squares and were thus not counted. Smears were prepared after centrifugation of the remaining 51 samples and spermatozoa were found in 6. Totally 7 flushings from heifers slaughtered 2 h and 9 from heifers slaughtered 12 h after insemination were erroneously counted as zero.

Results of the countings

Results of the spermatozoa countings in the genital tract flushings are summarized in Table 2 and Figure 2. The number of spermatozoa recovered from vagina includes spermatozoa in the flushing solution and mucus from the vagina plus spermatozoa found in the mucus and urine that drained from the vagina. In Table 2 the data concerning uterine and oviductal segments refer to both the left and the right sides.

Table 2. Number of spermatozoa ($\times 10^3$) recovered in flushing solution and mucus from different regions 2 and 12 h after insemination with 160×10^6 spermatozoa.

Heifer	Total	Vagina [a]	Cervix	Uterus	UTJ	Isthmus	Ampulla	Infundibulum
2 h								
A	10 559.0	10 250.2	9.3	266.1	21.7	7.3	4.4	0
B	7 736.4	7 196.8	454.4	79.9	2.6	0	2.6	0
C	65 388.8	65 004.9	99.0	244.8	14.1	15.6	4.5	5.9
D	9 842.1	9 766.9	48.1	16.1	3.1	5.2	2.6	0[c]
12 h								
E	183.4	54.0	15.9	98.4	0[c]	7.3	0	7.8
F[b]	192.6	190.9	0	0[c]	0	1.7	0	0
G[b]	3 436.4	3 142.4	46.9	202.8	0	21.7	20.0	2.6
H[b]	69.2	43.3	0	15.5	0	5.2	5.2	0

[a] The figures also include spermatozoa in the mucus and urine that drained from vagina.

[b] The heifer had ovulated at the time of slaughter.

[c] Spermatozoa were found outside the squares of the Bürker chamber.

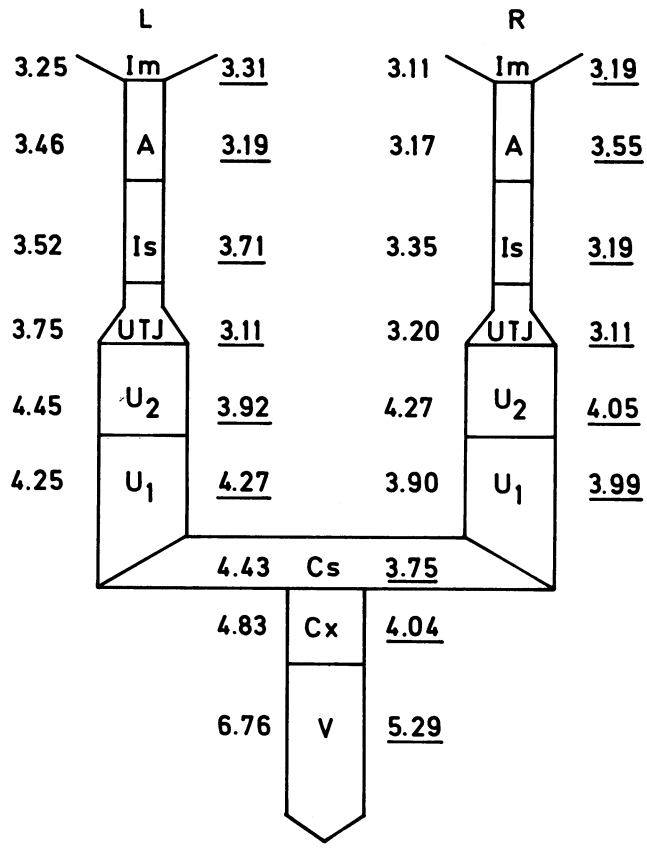

Figure 2. Mean $\log_{10}$ numbers of spermatozoa in different segments of the genital tract at 2 and 12 (underlined) h after insemination. The $\log_{10}$ numbers of spermatozoa in UTJ at 12 h is an estimate since no spermatozoa were found within the squares of the Bürker chamber in these segments (c.f. Materials and methods and Table 2).

The recovery rate of spermatozoa varied considerably among heifers. At 2 h after insemination it ranged from 6.2 to 40.9 % of the total number inseminated with an average of 14.6 % and at 12 h it ranged from 0.04 to 2.2 %, with an average of 0.6 %. In 7 of the heifers most spermatozoa were found posterior to the site of insemination and the majority of these spermatozoa were recovered in the mucus collected from the vagina. Spermatozoa recovered anterior to the site of semen deposition were mostly found in the uterus, both at 2 and 12 h after insemination.

Numbers of spermatozoa in vagina, cervix and the uterine

body declined from 2 to 12 h after insemination. In the posterior parts of uterus (U_1) the number of spermatozoa rose slightly between 2 and 12 h, whereas the number declined in the anterior part (U_2), so that the total number of spermatozoa recovered from the uterus was lower at 12 than at 2 h.

The average total number of spermatozoa recovered from the oviducts was nearly the same at 2 (89.6×10^3) and 12 h (71.5×10^3) after insemination but there were apparent differences in the distribution. At 2 h almost half of the spermatozoa recovered from the oviduct came from the UTJ, whereas at 12 h no spermatozoa were counted in the flushings from this part of the tract. At 12 h most (35.9×10^3) of the spermatozoa was recovered from the isthmus.

Numbers of spermatozoa recovered from right and from left sides of the uterus and the oviducts were compared (Table 3). In 6 of 8 heifers, most of the spermatozoa were recovered from the left part of the tract (uterus and oviducts), whereas the preovulatory follicle or corpus haemorrhagicum was in the right ovary in 5 of the heifers. Only 1 heifer (E) had the majority of spermatozoa in the oviduct ipsilateral to the folliclebearing ovary. None of the 3 heifers that had ovulated at slaughter 12 h after insemination (F, G and H) had more spermatozoa in the side adjacent to the ovary with the recent ovulation. However, the recovery of spermatozoa in heifer H was extremely low.

Table 3. Distributions of spermatozoa on the left and right sides of the uterus and oviducts in comparison to the ovary containing the largest follicle or corpus haemorrhagicum.

Heifer	% spermatozoa on the left side of uterus and oviducts of the total no. recovered from uterus and oviducts	% spermatozoa in the left oviducts of the total no. recovered from the oviducts	Occurrence of follicle or corpus haemorrhagicum in left ovary
D	56	24	+
E	61	100	+
H*	43	25	+
A	59	91	—
B	49	100	—
C	88	91	—
F*	100	100	—
G*	56	55	—

* The heifer had ovulated at the time of slaughter.

DISCUSSION

The method used for sperm recovery was not totally efficient since spermatozoa were found in deep folds of the mucus membrane, both at 2 and 12 h after insemination, in 15 % of the tissue specimens. Spermatozoa were found only outside the squares of the Bürker chamber in 10 samples and spermatozoa were also found in 6 of the 51 smears prepared from flushing solutions where no spermatozoa were recovered in the Bürker chamber. However, these samples, in which the number of spermatozoa were erroneously recorded as zero, were randomly distributed among heifers and times and did not influence the evaluation of the results.

Mattner et al. (1969) compared the number of spermatozoa recovered from the genital tract of ewes after artificial insemination with fresh and frozen semen and found significantly less spermatozoa when frozen semen was used. However, the recovery rate of spermatozoa in this study (14.6 and 0.6 % at 2 and 12 h after insemination) was in accordance with studies on sperm distribution in cattle using fresh semen (13.4 % at 1 h and 1.61 % at 16 h after semen deposition) (*El-Banna & Hafez* 1970 and *Dobrowolski & Hafez* 1970). Thus there was no evidence of lowered sperm recovery due to freezing of semen.

The largest number of spermatozoa was found in the vaginal mucus both at 2 and 12 h after insemination. As the semen was deposited in the uterine body this indicates a considerable backflow of semen through the cervix. This backflow apparently continued during the first 12 h after insemination. It could not be determined if a simultaneous influx of spermatozoa from the cervix to the uterus occurred.

The loss of spermatozoa from the genital tract may also have occurred through the infundibulum of the oviduct. Of the 8 inseminated heifers spermatozoa were found in the infundibulum of 4. An additional way of losing spermatozoa is by phagocytosis (*Austin* 1957). This phenomenon was not investigated in this study.

There was a considerable difference in the total number of recovered spermatozoa at 2 and 12 h after insemination. In the oviducts, however, the numbers were nearly the same at 2 (89.6×10^3) and at 12 h (71.5×10^3) after insemination. This indicates that a population of spermatozoa was established in

the oviducts early after insemination and then kept rather constant until and beyond the time of ovulation. Establishment of this population of spermatozoa seemed to occur without influence of the location of the preovulatory follicle. Only 1 of the 8 heifers in the experiment had the majority of spermatozoa in the oviduct ipsilateral to the ovary bearing the biggest follicle or the corpus haemorrhagicum. Thus there were no indications of a local regulatory function of the maturing follicle.

In cows, *Hunter & Wilmut* (1982/1983) used the technique of ligation of oviducts at different time intervals after natural mating to study when a population of spermatozoa competent to fertilize the egg entered the oviduct. They found no fertilized eggs when they separated the oviducts from the uterus within 6 h after mating early in estrus. In our experiments the number of spermatozoa recovered from the oviducts 2 h after insemination was considerable and probably large enough to allow fertilization. Further work needs to be done to fully clarify the importance of this early entrance of spermatozoa into the oviducts. The fact that we inseminated the heifers late in estrus could affect sperm transport and thereby make the establishment of a functional population of spermatozoa in the oviducts more rapid (*Turnbull* 1966 and *Hunter et al.* 1982).

ACKNOWLEDGEMENT

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REFERENCES

Austin, C. R.: Fate of spermatozoa in the uterus of the mouse and rat. J. Endocr. 1957, *14*, 335—342.

Dobrowolski, W. & E. S. E. Hafez: Transport and distribution of spermatozoa in the reproductive tract of the cow. J. Anim. Sci. 1970, *31*, 940—943.

Einarsson, S.: Site, transport and fate of inseminated semen. 9th Int. Congr. on Anim. Repr. and AI, Madrid 1980, Vol. 1, p: 147—158.

El-Banna, A. A. & E. S. E. Hafez: Sperm transport and distribution in rabbit and cattle female tract. Fertil. Steril. 1970, *21*, 534—540.

Hawk, H. W.: Sperm survival and transport in the female reproductive tract. J. Dairy Sci. 1983, *66*, 2645—2660.

Hunter, R. H. F., L. Barwise & R. King: Sperm transport, storage and release in the sheep oviduct in relation to the time of ovulation. Brit. vet. J. 1982, *138*, 225—232.

Hunter, R. H. F. & I. Wilmut: The rate of functional sperm transport into the oviducts of mated cows. Anim. Repr. Sci. 1982/1983, *5*, 167—173.

Lagerlöf, N.: Morphologische Untersuchungen über Veränderungen in Spermabild und in den Hoden bei Bullen mit verminderter oder aufgehobener Fertilität (Morphological studies on changes in sperm and testicular morphology in bulls with decreased fertility or sterility). Thesis, Uppsala 1934.

Mattner, P. E., K. W. Entwistle & I. C. A. Martin: Passage, survival and fertility of deep-frozen ram semen in the genital tract of the ewe. Aust. J. biol. Sci. 1969, *22*, 181—187.

Turnbull, K. E.: The transport of spermatozoa in the rabbit doe before and after ovulation. Aust. J. biol. Sci. 1966, *19*, 1095—1099.

SAMMANFATTNING

Fördelning av spermier i könsorganen hos kvigor efter artificiell insemination.

Åtta kvigor inseminerades i corpus uteri med sperma frusen i minipayetter, innehållande 160×10^6 spermier. Två (n = 4) eller 12 (n = 4) timmar efter inseminationen slaktades kvigorna och spermierna lokaliserades genom spolning av könsorganen. Kontroll av metodens effektivitet visade att det förelåg en viss underskattning av antalet återfunna spermier. Denna underskattning var dock slumpmässigt fördelad mellan kvigorna och mellan segmenten inom könsorganen.

Det totala antalet återfunna spermier var högre vid 2 timmar än vid 12 timmar efter inseminationen (14,6 resp. 0,6 % av antalet inseminerade spermier). Huvuddelen av spermierna återfanns i vagina både vid 2 och 12 timmar och i större mängd vid 2 timmar. I livmodern förelåg en viss sänkning av antalet spermier mellan 2 och 12 timmar. Antalet spermier i äggledarna var nästan det samma vid 2 ($89{,}6 \times 10^3$) och 12 ($71{,}5 \times 10^3$) timmar efter inseminationen. Vid 2 timmar sågs spermier i alla delar av äggledaren och med huvuddelen i övergången mellan livmoderhorn och äggledare, medan vid 12 timmar de flesta spermierna fanns i äggledarens isthmusdel.

Hos 6 av de 8 kvigorna återfanns fler spermier på vänster än på höger sida av könsorganen. Endast i 1 kviga sågs merparten av spermierna på samma sida som den follikelbärande äggstocken.

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