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In vivo imaging of *in situ* motility of fresh and liquid stored ram spermatozoa in the ewe genital tract

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Abstract

The fertility of ram semen after cervical insemination is substantially reduced by 24 h of storage in liquid form. The effects of liquid storage on the transit of ram spermatozoa in the ewe genital tract was investigated using a new procedure allowing direct observation of the spermatozoa in the genital tract. Ejaculated ram spermatozoa were double labeled with R18 and MitoTracker Green FM, and used to inseminate ewes in estrus either cervically through the vagina or laparoscopically into the base of the uterine horns. Four hours after insemination, the spermatozoa were directly observed *in situ* using fibered confocal fluorescence microscopy in the base, middle and tip of the uterine horns, the utero-tubal junction (UTJ) and the oviduct. The high resolution video images obtained with this technique allowed determination of the distribution of spermatozoa and individual motility in the lumen of the ewe's genital tract. The results showed a gradient of increasing concentration of spermatozoa from the base of the uterus to the UTJ 4 h after intra-uterine insemination into the base of the horns. The UTJ was shown to be a storage region for spermatozoa before their transfer to the oviduct. The *in vitro* storage of spermatozoa in liquid form decreased their migration through the cervix and reduced the proportion of motile spermatozoa and their straight line velocity at the UTJ and their transit into the oviduct.

Reproduction (2009) **138** 45–53

Introduction

Fertility in the ewe after cervical insemination has been shown to be reduced when semen have been stored in liquid form for several hours, despite any major reduction in semen quality as assessed by standard *in vitro* parameters such as motility (Salamon & Maxwell 2000). A substantial decrease in fertility was also reported when frozen–thawed semen was used for cervical insemination, and this effect was partially overcome by intra-uterine insemination (Gillan & Maxwell 1999), which suggested that impaired migration of spermatozoa through the cervix caused reduced fertility in ewes inseminated with preserved semen. Indeed, migration through the cervix appeared to be the critical limiting factor for migration of ram spermatozoa from the vagina to the oviducts (Lightfoot & Restall 1971, Hawk & Conley 1975, Hawk 1983). The migration of spermatozoa through the cervix may involve increased contractility of the cervix at estrus (Wergin 1985) because cervical myoelectrical activity was found to vary during the cycle and reach a peak around ovulation (Cavaco-Gonçalves *et al.* 2006). The morphology of the cervix is very variable, and differences between animals, during the cycle (Kershaw *et al.* 2005) and between breeds (Kaabi *et al.* 2006) have been reported. The differences in fertility reported between breeds of sheep after insemination with frozen–thawed semen might be related to differences in sperm transit in the genital tract of the ewe (Fair *et al.* 2005). The physiological study of sperm transit and quality in the female genital tract has to date been limited by the lack of techniques to observe the behavior of spermatozoa in the genital tract of the living female at a cellular level. A new technique, fibered confocal fluorescence microscopy (FCM), provides direct observation of the microscopic structure of living tissue (Laemmel *et al.* 2004, Thiberville *et al.* 2007). The technique is based on the principle of confocal microscopy, which provides an in-focus image of a thin section within a biological sample, where the microscope objective is replaced by a flexible fiber-optic miniprobe. With this technique it is possible to obtain images with endogenous or exogenous tissue fluorophores through a fiber-optic probe of 1 mm diameter or less. In this study, we developed a method based on FCM to observe the behavior of individual spermatozoa

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-09-0108
Online version via www.reproduction-online.org

and to determine quantitatively the *in situ* patterns of migration along the genital tract of ewes inseminated with fresh semen or semen preserved for 24 h at 15 °C.

Results

Validation of R18/MitoTrackerGreen FM labeling for observing spermatozoa in vivo

The ability to observe spermatozoa *in vivo* depended on their adequate labeling with bright and stable fluorescent probes. Several fluorochromes were, therefore, tested for their ability to label spermatozoa over several hours, both *in vitro* and *in vivo*. The fluorochrome MitoTracker Green FM is a vital stain that provided stable, green fluorescent labeling of the mitochondria of live spermatozoa (Fig. 1A). Fluorochrome R18 provided bright and stable orange labeling over the surface of the whole spermatozoon, including the flagella (Fig. 1B). Labeling with R18 was independent of sperm viability, and live and dead spermatozoa had the same orange fluorescence, but MitoTracker Green FM labeled only living spermatozoa (Fig. 2).

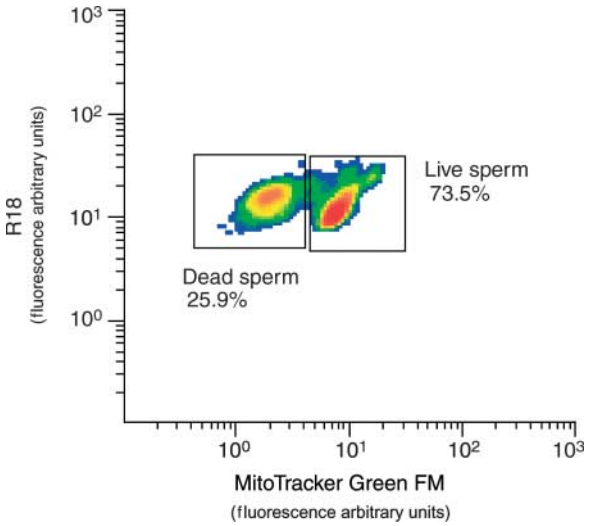

Figure 2 Quantification of fluorescence in ram spermatozoa. Fluorescence intensity after labeling spermatozoa with 300 µM R18 and 20 µM MitoTracker Green FM was quantified by flow cytometry. Live and dead populations could be discriminated by MitoTracker labeling, whereas R18 fluorescence was the same for live and dead spermatozoa.

Using FCM to image spermatozoa

Imaging of spermatozoa was first attempted *in vitro*. Spermatozoa were labeled with R18 and MitoTracker Green FM, diluted in PBS–BSA at 37 °C. The tip of the FCM microprobe was placed at the bottom of a tube containing spermatozoa and images were recorded. The 1.5 mm probe provided imaging of large populations of spermatozoa (Fig. 3A, Supplementary Video 1, which can be viewed online at www.reproduction-online.org/supplemental/) at a resolution of 3.3 µm, thus allowing spermatozoa to be counted and their motility analyzed. The probe with a diameter of 4 mm provided imaging of individual spermatozoa at high resolution (1.8 µm) so that individual parts of a spermatozoon such as the head, the intermediate piece and the flagella could be examined in detail (Fig. 3B and C, Supplementary Video 2, which can be viewed online at www.reproduction-online.org/supplemental/). One of the advantages of FCM is its ability to examine spermatozoa that are not constricted between a slide and a coverslip and thus, free to progress in a three dimensional environment. Some features of sperm motility such as rotation of the head can be clearly observed (Fig. 3D–H).

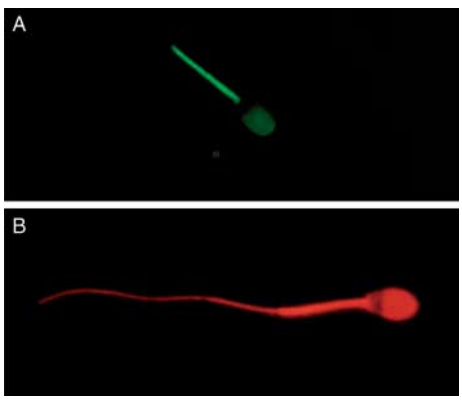

Figure 1 Ejaculated live ram spermatozoa labeled with MitoTracker Green FM (A) and R18 (B).

Although single labeling with R18 would have been sufficient for sperm imaging with FCM, we used double labeling with R18 and MitoTracker Green FM to provide data on the live/dead ratio *in vitro* by flow cytometry and *in vivo* using the FCM system.

Spermatozoa were imaged *in situ* in the genital tract of anesthetized ewes 4 h after intra-uterine insemination at the base of the uterine horn with spermatozoa labeled with R18/Mitotracker Green FM. The uterine horns were exteriorized at laparotomy and the FCM microprobe was inserted into several anatomical regions (Fig. 4A). The diameter of the probe (1.5 mm) allowed its insertion into all regions analyzed, including the oviduct, whereas use of the 4 mm probe was restricted to the uterine regions and the utero-tubal junction (UTJ). In preliminary experiments, we observed autofluorescence of the endometrial wall of the uterus. This was not due to fluorochrome leakage from spermatozoa because it was also observed in control ewes which had not been inseminated. Individual cells in the endometrial wall could be identified with the 4 mm probe on the basis of their autofluorescence (Fig. 4C).

However, the high signal-to-background ratio of fluorescing spermatozoa allowed their identification in the female tract. Populations of motile spermatozoa

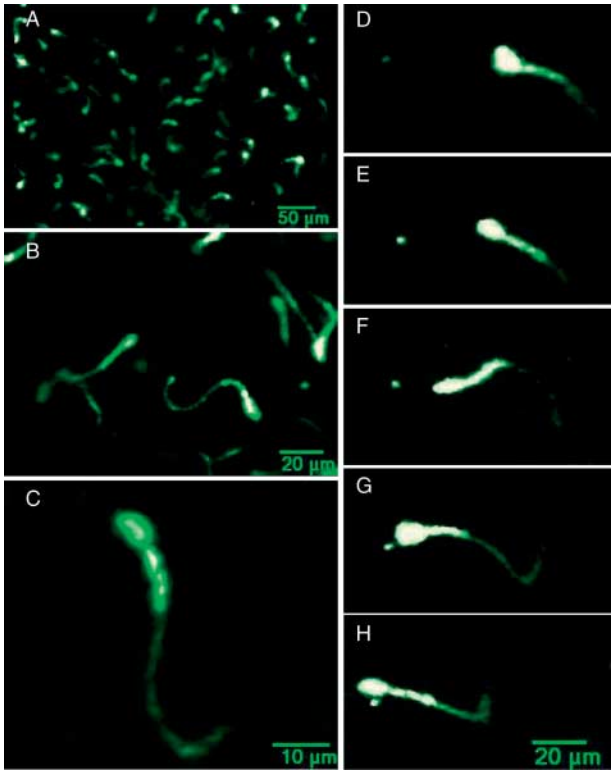

Figure 3 *In vitro* imaging of live spermatozoa with the FCM system. Ram spermatozoa labeled with R18 and MitoTracker Green FM were imaged in an eppendorf tube using a 1.5 mm probe (Panel A) or a 4 mm probe (Panels B and C). Populations of motile spermatozoa could be observed using a 1.5 mm probe with a resolution of 3.3 µm, whereas regions of individual spermatozoa could be observed with a 4 mm probe with a resolution of 1.8 µm. Individual motility could also be observed with the high resolution probe and features such as the rotation of the sperm head could be seen (Panels D–H). The time interval between two successive images of this figure was 83 ms.

were found in the lumen of the uterus (Fig. 4B, Supplementary Video 3, which can be viewed online at www.reproduction-online.org/supplemental/), and, individual parts of the spermatozoa (head, intermediate piece, flagella) could be observed when using the 4 mm probe (Fig. 4C).

In vivo *motility of spermatozoa*

Ewes in estrus were inseminated at the base of the uterine horns with D0 or D1 spermatozoa labeled with R18/MitoTracker Green FM, and examined using FCM 4 h later. The uterine horns have regular and strong contractions during estrus, and although sperm motility could be clearly observed in the female tract at this time, the rhythmic uterine contractions impeded the fine discrimination of sperm motion caused by the beating of the flagellum from motion induced by the uterine contractions. However, the UTJ is a region with limited contractile activity and sperm motility could therefore be

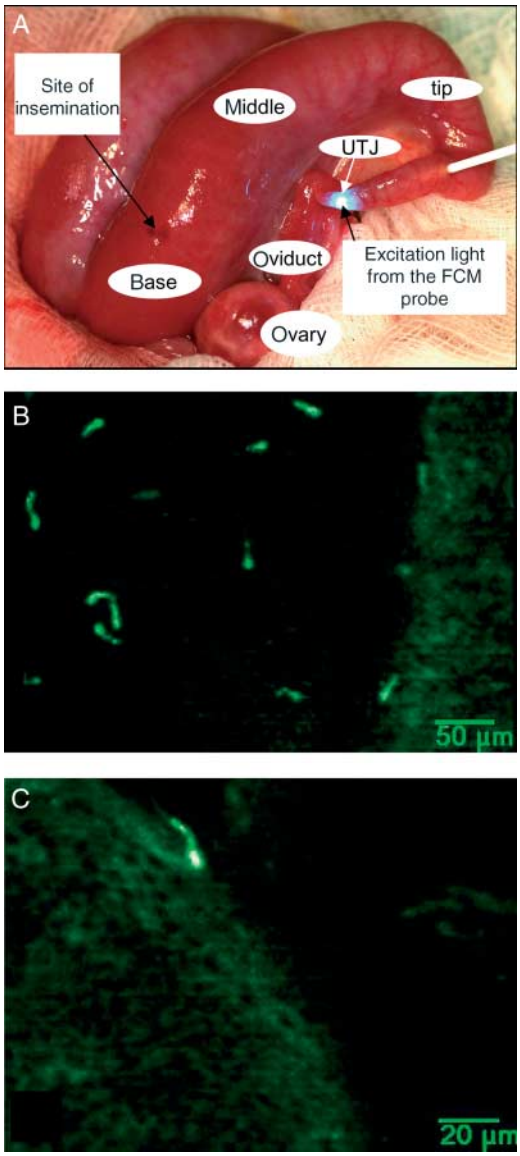

Figure 4 *In situ* imaging of ram spermatozoa. The uterine horns, oviducts and ovaries from adult ewes were exteriorized under general anesthesia (Panel A). A microprobe (in this example the 3.3 µm resolution probe) was inserted into the different regions of the female tract. Spermatozoa were imaged in the base, middle and tip of the uterine horns, in the utero-tubal junction and in the oviducts. The blue spot on the picture shows the excited light emitted by the tip of a probe inserted in the utero-tubal junction. Panel B shows spermatozoa *in situ* at the utero-tubal junction observed using the 3.3 µm resolution probe. The lumen of the tract can be clearly distinguished from the autofluorescencing epithelial wall of the uterus, revealing individual spermatozoa labeled with R18 and MitoTracker Green FM that can be easily seen because of their intense fluorescence. Panel C shows the *in situ* imaging of spermatozoa at the utero-tubal junction using a 1.8 µm resolution probe. The different regions (head, intermediate piece and flagella) of a single spermatozoon can be seen as well as the autofluorescencing cells of the epithelial wall of the utero-tubal junction.

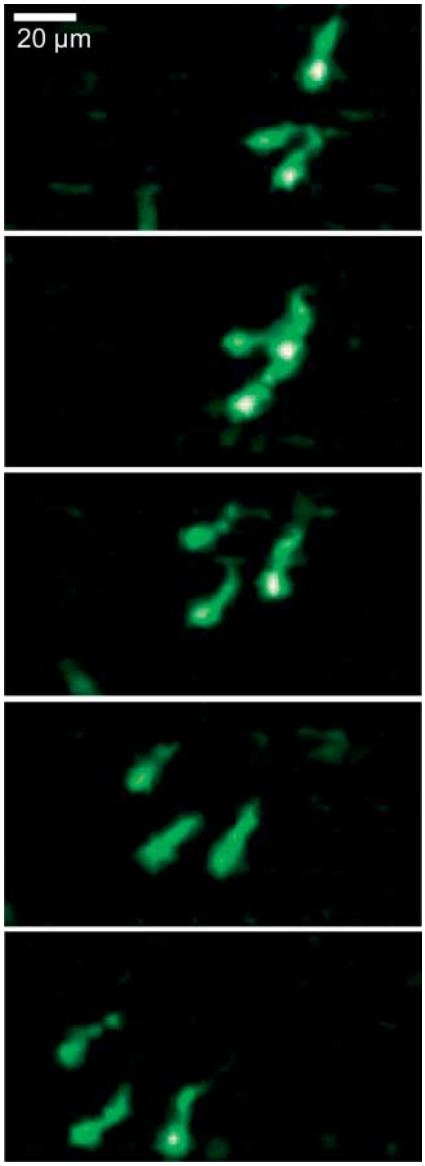

Figure 5 *In situ* motility of spermatozoa in the utero-tubal junction. Video images were recorded with fibered confocal fluorescence microscopy at a rate of 12 frames/s (1 image/83 ms) allowing the direct observation of motility of individual spermatozoa and estimation of the proportion of motile spermatozoa. The time interval between two successive images of this figure is 330 ms.

quantitatively evaluated in the lumen of the UTJ. Images of the motility of individual spermatozoa were recorded (Fig. 5, Supplementary Video 4, which can be viewed online at www.reproduction-online.org/supplemental/) and their tracks drawn (Fig. 6).

The storage of spermatozoa at 15 °C for 24 h in a milk-based diluent resulted in a significant ($P<0.05$) reduction in the proportion of motile spermatozoa but had no effect on their straight line velocity (VSL) in the *in vitro* tests (Fig. 7A). The proportion of motile spermatozoa after storage (D1) was reduced *in vivo* ($P<0.05$) compared to D0 spermatozoa (Fig. 7B). Similarly, the *in vivo* VSL of D1 spermatozoa was lower ($P<0.05$) compared to D0 spermatozoa (Figs 6 and 7B).

Storage in vitro *reduced sperm transit in the female genital tract after AI*

The concentration of spermatozoa in different regions of the female tract was quantified for D0 and D1 spermatozoa 4 h after intra-uterine insemination. For D0 and D1 spermatozoa, an increasing sperm concentration gradient was found from the base of the horn to the UTJ ($P<0.05$). The sperm concentration was significantly lower ($P<0.05$) in the oviduct compared to the UTJ (Fig. 8A). A marked effect of storage was observed on sperm transit and the sperm concentration was significantly lower ($P<0.05$ for all regions) for D0 spermatozoa compared to D1 spermatozoa in all the regions of the uterine horns, the UTJ and the oviduct (Fig. 8). The ability of spermatozoa to migrate through the cervix was also investigated by observing spermatozoa in the uterus after cervical insemination with D0 and D1 spermatozoa (Fig. 8B). Four hours after insemination, spermatozoa could only be found in the body of the uterus, very close to the cervix, regardless of storage. The number of D1 spermatozoa in the body of the uterus was significantly reduced ($P<0.05$) compared to D0 spermatozoa, suggesting that migration of spermatozoa through the cervix was affected by storage.

Discussion

In this study, using fibered confocal microscopy, we observed individual spermatozoa in the female genital tract *in vivo* and quantitatively followed their transit through the uterus and their migration into the oviduct. Quantitative studies of sperm transit in the female genital tract have previously been based on histology, electron microscopy or counting spermatozoa flushed from different regions of the female genital tract after ovariohysterectomy or slaughter (Scott 2000, Hunter 2005, Rodriguez-Martinez *et al*. 2005, Suarez & Pacey 2006). Spermatozoa have also been directly observed *ex vivo* in the hamster (Katz & Yanagimachi 1980) and the mouse (Suarez 1987) through the transparent wall of excised oviducts. Few studies have investigated sperm transit in the female genital tract using *in vivo* procedures. Scintigraphy after vaginal insemination with radiolabeled spermatozoa had indicated the portions of the female genital tract containing spermatozoa *in vivo* and provided indirect quantification of sperm distribution in the ewe (Bruckner *et al*. 1982, Balogh *et al*. 1995), rabbit (Bockisch 1993) and human (Özgür *et al*. 1997, Kadanali *et al*. 2001). In the study reported here, FCM provided high resolution images of spermatozoa and allowed the *in situ* observation of individual spermatozoa. However, the technique

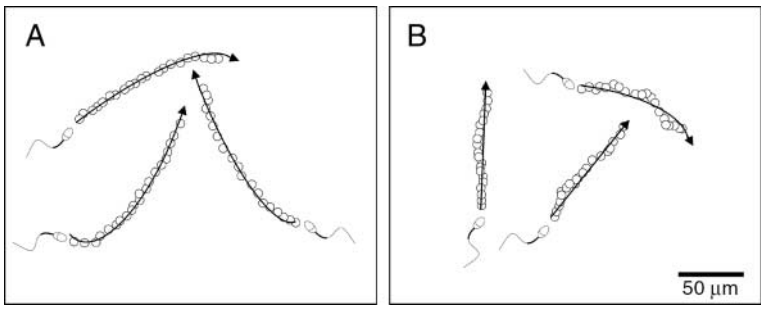

Figure 6 Effects of storage for 24 h at 15 °C on the velocity of spermatozoa in the utero-tubal junction. Individual tracks were determined from video images of spermatozoa recorded with fibered confocal fluorescence microscopy in the utero-tubal junction 4 h after intra-uterine insemination. The tracks were constructed from 20 successive positions of the sperm head over a total period of 1.6 s. Representative tracks of D0 (A) and D1 (B) spermatozoa exhibiting straight line velocity equal to the mean value of the whole sperm populations are shown, indicating reduced *in vivo* velocity of spermatozoa after storage.

depends on the stability of the fluorochromes used to label spermatozoa. MitoTracker Green FM labeled the mitochondria of live spermatozoa (Garner *et al*. 1997) and has been used previously to study the fate of the mitochondria in spermatozoa during *IVF* (Sutovsky *et al*. 1996). Given the resolution and the sensitivity of FCM, the restriction of MitoTracker Green FM labeling to the intermediate piece of the spermatozoa was a limiting factor for the *in situ* detection of spermatozoa in our preliminary studies. We therefore decided to double label spermatozoa with MitoTracker Green FM and another fluorochrome. The second fluorochrome required two main properties: 1) bright and stable fluorescence over the entire surface of the spermatozoa in order to detect them clearly *in situ* and 2) ability to label all spermatozoa *in situ* regardless of viability in order to quantify the whole sperm population *in situ*. When tested, many probes suitable for *in vitro* labeling of spermatozoa such as the DNA stain (SYTO 16) or lipid membrane stain (DiA) was unstable under *in vivo* conditions (data not shown). One stain, Octadecyl rhodamine B (R18), labeled the spermatozoa bright orange over the entire surface, irrespective of viability. This fluorochrome has been used to assess fusion between spermatozoa and R18-loaded prostasomes (Palmerini *et al*. 2006) or liposomes (He *et al*. 2001). Fusion with R18-loaded liposomes was not found to modify the viability of spermatozoa from boars before or after freezing (He *et al*. 2001), and in our preliminary tests, labeling with R18 did not modify the *in vitro* motility of fresh or preserved ram spermatozoa. We therefore used double labeling with R18 and MitoTracker Green FM and FCM to make direct observations of ram spermatozoa in the genital tract of the ewe. One striking and unexpected observation was the autofluorescence exhibited by the endometrial wall and the lining of the UTJ in ewes which had not been inseminated with fluorescent spermatozoa. Autofluorescence after excitation/emission with 450/495 nm wave length has been observed *in situ* in the human cervix (Brookner *et al*. 2000, Drezek *et al*. 2003). This fluorescence originated from both the epithelium and the stroma, and the autofluorescence from the epithelial cells is associated with the metabolism of NADH (Brookner *et al*. 2000). We used very similar conditions to measure fluorescence (excitation/emission of 488/525–575 nm) and the very short working distance of the microprobe (<10 µm) suggested that the fluorescence we observed was autofluorescence in the epithelium of the uterus. Overcoming this problem required a higher intensity of fluorescence labeling of spermatozoa compared to the autofluorescence of the uterine epithelium. This autofluorescence had an unexpected

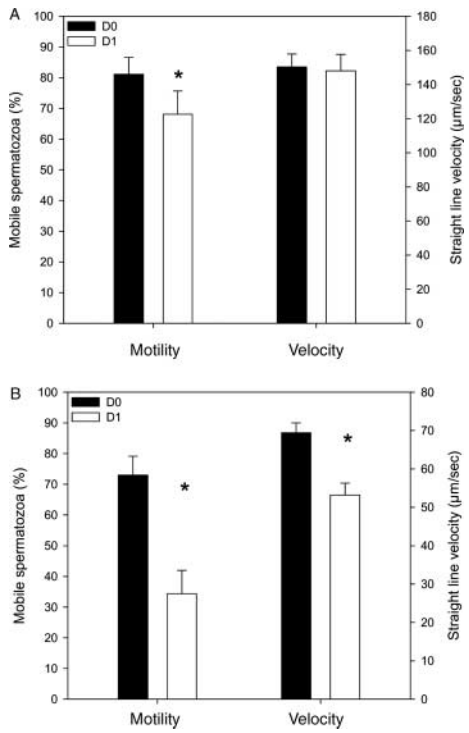

Figure 7 Effects of storage for 24 h at 15 °C on *in vitro* and *in utero* motility of ram spermatozoa. Proportions of motile spermatozoa were determined on either the day of collection (D0) or after storage for 24 h at 15 °C (D1). *In vitro* motility and straight line velocity were measured by CASA (A) and *in vivo* motility and straight line velocity were measured at the utero-tubal junction 4 h after intra-uterine insemination using video recording with fibered confocal fluorescence microscopy (B). Data are expressed as means±S.E.M. Asterisks indicate significant differences ($P<0.05$) between D0 and D1.

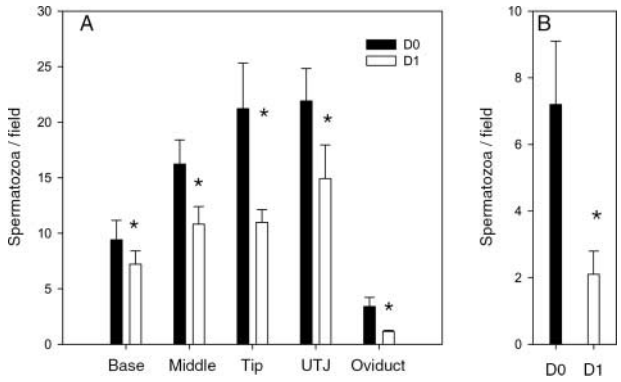

Figure 8 Effects of storing ram spermatozoa for 24 h at 15 °C on their transit in the genital tract of the ewe. (Panel A) Sperm concentrations were estimated in different regions of the genital tract (base, middle and tip of the uterine horn, the utero-tubal junction (UTJ) and the oviduct) using fibered confocal fluorescence microscopy 4 h after intra-uterine insemination at the base of each uterine horn. (Panel B) Sperm concentrations were determined in the body of the uterus 4 h after cervical insemination. Data are expressed as means ± S.E.M. Asterisks indicate significant differences ($P<0.05$) between D0 and D1.

advantage because it helped to define the lumen of the uterus and the spatial distribution of the spermatozoa in the uterus. By contrast, no autofluorescence was observed in the oviduct, suggesting differences in composition or metabolism between the epithelium of the uterus and oviduct.

Most free spermatozoa were found in the lumen of the genital tract. Binding of spermatozoa to the surface epithelium of the uterus was observed only rarely and then only for immotile spermatozoa. The quantification of spermatozoa in the different regions of the female genital tract 4 h after insemination in the base of each uterine horn revealed an increasing concentration gradient of spermatozoa from the base of the uterus to the UTJ. Given that the spermatozoa were deposited at the base of each uterine horn, we expected to observe a greater concentration of spermatozoa in this region. Surprisingly, few spermatozoa were found at the site of insemination, suggesting that spermatozoa that did not, or could not, migrate toward the oviduct were quickly flushed into the cervix and discarded, presumably by descending uterine contractions (Toutain *et al*. 1985).

Ram spermatozoa were found in the base of the uterine horn 4 h after cervical insemination and in the oviduct 4 h after intra-uterine insemination into the base of the each horn. This pattern of distribution over time agrees with previous studies that have shown that the migration of spermatozoa from the vagina to the oviduct requires about 8–10 h (Hunter *et al*. 1980).

The UTJ clearly appeared to be a barrier because the concentration of spermatozoa was lower in the oviduct than in the UTJ. Contractions were less frequent at the UTJ compared to the other regions of the uterus. Contractions were so frequent in the uterus that the individual motility of spermatozoa could not be precisely estimated. However, because the rhythm of contractions was slower in the UTJ, motility of individual spermatozoa could be assessed. Spermatozoa showed linear trajectories, with a motility pattern similar to the progressive motility observed *in vitro*. Quantification showed that the *in situ* motility of spermatozoa in the UTJ 4 h after intra-uterine insemination was significantly reduced by *in vitro* preservation for 24 h, with an overall decrease in the percentage of motile spermatozoa. This considerable decline in *in situ* motility contrasts strikingly and importantly with the small decline in *in vitro* motility for stored spermatozoa. Fertility after cervical insemination was markedly reduced after storage for 24 h at 15 °C or after freezing and thawing (Salamon & Maxwell 2000). However, fertility after intra-uterine insemination with stored or frozen and thawed semen that 'by-passed' the cervix was not reduced, suggesting that the cervix is a barrier limiting the success of fertilization when using stored or frozen and thawed spermatozoa. Indeed, the *in vivo* observation of spermatozoa, in this study, demonstrated that the ability of spermatozoa to traverse the cervix was markedly reduced after semen storage for 24 h. *In vitro* preservation of semen therefore decreased the ability of its spermatozoa to traverse the cervix, and decreased their intra-uterine motility and their ability to migrate to the oviduct. The available *in vitro* methods of measurement of semen quality such as motility and IVF do not satisfactorily account for the loss of fertility of ram spermatozoa following their preservation (Sanchez-Partida *et al*. 1999, Papadopoulos *et al*. 2005, O'Meara *et al*. 2008). Indeed, the *in vitro* parameters of semen quality have been reported to be little modified by *in vitro* storage for 24 h at 15 °C, whereas the loss of *in vivo* function of these spermatozoa as assessed in this study was of a similar magnitude to the loss of fertility seen in other studies (Maxwell & Salamon 1993). This suggests that storage and preservation of spermatozoa resulted in a proportionally much greater change in their *in utero* and cervical physiology in the female genital tract.

The preliminary images of *in vitro* motility observed with FCM suggest its potential for the study of sperm motility in a three dimensional environment. This could help overcome the drawbacks to the study of sperm motility induced by their restriction between slide and coverslip and hence refine the *in vitro* analysis of sperm motility. Studies could be performed *in situ* in the tube without interaction with the spermatozoa. However, this requires the development of new analytical methods to determine the parameters of motility in these new conditions.

This study was performed in ewes but the method of sperm imaging we have developed could be applied to other species. We have used the same procedure to label boar spermatozoa, to perform cervical insemination in sows and to observe spermatozoa in different regions of

the genital tract of the anesthetized sow (preliminary findings). Interestingly, boar spermatozoa could be found in the UTJ at times ranging from 15 min to at least 18 h after insemination. Moreover, a pregnancy of normal litter size was achieved after *in vivo* imaging of fluorochrome-labeled spermatozoa, suggesting that the fertilizing ability of spermatozoa from boars is not compromised by this technique. If this proves to be correct after more extensive investigation in sows and the females of other species, then sperm transit and survival in the female genital tract could be studied in various physiological conditions over extended periods of time after insemination. In other species such as cattle where the optical probe could be non-surgically inserted into the cervix or the uterus in the unanesthetized, standing cow, sperm transport could be studied over time in the same animal.

This study highlights the need for further investigation of the role of the cervix in the physiological functions of spermatozoa in the genital tract of the ewe and as a determinant of fertility following AI with semen that has been chilled and stored with a preservative or frozen and thawed. The method we have developed for the observation of spermatozoa in the female genital tract will help to understand the physiological processes involved in sperm transit and survival.

Materials and Methods

Reagents and media

Octadecyl rhodamine B chloride (R18, O-246) and MitoTracker Green FM (M-7514) were purchased from Molecular Probes (Eugene, OR, USA). Stock solutions of R18 and MitoTracker Green FM (30 and 2 mM respectively) were prepared by re-suspension of the dried products in DMSO and stored in aliquots at −20 °C. All other chemicals were of reagent grade and purchased from Sigma.

Rams and semen collection

Semen from three adult Lacaune rams was collected using an artificial vagina. The ejaculates were pooled and placed into a water bath (35 °C) immediately after collection. Mass motility of undiluted semen was assessed by wave motion using phase contrast microscopy (100×) and graded (scale 0–5). Sperm concentration was estimated using a spectrophotometer. Semen was used when the mass motility score was over 4 and the sperm concentration was at least 3×10^9 sperm/ml.

Labeling of spermatozoa and storage of semen

The semen was diluted in a 15 ml tube to a final concentration of 1×10^6 spermatozoa/ml in a warm (35 °C) milk-based extender prepared from skimmed milk powder (11% w/v) and distilled water, heated to 95 °C for 10 min and supplemented with gentamycin (50 μg/ml). The diluted semen was divided into two aliquots. One aliquot was supplemented with 1% (v/v) stock solution of R18 and MitoTracker Green FM (to final concentrations of 300 and 20 μM respectively) and incubated for 10 min at 37 °C just before insemination (Day 0 (D0) semen). The fluorochrome MitoTracker Green FM labels the mitochondria of live spermatozoa only, whereas R18 labels the surface of live and dead spermatozoa (see results). The other aliquot was allowed to cool to 15 °C in an incubator for storage for 24 h at 15 °C. After 24 h, the stored semen was also labeled using R18 and MitoTracker Green FM prior to insemination (Day1 (D1) semen) as described above.

Flow cytometry assessment

The intensity of labeling of D0 and D1 spermatozoa with fluorescent probes was measured with an EPICS-XL flow cytometer (Beckman-Coulter, Villepinte, France) using an air-cooled argon laser operating at 488 nm and 15 mW. Aliquots (10 μl) of D0 and D1 spermatozoa labeled with the R18 and MitoTracker Green FM fluorochromes, as described above, were diluted in 1 ml PBS with BSA (1mg/ml) and incubated at 37 °C for 10 min. Forward scattering and side scattering were recorded to define the size characteristics and provide gating parameters for selection of the sperm population. Green fluorescence (MitoTracker Green FM) and orange fluorescence (R18) were then quantified for a minimum of 10^4 cells for each analysis.

Cervical and intra-uterine insemination

Estrus was synchronized in 24 Romanov×Ile de France ewes with progestagen-impregnated intravaginal sponges containing 30 mg fluorogestone acetate. The sponges were removed after 13 days and the ewes were injected i.m. with 500 IU pregnant mare serum gonadotrophin.

Cervical and intra-uterine insemination with D0 and D1 spermatozoa was performed using standard techniques 55 h after removal of sponges (Colas 1984). Immediately after the spermatozoa had been labeled, mini straws (0.25 ml, IMV Technologies, L'Aigle, France) were filled with 250×10^6 or 150×10^6 spermatozoa and used for cervical or intra-uterine insemination respectively. Cervical insemination was performed on 12 ewes (six ewes per treatment with D0 and D1 semen) using two straws per ewe, resulting in an insemination dose of 500×10^6 spermatozoa per ewe. Semen was deposited as near as possible to the cervix. Intra-uterine insemination was performed using standard laparoscopic techniques (Colas 1984) except that the semen was placed at the base of each horn. Half of one straw was used for each horn, resulting in a dose of 75×10^6 spermatozoa per horn or 150×10^6 spermatozoa per ewe. Twelve ewes were used; six ewes per treatment (D0 and D1 semen).

Fibered confocal fluorescence microscopy

FCM, or FCM1000 (Leica Microsystems, Wetzlar, Germany) is based on Cellvizio technology (Mauna Kea Technologies, Paris, France), which was designed for *in vivo* imaging. FCM makes use of a laser scanning unit ($\lambda=488$ nm) associated with a range of miniaturized fiber-based optical microprobes, with

different tip diameters (300 μm to 4.2 mm) and lateral resolutions (1.4–3.3 μm). A dedicated image acquisition and post processing software drives the scanning unit and enables a real-time (12 frames/s) reconstruction of the images delivered by the bundle of optical fibers. Sperm quantification in the female genital tract was performed with the S1500 microprobe (1.5 mm ∅, 3.3 μm lateral resolution, 500×600 μm field of view), thanks to its size compatibility with the lumen of the uterus and the oviducts and its capability to record dynamic images of the motile spermatozoa. High resolution *in vitro* and *in vivo* images of individual spermatozoa were also acquired with the M microprobe (4.2 mm tip ∅, 1.4 μm lateral resolution).

In vivo *imaging of sperm*

The transit of spermatozoa in the female genital tract was quantified by *in vivo* imaging 4 h after insemination. Ewes were placed under general anesthesia and the genital tract (uterine horns, oviducts and ovary) exteriorized after laparotomy. In order to insert the 1.5 mm microprobe into the lumen of the genital tract, 2 mm openings were made through the wall of the genital tract using electrocautery at different levels; i.e. the uterine body near the cervix, the base of the uterine horn and the tip of the uterine horn, near the UTJ.The probe was carefully inserted through these different openings and directed towards the region being analyzed; i.e. the cervix, the base, middle and tip of the uterine horn, the UTJ and the isthmus of the oviduct. Image acquisition was standardized and video sequences of 2 min were recorded for the various regions of the genital tract. Data were acquired for both ipsi and contralateral horns of each ewe. Videos are provided as Supplementary Material (Videos (SV1–4)).

Analysis of in vivo *data*

Quantification was performed using the software provided with the imaging system as follows: 10 frames were selected from each video sequence and the number of spermatozoa in each frame was determined by manual counting. *In vivo* fluorescence of spermatozoa was also quantified using the software. Each of the 12 000 individual optical fibers in the miniprobe provided a single intense fluorescence. The area of a single spermatozoon was covered by a mean of 10 and 30 optical fibers for the 1.5 and 4 mm miniprobes respectively. The mean fluorescence of individual spermatozoa was then computed. Fluorescence was compared using a total of 100 D0 and D1 spermatozoa from the UTJ.

Assessment of motility

In vitro motility of D0 and D1 spermatozoa was assessed after labeling with R18/MitoTracker Green FM using a computer-assisted analysis system (Hamilton-Thorn, Beverly, MA, USA). A 10 μl aliquot of labeled semen was diluted in 1 ml PBS with BSA (1 mg/ml) and incubated at 37 °C for 10 min. Two 10 μl drops were then transferred to a preheated slide and four fields per drop were analyzed to determine the proportion and VSL of motile spermatozoa.

In vivo motility was calculated from video sequences acquired at the UTJ. Examination of 20 successive positions of the sperm head allowed motile spermatozoa to be distinguished from non-motile spermatozoa so that the proportion of motile spermatozoa could be calculated for a minimum of 100 spermatozoa. Trajectories of spermatozoa were drawn from 20 successive positions of the head. The straight line distance between the first and the last point was measured using the scale of the images provided by the IC-viewer software. Given the rate of acquisition of 12 images per second, two successive positions were separated by 83 ms. The VSL was calculated by dividing the straight line distance by the time elapsed between the first and the last points.

Statistical analyses

Statistical analyses were carried out using SAS software (SAS Institute, Cary, NC, USA). The data for *in vitro* and *in vivo* motility were analyzed by ANOVA using the GLM procedure to determine the effects of semen storage on these parameters. The effects of semen storage on sperm transit in the female genital tract were estimated using the generalized estimated equation method in the GENMOD procedure. As the number of spermatozoa per field followed a Poisson distribution, a log link between the number of spermatozoa and the different factors of the model was used. The model was:

$$g(y_{ij}) = b_i + c_j$$

where g is the log function and y_{ij} is the mean number of spermatozoa per field associated with the method of semen preservation (i), and the region of the female genital tract (j). Thus, b_i is the fixed effect of the method of semen preservation (D0 or D1) and c_j is the fixed effect of the region of the female genital tract (base, middle and tip of the horn, the UTJ, and the oviduct). Ewes were included in the model as a repeated measure.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by funds from the PHASE department of INRA.

Acknowledgements

The authors wish to thank Mauna Kea Technology and Leica for providing the FCM1000 system, Guillaume Tsikis for technical assistance with the evaluation of spermatozoa, Christelle Hennequet for help with the statistical analyses, Dr Rex Scaramuzzi for critical review of the manuscript and Doreen Raine for editing the English text.

References

Balogh L, Szasz F, Zoldag L, Huszenicza G, Toth L, Dabasi G & Janoki G 1995 *In vitro* and *in vivo* motility studies of 99mTc HM-PAO labelled sperm cells. *Acta Veterinaria Hungarica* **43** 63–77.

Bockisch A 1993 Sperm cell dynamics in the female rabbit genital tract after insemination monitored by radiolabeled spermatozoa. *Journal of Nuclear Medicine* **34** 1134–1139.

Brookner CK, Follen M, Boiko I, Galvan J, Thomsen S, Malpica A, Suzuki S, Lotan R & Richards-Kortum R 2000 Autofluorescence patterns in short-term cultures of normal cervical tissue. *Photochemistry and Photobiology* **71** 730–736.

Bruckner G, Kampfer I, Rummer HJ, Menger H & Schneider G 1982 Use of ^{131}I labeling for studying the distribution and migration of spermatozoa in the genital tract of female sheep following artificial insemination. 1. Gamma camera studies of the course of migration. *Archiv für Experimentelle Veterinärmedizin* **36** 297–305.

Cavaco-Gonçalves S, Marques CC, Horta AEM & Figueroa JP 2006 Increased cervical electrical activity during oestrus in progestagen treated ewes: possible role in sperm transport. *Animal Reproduction Science* **93** 360–365.

Colas G 1984 Semen technology in the ram. In *The Male in Farm Animal Reproduction*, pp 219–236. Ed. M Courot. Dordrecht: Martinus Nijhoff Publishers.

Drezek RA, Richards-Kortum R, Brewer MA, Feld MS, Pitris C, Ferenczy A, Faupel ML & Follen M 2003 Optical imaging of the cervix. *Cancer* **98** 2015–2027.

Fair S, Hanrahan JP, O'Meara CM, Duffy P, Rizos D, Wade M, Donovan A, Boland MP, Lonergan P & Evans ACO 2005 Differences between Belclare and Suffolk ewes in fertilization rate, embryo quality and accessory sperm number after cervical or laparoscopic artificial insemination. *Theriogenology* **63** 1995–2005.

Garner D, Thomas C, Joerg H, DeJarnette J & Marshall C 1997 Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. *Biology of Reproduction* **57** 1401–1406.

Gillan L & Maxwell WM 1999 The functional integrity and fate of cryopreserved ram spermatozoa in the female tract. *Journal of Reproduction and Fertility Supplement* **54** 271–283.

Hawk HW 1983 Sperm survival and transport in the female reproductive tract. *Journal of Dairy Science* **66** 2645–2660.

Hawk HW & Conley HH 1975 Involvement of the cervix in sperm transport failures in the reproductive tract of the ewe. *Biology of Reproduction* **13** 322–328.

He L, Bailey JL & Buhr MM 2001 Incorporating lipids into boar sperm decreases chilling sensitivity but not capacitation potential. *Biology of Reproduction* **64** 69–79.

Hunter RH 2005 The fallopian tubes in domestic mammals: how vital is their physiological activity? *Reproduction, Nutrition, Development* **45** 281–290.

Hunter RH, Nichol R & Crabtree SM 1980 Transport of spermatozoa in the ewe: timing of the establishment of a functional population in the oviduct. *Reproduction, Nutrition, Development* **20** 1869–1875.

Kaabi M, Alvarez M, Anel E, Chamorro CA, Boixo JC, de Paz P & Anel L 2006 Influence of breed and age on morphometry and depth of inseminating catheter penetration in the ewe cervix: a postmortem study. *Theriogenology* **66** 1876–1883.

Kadanali S, Varoglu E, Komec D & Uslu H 2001 Evaluation of active and passive transport mechanisms in genital tracts of IUD-bearing women with radionuclide hysterosalpingoscintigraphy. *Contraception* **63** 41–45.

Katz DF & Yanagimachi R 1980 Movement characteristics of hamster spermatozoa within the oviduct. *Biology of Reproduction* **22** 759–764.

Kershaw CM, Khalid M, McGowan MR, Ingram K, Leethongdee S, Wax G & Scaramuzzi RJ 2005 The anatomy of the sheep cervix and its influence on the transcervical passage of an inseminating pipette into the uterine lumen. *Theriogenology* **64** 1225–1235.

Laemmel E, Genet M, Le Goualher G, Perchant A, Le Gargasson JF & Vicaut E 2004 Fibered confocal fluorescence microscopy (Cell-viZio) facilitates extended imaging in the field of microcirculation. A comparison with intravital microscopy. *Journal of Vascular Research* **41** 400–411.

Lightfoot RJ & Restall BJ 1971 Effects of site of insemination, sperm motility and genital tract contractions on transport of spermatozoa in the ewe. *Journal of Reproduction and Fertility* **26** 1–13.

Maxwell WM & Salamon S 1993 Liquid storage of ram semen: a review. *Reproduction, Fertility, and Development* **5** 613–638.

O'Meara CM, Hanrahan JP, Prathalingam NS, Owen JS, Donovan A, Fair S, Ward F, Wade M, Evans ACO & Lonergan P 2008 Relationship between *in vitro* sperm functional tests and *in vivo* fertility of rams following cervical artificial insemination of ewes with frozen–thawed semen. *Theriogenology* **69** 513–522.

Özgür K, Yildiz A, Üner M, Erkiliç M, Trak B & Erman O 1997 Radionuclide hysterosalpingography with radiolabeled spermatozoa. *Fertility and Sterility* **67** 751–755.

Palmerini C, Cametti C, Sennato S, Gaudino D, Carlini E, Bordi F & Arienti G 2006 Role of cholesterol, DOTAP, and DPPC in prostasome/spermatozoa interaction and fusion. *Journal of Membrane Biology* **211** 185–190.

Papadopoulos S, Hanrahan JP, Donovan A, Duffy P, Boland MP & Lonergan P 2005 *In vitro* fertilization as a predictor of fertility from cervical insemination of sheep. *Theriogenology* **63** 150–159.

Rodriguez-Martinez H, Saravia F, Wallgren M, Tienthai P, Johannisson A, Vazquez JM, Martinez E, Roca J, Sanz L & Calvete JJ 2005 Boar spermatozoa in the oviduct. *Theriogenology* **63** 514–535.

Salamon S & Maxwell WMC 2000 Storage of ram semen. *Animal Reproduction Science* **62** 77–111.

Sanchez-Partida LG, Windsor DP, Eppleston J, Setchell BP & Maxwell WM 1999 Fertility and its relationship to motility characteristics of spermatozoa in ewes after cervical, transcervical, and intrauterine insemination with frozen–thawed ram semen. *Journal of Andrology* **20** 280–288.

Scott MA 2000 A glimpse at sperm function *in vivo*: sperm transport and epithelial interaction in the female reproductive tract. *Animal Reproduction Science* **60–61** 337–348.

Suarez SS 1987 Sperm transport and motility in the mouse oviduct: observations *in situ*. *Biology of Reproduction* **36** 203–210.

Suarez SS & Pacey AA 2006 Sperm transport in the female reproductive tract. *Human Reproduction Update* **12** 23–37.

Sutovsky P, Navara CS & Schatten G 1996 Fate of the sperm mitochondria, and the incorporation, conversion, and disassembly of the sperm tail structures during bovine fertilization. *Biology of Reproduction* **55** 1195–1205.

Thiberville L, Moreno-Swirc S, Vercauteren T, Peltier E, Cave C & Bourg Heckly G 2007 *In vivo* imaging of the bronchial wall microstructure using fibered confocal fluorescence microscopy. *American Journal of Respiratory and Critical Care Medicine* **175** 22–31.

Toutain PL, Marnet PG, Laurentie MP, Garcia-Villar R & Ruckebusch Y 1985 Direction of uterine contractions during estrus in ewes: a reevaluation. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **249** R410–R416.

Wergin WP 1985 Interactions between spermatozoa and the crypts, cilia, and mucus of the cervix in the ewe. *Scanning Electron Microscopy* **3** 1191–1199.

Received 24 March 2009
First decision 15 April 2009
Accepted 7 May 2009