Sperm Cell Dynamics in the Female Rabbit Genital Tract After Insemination Monitored by Radiolabeled Spermatozoa

Andreas Bockisch

Johannes Gutenberg-Universität, Mainz, Germany

Dynamic in vivo imaging of spermatozoa radiolabeled with ^{99m}Tc-HMPAO in the female genital tract was performed noninvasively. Animal studies were performed under a variety of insemination conditions and their influence on the dynamics and number of spermatozoa in ascent was demonstrated. Spermatozoa motility was not significantly reduced by radiolabeling. In vitro and in vivo labeling stability was >93% after 18 hr. Female rabbits were inseminated with labeled spermatozoa and dynamic scintigraphic studies were performed up to 12 hr after insemination. The in vivo behavior of labeled spermatozoa was similar to native spermatozoa. Absolute spermatozoa numbers could be determined noninvasively and reliably at different parts in the female genital tract at various timepoints after insemination.

J Nucl Med 1993; 34:1134–1139

There is an increasing awareness of the problem of infertility. Some studies have suggested that up to 40% of men or women may be infertile (*1*). Several methods of evaluating infertility are now available, including radionuclide hysterosalpingography (*2,3*) which evaluates the patency of the Fallopian tubes. Since it is known that cervical mucus may be penetrated by particles but not by fluids (*3,4*), a physiological test for spermatozoa migration could also be of great utility.

Investigations in this direction, however, have been performed almost exclusively in animals (*5,6*). There are only a few preliminary descriptions in the literature concerning spermatozoa numbers and transit times in the human female genital tract (*7–12*). These studies, however, were not standardized and were investigated only at one timepoint: post coitum (*13*).

More precise studies conducted in animals were all invasive, wherein animals were killed at defined timepoints after insemination or copulation. The genital tract then was resected and different parts isolated. From these segments, spermatozoa recovery was attempted. This approach is labor-intensive and is also prone to significant errors.

Fertilization, which involves the fusion of a spermatozoum and the ovum, is believed to take place in the ampullic part of the Fallopian tube (*6,14*) and requires quite well defined conditions. The ovulum must come in contact with the spermatozoa in the right number and at the right place and time. Spermatozoa must reach the ampullic region at that time after ovulation when the ovulum passes through. During ascension, spermatozoa need to undergo a complicated change called the capacitation, which is a prerequisite for fertilization.

The number of spermatozoa entering the Fallopian tube, the order of magnitude and migration speed have not been reliably established. In addition, passive transport reportedly occurs soon after insemination (*15,17*).

In this paper, we describe a method for radiolabeling spermatozoa without significant damage. By using radiolabeled spermatozoa, spermatozoa transport and migration dynamics in the female rabbit genital tract were noninvasively and precisely measured using scintigraphic techniques.

METHODS

Radiolabeling of Spermatozoa

Technetium-99m-hexamethyl propylene amine oxime (HMPAO) is a radiopharmaceutical that allows gentle and stable labeling of spermatozoa. The labeling technique for human spermatozoa has been described elsewhere (*18*). Maximum HMPAO concentration and maximum specific activity resulted in the highest labeling yield. In this study, the same finding was established for rabbit spermatozoa obtained from five male rabbits with normal spermiogram.

The ejaculate was centrifuged at 500 g and the spermatozoa pellet resuspended in and incubated with ^{99m}Tc-HMPAO immediately after its preparation. After incubation, the spermatozoa were washed three times using 1 ml of Ham's F-10 solution with intermediate centrifugations and resuspension in a fresh solution (*19*). Finally, the spermatozoa were suspended in 1 ml of nutrition solution (Ham's F-10 plus serum). Spermatozoa motility was checked at different points during the preparation by counting the fraction of motile spermatozoa in a counting chamber. The num-

Received Aug. 18, 1992; revision accepted Mar. 30, 1993.

For correspondence or reprints contact: Andreas Bockisch, MD, PhD, Johannes Gutenberg-Universität, Langenbeckstrasse 1, D-6500 Mainz 1, Germany.

Note: This paper contains the essentials of the author's "Habilitationsschrift" performed at the Klinik für Nuklearmedizin der Universität Bonn (Head of Department: Prof. Biersack).

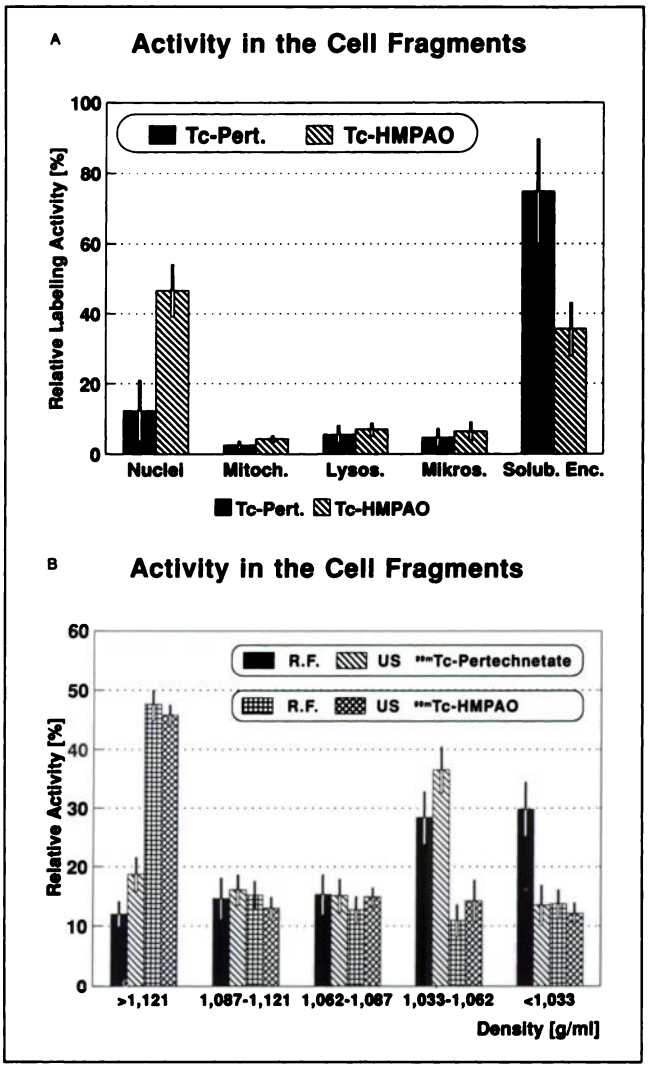

FIGURE 1. (A) Repeat ultracentrifugation and (B) Percoll® density gradient. Density >1.12 g/cm^3 corresponds to nuclei >1.087 and <1.121 to solid cell fragments. Enzymes and liquids have a density <1.033 g/cm^3. HMPAO activity is mostly bound to solid fragments of the spermatozoa, nuclei and mitochondria. In contrast, incubation of spermatozoa with [^{99m}Tc]pertechnetate leads to high activity in the fraction of soluble enzymes. Cell cracking by repeat freezing (R.F.) or ultrasound (US).

ber of motile spermatozoa decreased by about 10% when compared to untreated controls of the same ejaculate.

The labeling yield (percentage of incubation activity bound to the triple washed spermatozoa) was 52% ± 6% and the in vitro labeling stability was 93% ± 2% after 18 hr of incubation at 37°C. To determine labeling stability, spermatozoa were separated from the nutrition solution by centrifugation and the activity of both the pellet and the supernatant was measured. Spermatozoa motility nor semen quality were not significantly reduced by radiolabeling. Therefore, labeled spermatozoa represented whole ejaculate. These results coincide with those previously established for human spermatozoa.

For the animal studies, the HMPAO vial (Ceretec®, Amersham) was labeled with 3700 MBq in 5 ml of [^{99m}Tc]pertechnetate generator eluate in a standard procedure in order to guarantee standard conditions, although higher specific activities would increase the labeling yield. Usually, $100 \cdot 10^6$ spermatozoa were labeled with 75 MBq ^{99m}Tc-HMPAO. The incubation of spermatozoa was restricted to 10 min, since rabbit spermatozoa are more vulnerable than human spermatozoa. After labeling, the number of motile rabbit spermatozoa was reduced by about 10% when compared to untreated controls of the same ejaculate.

In addition, spermatozoa labeled with ^{111}In-oxine were studied and optimized to obtain a double nuclide investigation. Labeling with ^{111}In-oxine also resulted in a high labeling yield, but the stability is much lower. Indium-111-oxine labeling reportedly induces a considerable decrease in spermatozoa motility (*20*). Because ^{111}In-oxine was used for dead spermatozoa only, this aspect was not investigated here.

In order to identify the subcellular structures to which ^{99m}Tc-HMPAO binds, labeled spermatozoa were fragmented by repetitive freezing or ultrasound. Fragments were separated using repeat ultracentrifugation or density gradients. Ultracentrifugation employed 600 g for 10 min to separate the nuclei, 8000 g for 10 min for mitochondria, 25,000 g for 10 min for lysosomes and 150,000, for 60 min for the microsomes. After each centrifugation, the pellet containing the structures mentioned above was collected and the supernatant was centrifuged in the next step. The final supernatant contained soluble enzymes only. The activities in the individual pellets and the final supernatant were measured and the ratio of the activity in each fraction over the total activity is displayed in Figure 1. Ultracentrifugation using a density gradient resulted in a "stack" of different density bands defined by the characteristics of the medium (Percoll®) containing the cell fragments of corresponding density.

Technetium-99m-HMPAO was bound mostly to solid structures, predominately to the nucleus, of the cell. Only about one-third was found in the cytosol, which explains the high labeling stability.

In Vivo Studies

This study (Table 1) was designed to determine whether:

1. The label is stable in vivo. This means that activity distribution is identical to spermatozoa distribution.
2. Labeled spermatozoa behave in vivo in similar fashion to unlabeled spermatozoa.

Therefore, female rabbits were inseminated after synchronization by intravenous injection of 100 IE HCG (Primagonyl®, Schering AG Berlin/Bergkamen) 2 wk before the corpus luteum phase and 3 wk before the study in ovulating animals. For the latter, an additional HCG injection was added at 10 or 5 hr before or at the time of insemination (corresponding to insemination at the time of 5 or 10 hr prior to ovulation), which was performed in the posterior vagina using standard techniques. Imaging was initiated within 60 sec of insemination and the animals were kept immobile

TABLE 1
Study Design

1. $100 \cdot 10^6$ evenly labeled spermatozoa were inseminated 10 and 5 hr before and at time of ovulation.
2. Additional studies served for quality controls:
   $100 \cdot 10^6$ spermatozoa were inseminated during the corpus luteum phase.
   $50 \cdot 10^6$ spermatozoa were evenly labeled and $50 \cdot 10^6$ untreated spermatozoa were applied as a mixture.
3. A double nuclide study was performed using $50 \cdot 10^6$ spermatozoa labeled with ^{99m}Tc-HMPAO and $50 \cdot 10^6$ spermatozoa labeled with ^{111}In-oxine which were killed by heat after labeling.

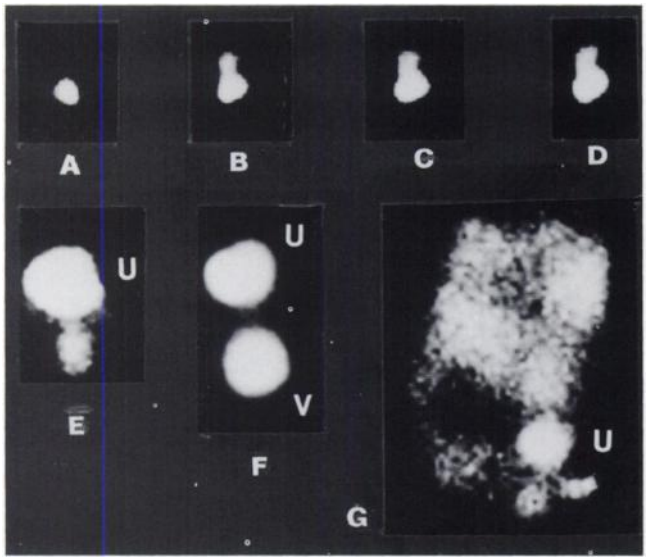

FIGURE 2. Typical dynamic spermatozoa scintigram (ventral view of the rabbit). Images of the same animal at different times after insemination of $100 \cdot 10^6$ rabbit spermatozoa labeled with 40 MBq ^{99m}Tc-HMPAO. Images A–D were acquired for 1 min, E and F for 5 min and G for 15 min. Image A was started immediately; images B–G, 5, 10, 20, 30, 120 and 720 min, respectively, after insemination. Image A displays only intravaginal activity; images B–D show increasing uterine activity. On D, a separation of the two spermatozoa pools (U = uterus; V = vagina) is seen, which is due to a retrograde passive transport of the vaginal spermatozoa. Two hours after insemination, most of the intracorporal remaining spermatozoa are intrauterine. After 12 hr, a considerable amount of spermatozoa have migrated through the genital tract and are found intraperitoneally.

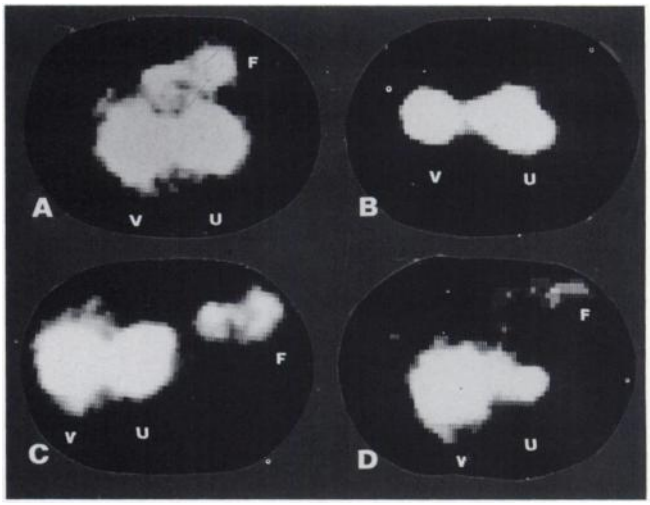

FIGURE 3. Ventral views of the rabbit visualize dynamic development of spermatozoa distribution. Double nuclide imaging after insemination of $50 \cdot 10^6$ ^{111}In-labeled dead and ^{99m}Tc-HMPAO-labeled motile spermatozoa. The motile spermatozoa (A) are visualized in the right Fallopian tube 6 hr after insemination in contrast to the dead spermatozoa (B). Twelve hours after insemination, only a few dead spermatozoa (D) are detected intrauterine. However, more than 50% of the motile spermatozoa (C) remaining in the animal at that time are intrauterine. In addition, there are many spermatozoa in the Fallopian tubes and many are intraperitoneal. The left image is caudal; the right is cranial. F = Fallopian tube; U = uterus; V = vagina.

in a special low absorption cage during imaging. Scintigrams were taken at ventral views: the first 30 min as dynamic scans, 5 sec/frame during the first minute and 15 sec/frame thereafter. Static scans were added at 45, 60, 90, 120, 240, 480 and 720 min after insemination. Figure 2 demonstrates the development of activity distribution in a typical rabbit study.

RESULTS

There was no suggestion of significant labeling loss in the in vivo studies. Animal blood contained minimal amounts of activity. Maximum blood activity was reached about 1 hr after insemination and was ≤2 ppm of inseminated activity/ml blood, which corresponds to a >99% in vivo labeling stability. At no time was the thyroid visible. Animals inseminated in the corpus luteum phase showed no activity outside the vagina. This finding is in agreement with established data, in that spermatozoa do not penetrate into the cervix uteri in that phase of the cycle, and confirms that there is neither activity resorption nor transport of free activity.

The vitality and presence of active motion of ^{99m}Tc-HMPAO labeled spermatozoa was proven in the double nuclide study. A mixture of $50 \cdot 10^6$ spermatozoa labeled with ^{99m}Tc-HMPAO and $50 \cdot 10^6$ spermatozoa, killed after labeling with ^{111}In-oxine underwent simultaneous dynamic imaging in the ^{99m}Tc and ^{111}In energy windows, respectively, after insemination. Figure 3 compares the distribution of the two spermatozoa populations. For the first hour, the ^{111}In-labeled dead spermatozoa remain mostly intravaginal and there was no major transport into the uterus. In contrast, motile spermatozoa demonstrated a continuous migration into the uterus. Equilibrium was reached after about 4 hr between the vaginal sperm cells migrating into the uterus and those leaving the abdominal cavity. Obviously, motile ^{99m}Tc-HMPAO labeled spermatozoa are found earlier and in much higher numbers in upper parts of the female genital tract (Fig. 4).

These results demonstrate a clear difference between the behavior of the dead (^{111}In-labeled) and in vitro motile (^{99m}Tc-labeled) spermatozoa. Moreover, technetium-labeled spermatozoa retained their motility in vivo. In addition, these studies demonstrate an apparent preference of motile over amotile spermatozoa in ascent. Therefore, evenly labeled spermatozoa were compared with a 50% mixture of labeled and unlabeled spermatozoa. No significant difference between these groups was found (Fig. 5). Along with the established "sorting mechanism," one may conclude that labeled spermatozoa behave like native spermatozoa.

Besides the basic methodologic aspects, these studies established important results concerning spermatozoa ascension:

1. Insemination of a small number of spermatozoa ($1 \cdot 10^6$) results in a 5–10 times higher portion of ascending spermatozoa than a larger amount ($100 \cdot 10^6$).

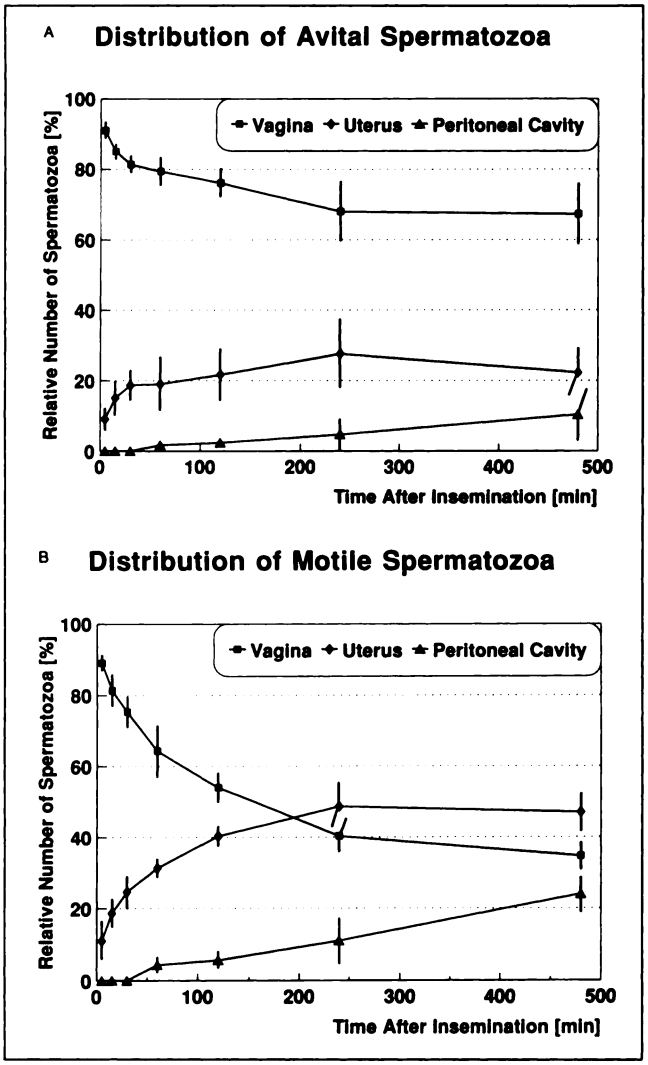

FIGURE 4. Time development of spermatozoa numbers for representative parts of the female genital tract normalized to the total number of spermatozoa remaining in the animals at respective times. Simultaneous examination after insemination of each $50 \cdot 10^6$ ^{111}In-labeled dead and ^{99m}Tc-HMPAO-labeled motile spermatozoa. Averaged data for three animals are presented.

2. The closer to ovulation that insemination occurred the faster the spermatozoa ascended. Rapid transport into upper segments of the genital tract after insemination was demonstrated, even with dead spermatozoa.

The number of spermatozoa accumulated in the Fallopian tubes was found to be high, usually in the tens of thousands, and ranged from undetectable to exceeding $300 \cdot 10^3$ in some animals. The earliest detection of spermatozoa in the Fallopian tube was 5 min after insemination and 30 min for the peritoneal cavity. Figure 6 compares results gained from $100 \cdot 10^6$ evenly labeled spermatozoa, including seminal plasma, inseminated 10 and 5 hr before and at the time of ovulation.

Radiation exposure was calculated according to the procedure reported by Makrigiorgos (*21*) and was found to be about 350 mGy for labeled spermatozoa, assuming $100 \cdot 10^6$

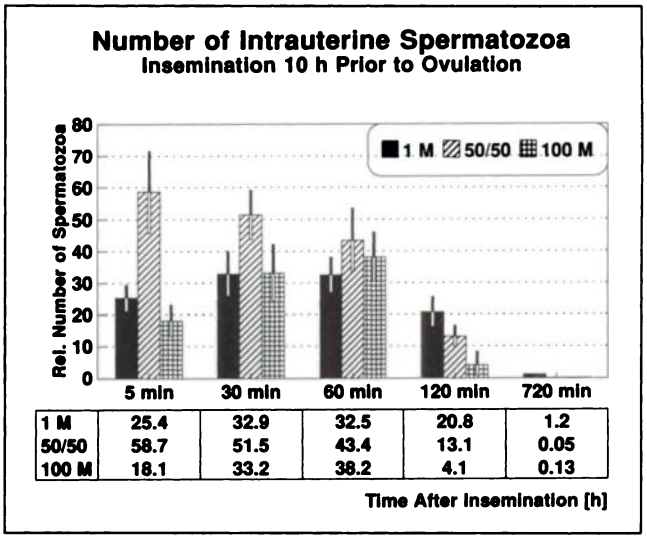

FIGURE 5. Development of spermatozoa populations after insemination during ovulation for different preparations of the inseminate. 1 M = $1 \cdot 10^6$ spermatozoa labeled even with ^{99m}Tc-HMPAO; 100 M = $100 \cdot 10^6$ spermatozoa evenly labeled with ^{99m}Tc-HMPAO; and 50/50 = $50 \cdot 10^6$ ^{99m}Tc-HMPAO labeled and unlabeled spermatozoa. Data are averaged for five animals.

spermatozoa labeled with 4 MBq, and <2 mGy for the ovaries.

DISCUSSION

HMPAO allows stable and gentle high yield labeling of spermatozoa. Thus, ^{99m}Tc-HMPAO-labeled spermatozoa allow the physiological investigation of spermatozoa ascension in the female genital tract after insemination. Besides dynamic imaging, numerical results can be obtained with high accuracy. If the number of inseminated sperma-

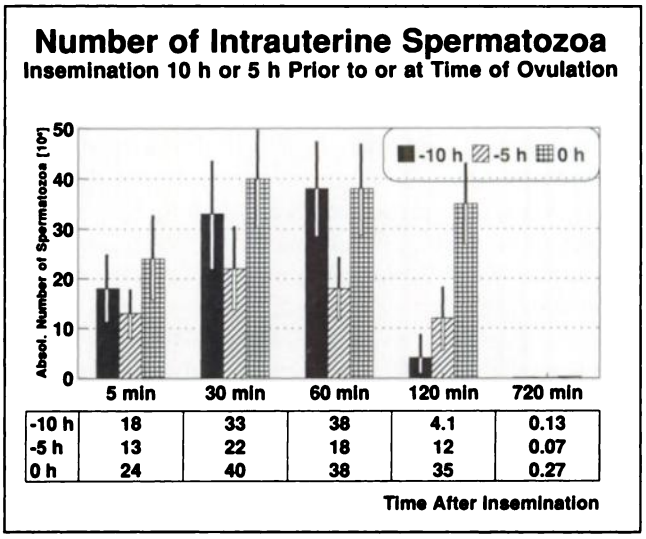

FIGURE 6. Development of spermatozoa populations after insemination of $100 \cdot 10^6$ evenly labeled spermatozoa at different times in relation to ovulation. −10 h = insemination about 10 hr prior to induced ovulation; −5 h = insemination about 5 hr prior to induced ovulation; 0 h = insemination at about time of induced ovulation.

tozoa is known, even the absolute number of spermatozoa can be determined at any segment in the genital tract at any time.

Other studies to date required killing the animals at a given time. Therefore, dynamic information in the same animal was never available. After killing, the genital tract was resected and spermatozoa recovery was attempted. This procedure is difficult and prone to errors. There may be a redistribution during death or contamination of parts of originally low spermatozoa concentrations during preparation. In addition, spermatozoa recovery has a low and varying yield (*14,22,23*), which may explain contradictory spermatozoa numbers in the genital tract. In previous studies, the uterine section of the Fallopian tube was difficult to investigate (*24*) and was often not taken into account, although high spermatozoa numbers would have been expected (*6*).

To date, studies have focused on spermatozoa discovered in the Fallopian tubes and in the cervix uteri and have not addressed the abdominal cavity. Tempelton and Mortimer (*11*) did attempt quantification of postcoital spermatozoa in the Douglas secrete and estimated about 10,000. Information concerning vaginal spermatozoa numbers is also scarce. Morton and Glover (*25*) reported a loss of ~98% of inseminated spermatozoa within 4 hr. That result is in conflict with those of the present study and might be explained by a low recovery efficiency. For all parts of the genital tract, the spermatozoa numbers detected in this paper are much higher than those reported in conventional studies. Four hours after insemination of $100 \cdot 10^6$ spermatozoa, we detected up to $25 \cdot 10^6$ in or close to the cervix uteri, in contrast to $2 \cdot 10^6$ described by Morton and Glover (*26*). The number of tubal spermatozoa is so far uncertain, even with respect to the order of magnitude. Morton and Glover (*25*) recovered <3,000 of $5{,}000 \cdot 10^6$ inseminated spermatozoa in the Fallopian tubes of rabbits. Similar studies by Hunter and Nichol (*14*) yielded <2,000. Ahlgren's post-coitum study of women (*13*) found no more than 24 intratubal spermatozoa. At the high end, Harper reports 800 to 55,000 (*27*) and Mroueh (*28*) $120 \cdot 10^3$ spermatozoa in the Fallopian tubes of rabbits. In the present study, reliable numbers could be measured reaching up to $300 \cdot 10^3$ after insemination of $100 \cdot 10^6$ spermatozoa. Again, the difference is most easily explained by insufficient recovery in the conventional studies.

With radionuclide investigation, abdominal spermatozoa could be reliably detected. Our numerical analysis demonstrated up to $200 \cdot 10^3$ intra-abdominal spermatozoa at 12 hr postinsemination, which corresponds to about 25% of intracorporally remaining spermatozoa. Because of tremendous recovery problems, there are no comparable results available from conventional studies.

The double nuclide studies demonstrated transportation of dead spermatozoa to upper parts of the genital tract, which confirms passive transport (*4,29,31*). This transport was not restricted to the first minutes after insemination but also occurred spontaneously at later times. This finding is in agreement with Overstreet and Tom (*17*), who report muscular contractions in the first 5 min after insemination and again several hours later. Dandekar et al. (*15*) and Noyes et al. (*16*) neglected to note fast transport or the transport of dead spermatozoa through the cervix uteri.

In a comparison of ascension speed, it was found that late insemination, in respect to ovulation, results in more rapid ascendency. This would seem to be a reasonable physiologic mechanism and was previously observed by Yanagimachi and Chan (*32*) in chinese hamsters.

In conclusion, ^{99m}Tc-HMPAO appears to be an ideal radiopharmaceutical for the gentle and stable radiolabeling of spermatozoa. The labeling yield was high, in vivo animal studies demonstrated preserved motility and there was no evidence of significant degradation when compared to unlabeled spermatozoa. By using this tool in fertility research, dynamic spermatozoa distribution in the female genital tract after insemination may be followed noninvasively, relevant structures of the tract can be scintigraphically defined and reliable absolute quantification of spermatozoa numbers in these structures can be established.

REFERENCES

1. Kepp R, Staemmler HJ. Lehrbuch der Gynäkologie. Stuttgart—New York: Georg Thieme Verlag; 1980.
2. Iturralde M, Venter PF. Hysterosalpingo-radionuclide scintigraphy (HERS). *Semin Nucl Med* 1981;11:301–314.
3. Stone SC, McCalley M, Braunstein PH, Egbert R. Radionuclide evaluation of the tubal function. *Fertil Steril* 1985;43:757–760.
4. De Boer CH. Transport of particulate matter through the human female genital tract. *J Reprod Fertil* 1972;28:295–297.
5. Blandau RJ. Gamete transport—comparative aspects. In: Hefez ESE, Blandau RJ, eds. *The mammalian oviduct*. Chicago: The University of Chicago Press; 1969:129–162.
6. Smith TT, Koyanagi F, Yanagimachi R. Distribution and number of spermatozoa in the oviduct of the golden hamster after natural mating and artificial insemination. *Biol Reprod* 1987;37:225–234.
7. Ahlgren M. Migration of spermatozoa to the Fallopian tubes and the abdominal cavity in women, including some immunological aspects. PhD dissertation, Lund, Sweden, 1969.
8. Brown JR. Rate of transport of spermia in human uterus and tubes. *Am J Obstet Gynecol* 1944;47:407–411.
9. Croxatto HB, Faundes A, Medel M, et al. Studies on sperm migration in the human female genital tract. In: Thibault C, Hafez ESE, eds. *Transport, survie et pouvoir fécondant des spermatozoides chez les vertébrés*. Paris: INSERM; 1974.
10. Rubenstein BB, Strauss H, Lazarus ML, Hankin H. Sperm survival in woman. Motile sperm in the fundus and the tubes of surgical cases. *Fert Steril* 1951;2:15–19.
11. Templeton AA, Mortimer D. Laparoscopic sperm recovery in infertile women. *Br J Obstet Gynaecol* 1980;87:1128.
12. Templeton AA, Mortimer D. The development of a clinical test of sperm migration to the site of fertilization. *Fertil Steril* 1982;37:410–415.
13. Ahlgren M. Sperm transport to and survival in the human fallopian tube. *Gynecol Invest* 1975;6:206–214.
14. Hunter RHF, Nichol R. Post-ovulatory progression of viable spermatozoa in the sheep oviduct and the influence of multiple mating on their pre-ovulatory distribution. *Br Vet J* 1986;142:52–58.
15. Dandekar P, Vaidya R, McMorris J. The effect of coitus on transport of sperm in the rabbit. *Fertil Steril* 1972;23:759–762.
16. Noyes RW, Adams CE, Walton A. Transport of spermatozoa into the uterus of the rabbit. *Fertil Steril* 1958;9:288.
17. Overstreet JW, Tom RA. Experimental studies of rapid sperm transport in rabbits. *J Reprod Fertil* 1982;66:601–606.
18. Bockisch A, Al-Hasani S, v.d. Ven H, et al. Markierung von Spermatozoen mit ^{99m}Tc-HMPAO und in-vivo-Darstellung der Spermatozoenmigration beim Kaninchen. *NucCompact* 1989;2:65–70.

19. Ham RG. An improved nutrient solution for diploid chinese hamster and human cell lines. *Exp Cell Res* 1963;29:515.
20. Olive DL, Weinberg JB, Haney AF. Sperm-macrophage interaction in the mouse: a quantitative assay in vitro using ^{111}In-oxine-labeled sperm. *Biol Reprod* 1987;37:1170–1178.
21. Makrigiorgos GM, Adelstein SJ, Kassis AI. Limitations of conventional internal dosimetry at the cellular level. *J Nucl Med* 1989;30:1856–1864.
22. Overstreet JW, Cooper GW. Sperm transport in the reproductive tract of the female rabbit. I. The rapid transit phase of transport. *Biol Reprod* 1978;19:101–114.
23. Thibault C. Sperm transport and storage in vertebrates. *J Reprod Fertil* 1973;18:39–53.
24. Hunter RHF. The timing of the capacitation in mammalian spermatozoa—a reinterpretation. *Res Reprod* 1987;19:3–4.
25. Morton DB, Glover TD. Sperm transport in the female rabbit: the role of the cervix. *J Reprod Fertil* 1974;38:131–138.
26. Morton DB, Glover TD. Sperm transport in the female rabbit: the effect of inseminate volume and sperm density. *J Reprod Fertil* 1974;38:139–146.
27. Harper MJK. Stimulation of sperm movement from the isthmus to the site of fertilization in the rabbit oviduct. *Biol Reprod* 1973;8:369–377.
28. Mroueh A. Oxytocin and sperm transport in rabbits. *Obstet Gynecol* 1967; 29:671–673.
29. Ahlgren M, Boström K, Malmqvist R. Sperm transport and survival in women with special reference to the Fallopian tube. In: Thibault C, Hafez ESE, eds. *Transport, survie et pouvoir fécondant des spermatozoïdes chez les vertébrés*. Paris: INSERM; 1973.
30. Davajan V, Kunitake GM. Fractional in vivo and in vitro examination of postcoital cervical mucus in the human. *Fertil Steril* 1969;20:197–202.
31. MacLeod J, Masten F, Silberman C, Sobrero AJ. The post-coital and post-insemination cervical mucus and semen quality. *Fertil Steril* 1963;10: 41–47.
32. Yanagimachi R, Chang MC. Sperm ascent through the oviduct of the hamster [redacted]abbit in relation to the time of ovulation. *J Reprod Fertil* 1963;6: 413