Transport of spermatozoa in the ewe : timing of the establishment of a functional population in the oviduct

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Summary. The technique of post-coital transection of the oviducts from the uterus at various intervals after mating has been used to establish how soon a population of spermatozoa competent to fertilise the egg(s) first appears in the oviduct of the ewe. Fertilised eggs were not found as a sequel to transection at 4 or 6 hrs after mating, whereas the incidence of fertilisation in a small series of ewes was 30 p. 100 following transection at 8 hrs and 100 p. 100 at 10 hrs. The mean number of spermatozoa associated with the zona pellucida at these later times increased from 2 to 13.4. The results are interpreted as indicating only a gradual progression of viable spermatozoa from the cervix to the oviduct, although the possible contribution of a rapid phase of sperm transport to the events of fertilisation is discussed. Finally, the isthmus rather than the cervix is reasoned to act as the functional sperm reservoir at the time of ovulation, with some form of peri-ovulatory programming of sperm release from this region of the tract.

Introduction.

Ovulation generally occurs some 24-30 hrs after the onset of œstrus in sheep. Since mating could therefore take place many hours before release of the egg from the Graafian follicle, it is reasonable to expect one or more sites of sperm storage in the female reproductive tract. The cervix is usually stated to be the site of formation of the primary sperm reservoir in the ewe (Quinlan, Maré and Roux, 1933), with some emphasis being placed on the condition of its mucous secretion in successfully fulfilling this rôle (Mattner, 1963, 1966 ; Raynaud, 1973 ; Robinson, 1973). In support of this contention, diminished fertility after treating sheep with progestagens for synchronisation of œstrus is attributed in part to an altered condition of the cervical mucus and thus the failure to establish or maintain adequate reserves of spermatozoa (see Robinson, 1967, 1973 ; Quinlivan and Robinson, 1969 ; Hawk and Conley, 1972 ; Allison, 1975).

Despite this suggested rôle of the cervix as a major sperm reservoir, there is a related and persistent controversy over the rate of transport of spermatozoa to the oviducts of unstressed, spontaneously mating ewes. In several studies, a phase of extremely rapid transport has been noted with spermatozoa apparently arriving in the upper oviducts within 6-30 min of coitus (Schott and Phillips, 1941 ; Starke, 1949 ;

Mattner, 1963 ; Mattner and Braden, 1963) or artificial insemination (Phillips and Andrews, 1937). Although these reports were not in agreement with the results of Green and Winters (1935), Dauzier and Wintenberger (1952) and Dauzier (1958), nor could they be substantiated by the later studies of Thibault and Wintenberger-Torres (1967), they do raise the important question as to whether the vanguard spermatozoa reaching the oviduct are those that participate in the actual process of fertilisation. In the pilot study described below, the technique of post-coital separation of the oviducts from the uterus has been used to isolate the cellular contents of the oviducts at various intervals after mating. In this manner, preliminary conclusions have been drawn as to how soon after mating a population of spermatozoa competent to ensure fertilisation might have entered the oviducts. This general approach has been used previously in a small number of sheep (Dauzier, 1958), but ligation of the oviduct was not used to judge when a population of fertilising spermatozoa first becomes established at that level. Rather, it was used to emphasize that very rapid sperm transport normally did not occur or was not associated with subsequent fertilisation.

Materials and methods.

Animals. — Twenty-six Scottish Blackface ewes and two Suffolk × Border-Leicester crosses aged 6-9 years and weighing 43-68 kg were used in this experiment. They had all lambed successfully in previous seasons and were representative of animals in the School of Agriculture flock. In late December 1979, they were assembled and housed as a single group in open-fronted covered yards, and fed on « hay cubes » supplemented with concentrates. A vasectomized ram was introduced twice daily to check for the occurrence of œstrus. Hormonal treatments for regulation of the œstrous cycle were not applied.

Mating procedure. — Ewes were assigned at random to one of four experimental groups based on the time of surgical intervention after mating, the intervals being 4, 6, 8 or 10 hrs $\pm$ 12 min (see table 1). All matings were made by two mature stud rams of proven fertility, one being a purebred Blackface, the other a purebred Suffolk. Ewes were usually mated twice, once by each ram, although on occasions a third or fourth mating occurred. Matings were performed between 08.00-09.00 hrs and, in order to avoid the effects of prolonged stress, mated ewes were left housed with the original group until 15 min before operation and 1-2 min before commencing anaesthesia. Because of this situation, neither starving nor pre-operative sedation were practised.

Surgical intervention. — Animals were moved from the covered yard into the neighbouring surgical building, and were immediately subjected to halothane-nitrous oxide-oxygen anaesthesia administered by means of a face mask. They were normally sufficiently relaxed within 2-3 min of commencement of this procedure to enable abdominal shearing. They were next placed on the operating table, the skin washed and sterilised, and endotracheal intubation achieved in 26 of the 28 animals. Using aseptic procedures, the reproductive tract was exposed through a mid-ventral laparotomy and the ovaries briefly examined for mature Graafian follicles. Double liga-

tures of No. 2.5 gauge metric braided silk (Ethicon Ltd) were placed 1-2 mm caudal to the utero-tubal junction on the side of the tract with the largest ovarian follicle, and the tissue transected between the ligatures. Care was taken to avoid major blood vessels during positioning of the ligatures, which involved their placement very close to or in the wall of the utero-tubal junction. In five animals in which a mature Graafian follicle was conspicuous on each ovary, the ligatures were positioned on and then removed from the second oviduct as a control procedure. Handling of the reproductive tissues was restricted to the minimum necessary to complete these manipulations, after which the abdominal wall was closed in two layers with interrupted sutures of No. 4 gauge braided silk (Ethicon Ltd). Animals were placed in individual recovery pens in the post-operative room adjoining the surgery.

Procedure at autopsy. — Ewes were killed at the Edinburgh City Abattoir one to three days after mating, usually on the second day, and the reproductive tract returned to the laboratory within 20 min of evisceration. Smears were prepared from the cervical and/or uterine contents to confirm the presence of spermatozoa. The oviducts were dissected free of their supporting ligaments, the ligatures removed, and the luminal contents flushed downwards from the fimbriated extremity with Eagle's medium (Flow Laboratories). The flushings were collected in plastic Petri dishes, examined under a dissecting microscope, and whole mounts made of recovered eggs by the method of Chang (1952). The eggs were fixed in 25 p. 100 acetic-alcohol for 24-48 hrs, stained with 1 p. 100 orcein in 45 p. 100 acetic acid, and examined by phase-contrast microscopy. Counts were made of the number of spermatozoa attached to or embedded in the zona pellucida as this membrane slowly dissolved upon addition of the 45 p. 100 acetic acid, and nuclear structures in the eggs were then examined in detail.

The experiment was completed in the first week of February, 1980.

Results.

No anatomical abnormalities of the reproductive tracts were observed at autopsy, but one tract was found to have pyometritis and the tubal flushings were therefore discarded. Spermatozoa were detected in the cervical or uterine smears of all animals, although a high proportion of the cells showed detachment of the sperm head from the tail or were in the process of being phagocytosed. The results from 27 animals are summarised in table 1. On the basis of the number of developing corpora lutea, 34 of the 36 eggs expected were recovered from the ligated and sectioned oviducts and all were denuded of any investment of granulosa cells. Five eggs were flushed from oviducts that had been treated as controls.

Fertilised eggs were recovered from the transected tubes of 9 animals, but no instance of fertilisation was observed in eggs recovered from animals operated upon at 4 or 6 hrs *post coitum*, nor were spermatozoa found in association with eggs from the transected oviduct in such animals. However, fertilised eggs were recovered from the control oviduct of two animals in each of these two groups. A low incidence of fertilisation (30 p. 100) was found following transection at 8 hrs, whereas all eight eggs recovered from tubes isolated at 10 hrs *post coitum* were fertilised (table 1).

TABLE 1

Summary of results from 27 ewes to show the proportion of animals yielding fertilised eggs and the incidence of fertilisation when the interval from mating to transection at the utero-tubal junction increased from 4 to 10 hrs ($\pm$ 12 min).

Interval from mating to transection of oviduct (hrs)	No. of animals yielding		Eggs recovered from ligated and sectioned tubes		Eggs fertilised		No. of spermatozoa per egg	
	eggs	fertilised eggs	potential No. ([1])	actual No.	No.	p. 100	range	mean
4	8	0	9	9	0 ([2])	0	0	0
6	6	0	8	7	0 ([2])	0	0	0
8	7	3	11	10	3 ([3])	30	1-14	2
10	6	6	8	8	8	100	1-47	13.4
Total.........	27	9	36	34	11	33	0-47	—

([1]) The number of developing corpora lutea was equated with the number of eggs shed.
([2]) 2 additional eggs recovered from control oviducts were fertilised.
([3]) 1 additional egg recovered from a control oviduct was fertilised.

The stages of development of the fertilised eggs ranged from 2- to 8-cells, and all appeared to be normal when examined after staining. The number of spermatozoa associated with the zona pellucida of these eggs was generally small (see table 1), but there was a clear increase in the mean number between the 8 and 10 hrs groups. Fragmentation of the cytoplasm was not observed in the unfertilised eggs although signs of degeneration could usually be detected, especially in the arrangement of chromosomes on the second meiotic spindle.

Discussion.

The results of this preliminary study suggest that a population of spermatozoa competent to promote fertilisation in the ewe is first found in the oviducts approximately eight hours after mating during a spontaneous oestrus. This is not to infer that, in a larger study, some fertilised eggs would not have been found as a sequel to post-coital transection at six hours, nor that fertilisation would always follow separation of the oviducts and uterus at ten hours after mating. Nonetheless, the most straightforward interpretation of the results is one of a gradual progression of viable spermatozoa from the uterus into the isthmus of the oviduct and, as judged from counts of spermatozoa attached to the zona pellucida, this process would not have achieved functional significance in the first four to six hours after mating. These views are therefore in accord with the observations of Dauzier and Wintenberger (1952), Dauzier (1958) and Lang and Oh (1970). The present interpretations are of course offered on the basis of techniques involving surgical intervention and the placement of ligatures on the reproductive tract, procedures which could undoubtedly modify the physiological functions of the oviduct both in terms of the pattern of muscular contractions and in the nature of the tubal secretions. But even with these reservations in mind — which must clearly apply to all four experimental groups — the results provide a useful

pointer to the timetable of events in the first hours after mating. Furthermore, the transection procedure itself would not be expected to alter the number of spermatozoa already in the isthmus and, in addition, all four control oviducts manipulated at four and six hours after mating yielded fertilised eggs.

If an interval of eight to ten hours needs to elapse after mating before a functional population of spermatozoa is established in the oviducts, then this situation must cast some doubt on the rôle of a rapid phase of sperm transport in the events of fertilisation or, alternatively, raise some interesting and provocative questions. Rather than become involved in a controversy over the extent to which technical artifacts may underlie reports of sperm transport to the upper oviducts in intervals as short as 6-30 min after mating of ewes (see Schott and Phillips, 1941 ; Starke, 1949 ; Mattner, 1963 ; Mattner and Braden, 1963), it would seem more fruitful to ask what functions might be served by a putative phase of rapid transport — if it is accepted in the light of the present observations that such vanguard spermatozoa do not participate directly in the fertilisation process. A phase of rapid transport from the anterior vagina to the upper region of the oviducts has also been reported in rabbits with spermatozoa arriving within minutes of coitus, but such cells were predominantly dead, moribund or immotile (Overstreet and Cooper, 1978). In a recent review of the subject (Hunter, 1980a), two possible rôles have been proposed for these vanguard spermatozoa, and these may also apply in the case of the ewe. First, dead or dying sperm cells could release products into the tubal lumen that interact with the epithelium and the later-arriving sperm and eggs to facilitate maturational changes in the gametes, or perhaps they function to sensitise the peritoneal phagocytosis system for the subsequent arrival of greater numbers of motile spermatozoa. These suggestions are amenable to further experimentation, and are currently being examined in our laboratory.

Whilst quantitative aspects of the relationship between depletion of the cervical sperm reservoir and establishment of a secondary sperm reservoir in the isthmus could be discussed at length (see Quinlan et al., 1933), observations on this relationship in the ewe have been analysed by Quinlivan and Robinson (1969) and Robinson (1973). Our own studies are less concerned with sperm transport from the site of ejaculation and more interested in the function of the oviducts themselves in the peri-ovulatory interval in farm animals. In particular, we are studying the possibility that ovarian secretions may regulate the activation and release of a population of spermatozoa that has arrived in the isthmus earlier in the period of oestrus ; there is good evidence for such control in the rabbit oviduct (Harper, 1973 ; Overstreet and Cooper, 1975, 1978). Moreover, it could be argued that since ewes would normally mate 24-30 hrs before ovulation, and because there is a functional population of spermatozoa already in the isthmus by 10 hrs after mating, then any peri-ovulatory programming of sperm movement would be exerted on the reservoir in the isthmus and not on those reserves in the cervix or uterus. Although Quinlan et al. (1933) reported a relatively short lifespan for ejaculated ram spermatozoa within the environment of the oviducts, their observations were unphysiological in that aliquots of whole semen contaminated with vaginal secretions were instilled directly into the ampulla of the oviducts. In any event, some form of peri-ovulatory control of sperm movement through the isthmus would provide one explanation for an accelerated rate of sperm transport close to

the time of ovulation which has been reported not only for rabbits (Braden and Austin, 1954 ; Turnbull, 1966) and hamsters (Yanagimachi and Chang, 1963) but also for sheep (Dauzier and Wintenberger, 1952) and pigs (Du Mesnil du Buisson and Dauzier, 1955 ; Hunter, 1980*b*). The case therefore is strengthened for the isthmus acting as the functional sperm reservoir at the time of ovulation.

Résumé. Pour trouver la vitesse d'établissement d'une population de spermatozoïdes capables de féconder les œufs dans l'oviducte de brebis, nous avons utilisé la technique de section des oviductes au niveau de la jonction utéro-tubaire, à des temps croissants après l'accouplement. Aucun œuf n'est fécondé quand la section est réalisée 4 ou 6 h après accouplement ; 30 p. 100 des œufs sont fécondés quand l'intervalle atteint 8 h et 100 p. 100 lorsqu'il atteint 10 h. Le nombre moyen de spermatozoïdes associés à la zone pellucide passe de 2 à 13,4 pour les intervalles 8 et 10 h. Ces résultats indiquent un passage progressif des spermatozoïdes fécondants du cervix vers l'oviducte ; la contribution éventuelle d'une phase de transport rapide des spermatozoïdes aux événements de la fécondation est discutée. En conclusion, l'isthme plutôt que le cervix semble être le réservoir des spermatozoïdes au moment de l'ovulation, avec peut-être un programme de libération des spermatozoïdes de cette région des voies génitales à la période de l'ovulation.

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