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Estudio sobre la estructura y función de la mucosa oviductal y el mesotelio peritoneal de la vaca

Memoria de Tesis presentada para la obtención del título de Doctor

por

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CAPÍTULO 5

A Scanning Electron Microscopie Study of the Peritoneal Mesothelium Covering the Genital Tract and its Ligaments in the Cow

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5 A Scanning Electron Microscopie Study of the Peritoneal Mesothelium Covering the Genital Tract and its Ligaments in the Cow

SUMMARY

The surface features of mesothelial cells of the reproductive tract and adjacent ligaments from 25 cyclic cows were examined by scanning electron microscopy and images analysis. In the external side of the infundibulum, the oviductal mucosa exceeds the free margin, forming a continuous band measuring 0.25 to 1 cm in width. This oviductal epithelium shows cyclical variations with a predominance of ciliated cells during the follicular phase. Peritoneal surfaces of the uterus and mesometrium have a higher microvilli density and length and a smaller cell surface area than in the oviduct and adjacent structures. We also observed the presence of micropores in the cell plasma membrane, of solitary cilia in the mesosalpinx and infundibulo-cornual ligament of some specimens, and of stomas in the mesosalpinx of one cow. When samples were processed without postfixation in osmium tetroxide, a layer of amorphous material covered all surfaces. No clear differences were observed associated with oestrus cycle, laterality or the side of ovarian bursa (internal vs. external). After intrauterine insemination of five cows, no spermatozoa were found on their peritoneal mesothelium. Numerous spermatozoa were found after intraperitoneal insemination being attached throughout mesothelial surfaces. These results indicate that there are regional variations but no cyclic changes in the surface features of mesothelial cells covering the genital area in the cow.

Key words: bovine, mesothelial cells, intraperitoneal insemination.

INTRODUCTION

Organs and tissues of the peritoneal cavity are lined with a continuous serous membrane, which consists of loose connective tissue covered by a single layer of mesothelial cells. The peritoneal mesothelium is involved in the formation and absorption of peritoneal fluid (Odor, 1954; Gosselin and Berndt, 1962; Tsilibary and Wissing, 1977; Li et al., 1996). It has a slippery surface which protects internal body organs from surface abrasion (Andrews and Porter, 1973), and plays an important role in the regulation of peritoneal inflamation and tissue regeneration (Oral et al., 1996; Hall et al., 1998). The functions of mesothelial cells have been associated with several superficial features. Odor (1954) suggested that their microvilli might increase surface area in order to facilitate exchange of soluble substances between cells and the body cavity. Andrews and Porter (1973) proposed that serosal microvilli, together with their glycocalyx, might harbor a layer of serous exudate and thereby create a slippery cushion designed to protect the thin mesothelium from frictional damage.

Successful intraperitoneal insemination (Hunter, 1988) and peritoneal oocyte and sperm transfer (POST) (Sharma et al., 1987; Gentry et al., 1989) reflect the participation of mesothelial cells in other processes than those described above. The characteristics of these cells may influence the efficiency of sperm transport to the oviductal lumen after intraperitoneal insemination and the pick-up process after POST. This work was undertaken to study by SEM the peritoneal mesothelium of the cow genital area during the estrus cycle. The presence of spermatozoa on the mesothelial surface was also evaluated following intrauterine or intraperitoneal insemination.

MATERIALS AND METHODS

Animals

The genital organs and adjacent ligaments from 25 mature Friesian cows were

obtained 15-30 min after slaughter in an abattoir. According to ovarian and genital tract findings and serum progesterone levels, the animals were classified to one of the following phases of the cycle: estrus (day 1), postestrus (days 2-4), diestrus (days 5-15) and proestrus (days 16-21) (Grunert, 1982). Seven animals in estrus, seven in postestrus, eight in diestrus and three in proestrus were examined.

Scanning Electron Microscopy

Tissue blocks from both the right and left sides of the genital area were prepared for scanning electron microscopy (SEM) from each cow. Tissue specimens 0.5 to 1 cm^2 were taken from the mesometrium and uterus at the uterine body level; from the mesosalpinx, infundibulo-cornual ligament and isthmus 5-6 cm proximal to the tubo-uterine junction, at the ovarian bursa level; and from the infundibulum, at a point adjacent to the free margin and another 1-2 cm distal. We studied both the internal and external surfaces of the samples taken at the isthmic level but only the dorsal surface of the remaining samples. Therefore, 16 samples were obtained from each cow.

The pieces were spread on a flat surface, and totally immersed in 2.5% glutaraldehyde (Prolabo, Fontenay S/ Bois, France) in 0.1 M phosphate buffer (pH 7.4) for 24 h (Sabatini et al., 1963). Fixed tissues were rinsed in 0.1 M phosphate buffer (pH 7.4), postfixed for 2h in 1% osmium tetroxide (Merk, Darmstadt, Germany) and washed again in phosphate buffer. Fixation and washing were carried out at 4° C and the tissues were then dehydrated in graded ethanol (25-100%) and substituted with acetone. Specimens were then subjected to critical-point drying using liquid CO_2 substitution (Anderson, 1951). The dried specimens were mounted on aluminum stubs, coated with gold in a Balzers Sputter Coater (Liechtenstein), and examined and photographed in a Zeiss DSM 940 (Oberkochen, Germany) scanning electron microscope at 15 kV. Four hundred tissue blocks were examined in this study.

Optical microscopy

Immediately after slaughter, segments adjacent to SEM samples were rapidly cut, fixed with Bouin's fixative, dehydrated and embedded in paraffin. The segments were cut into thickness of 5 μm, stained with hematoxylin and eosin, and observed and photographed with an Olympus BX50 (Tokyo, Japan) microscope.

Quantitative study

To obtain quantitative data on microvilli densities, microvilli length and measurements of cell surface area, SEM micrographs were studied using a computer image analyzer (UTHSCSA Image Tool 2.0 program, developed at the University of Texas Health Science Center at San Antonio, Texas, and available via Internet by anonymous FTP from maxcad6.uthscsa.edu). Measurements were determined for at least six different blocks from each peritoneal surface: mesometrium, uterus, mesosalpinx, infundibulo-cornual ligament, isthmus and infundibulum. Three micrographs were analyzed per tissue block. For microvilli length evaluation, values were average from 90 measurements performed per tissue block. Only horizontal and clearly distinguishable microvilli were measured. The surface area of mesothelial cells was measured in 50-75 cells per block. Statistical analysis was performed using SPSS statistical package (SPSS, 1999). Differences of microvilli density, microvilli length and cell surface area on the peritoneal surface were analyzed by general linear model and post-hoc pair-wise comparisons of means were made with Tukey's HSD. The Pearson correlation coefficient was calculated between microvilli density, microvilli length and surface area of mesothelial cells. $P<0.05$ was considered to reflect the presence of statistical significance.

Sperm transport

The presence of spermatozoa over the peritoneal surface and their relationship with mesothelial cells after intrauterine or intraperitoneal insemination was studied in

seven cows. Estrus was synchronized by a luteolytic dose of 500 mg of cloprostenol (im; Estrumate, Shering Plough Animal Health, Madrid, Spain) and 500 IU hCG plus 2 mg estradiol benzoate (im, Neonida, Smith Kline, Barcelona, Spain) 12 h later (Lopez-Gatius, 1989; Lopez-Gatius and Vega-Prieto, 1990). Estrus, detected by direct observation of animal behavior, was confirmed by palpation per rectum at the time of insemination (López-Gatius and Camon-Urgel, 1991). Cows were artificially inseminated at 12 to 24 h after onset of estrus, using semen from a bull of proven fertility packaged in 0.25 French straws. Five cows received 180 million sperm deposited deep into the uterine horns, and two cows received 45 million sperm deposited in the peritoneal cavity. For intraperitoneal insemination, semen was injected into the peritoneal cavity through the vaginal wall at the dorsal fornix level, using a sterile 19-gauge needle 4 cm long and a glass speculum 44 cm long and 10 mm in diameter and with a conical tip, as additional accessories to standard AI equipment, as described previously (López-Gatius, 1995).

After insemination, the cows were left undisturbed before being transported to a local abattoir for slaughter 24-26 h after insemination. Three animals in the intrauterine group and one in the intraperitoneal group had ovulated by the time of slaughter. The genital tracts and adjacent ligaments of the previously inseminated cows were processed by SEM, as above, and the samples were examined for sperm presence. Care was taken to avoid contamination samples with cervical mucus.

RESULTS

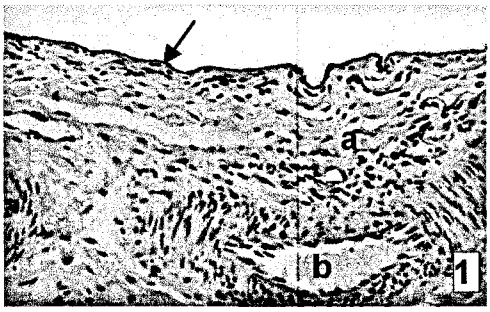
The tunica serosa covering the reproductive tract and adjacent ligaments consists of mesothelium, basal lamina and a layer of loose connective tissue. The mesothelium is a single layer of loosely attached squamous epithelial cells, either with a flattened or cuboidal appearance (fig.1,2).

Observations of the external side of the infundibulum revealed that the oviductal mucosa exceeded the free margin, forming a continuous band (fig.3). Transition between the mucosa and serosa was abrupt, and clearly distinguishable 0.25 to 1 cm from the free margin (fig.4). The oviductal epithelium forming this continuous band was densely ciliated at the follicular phase (fig 4); while the pool of ciliated cells decreased and bulbous processes of secretory cells were predominant throughout the epithelial surface in the luteal phase.

A surface film of amorphous material covered all the structures when the samples were processed without postfixation in osmium tetroxide and observed using SEM. This layer was frequently associated with red blood cells trapped beneath the dark material (fig.5). Postfixation with osmium tetroxide cleaned this smooth layer and enabled appreciation of the mesothelial cell surface, which was provided with microvilli (fig 6).

Anatomical findings revealed closely comparable structures covering both the serosal surface of the reproductive tract and the adjacent ligaments. Similarly, there were no clear differences attributable to the estrus cycle, laterality or the side of the ovarian bursa (internal vs. external).

The mesothelial cell surface was covered by numerous microvilli of different length (range 0.5 to 3.9 μm) and diameter (range 0.15 to 0.25 μm). The number of microvilli in 10 μm^2 of surface area ranged between 24 and 113. A direct correlation was found between microvilli density and length ($r = 0.49$; $P<0.001$). The surface area of cells ranged between 36 and 324 μm^2. An inverse correlation was found between microvilli density and cell surface area ($r = - 0.57$; $P<0.001$) and between the length of microvilli and cell surface area ($r = - 0.25$; $P<0.001$). The average number of microvilli in a 10 μm^2 surface area, microvilli length and cell surface area all varied among different peritoneal areas, so that on the mesothelial surfaces of the uterus and mesometrium microvilli were larger and more numerous, and serosal cell surface area was smaller than of the oviduct and adjacent structures (Table 1).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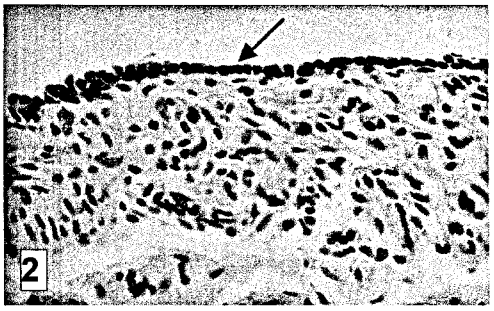
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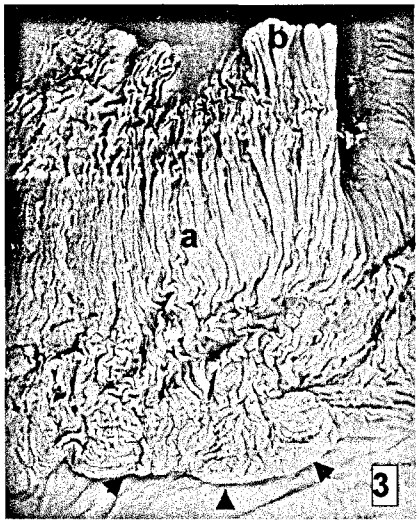
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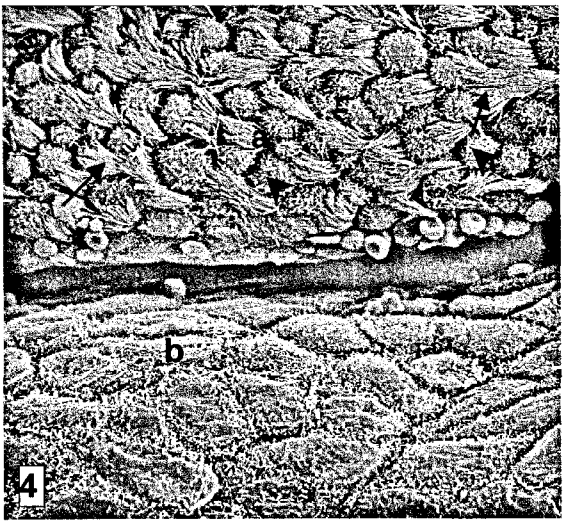
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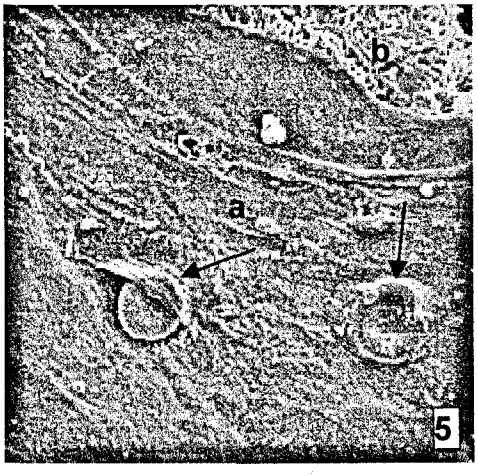
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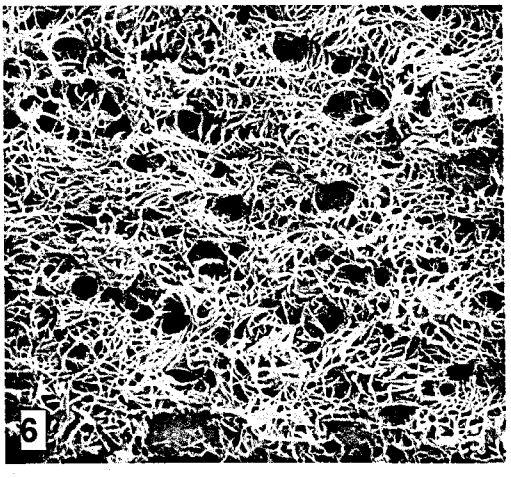
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Fig. 1. Cross section from the isthmus. The serosa contains loose connective tissue (a) and a large blood vessel (b). The mesothelium (arrow) consists of a single layer of flattened cells covering the external surface. Hematoxylin-eosin. x 1500.

Fig. 2. Hematoxylin-eosin stained section showing a group of cuboidal mesothelial cells (arrow) in the uterine serosa. x 300.

Fig. 3. Portion of the fimbrial margin from the external side of the infundibulum. A continuous band of oviductal epithelium 1 cm in width (a) may be seen adjacent to the undulated end (b). The boundary with the mesothelium is also visible (arrowheads). x 8

Fig. 4. More detailed view of the boundary between the oviductal epithelium (a), containing ciliated cells (arows) and secretory cells (arrowheads), and the mesothelium (b). Transition between both epithelia is abrupt (c). Note also the presence of red blood cells. x 800

Fig. 5. This micrograph of the mesometrium illustrates the dark surface film (a), covering the microvilli (b) in samples processed without osmium tetroxide. Note also the outline of red blood cells trapped beneath the dark material (arrows). x 2400

Fig. 6. After fixation with osmium tetroxide, a dense mat of long microvilli may be observed on the mesothelial surface of the mesometrium. The numerous microvilli conceal the limits between cells. x 2100

Table 1.- Morphometry of the mesothelium of the genital area in the cow

Peritoneal surface	No. Microvilli/10 μm² Mean ± s.e.m	Length of microvilli (μm) mean ± s.e.m	Cell surface area (μm²) mean ± s.e.m
Mesometrium	74.3 ± 5.35 [a]	3.1 ± 0.15 [a]	47.0 ± 4.78 [a]
Uterus	65.6 ± 4.63 [a]	2.5 ± 0.14 [a b]	46.2 ± 3.10 [a]
Mesosalpinx	43.2 ± 1.67 [b]	1.6 ± 0.13 [c]	152.5 ± 19.09 [a b]
Infundibulo-cornual lig.	39.9 ± 1.26 [b]	1.5 ± 0.08 [c]	184.0 ± 9.68 [b]
Isthmus	43.5 ± 3.58 [b]	1.4 ± 0.09 [c]	197.4 ± 29.40 [b]
Infundibulum	48.2 ± 4.99 [b]	2.0 ± 0.10 [b c]	120.5 ± 3.52 [a b]

[a, b, c] Means within the same column with different superscripts differ ($P<0.05$)

The serosal surface of the uterus and mesometrium showed a dense mat of long microvilli. The surface was frequently so densely covered by long microvilli that the limits between cells were indistinguishable (fig 7). When individual mesothelial cells were discernible, their boundaries were prominent, and the central region of each cell protruded towards the peritoneal cavity (fig.8).

Serous membranes of the oviduct and adjacent structures exhibited lower microvilli density and length (fig 9) and higher mesothelial cell surface area (fig 10). As a result, the cell plasma membrane was usually visible. The serosal surface was delicate and uniformly flat and the outlines of individual cells were usually discernible. Solitary cilia or long microvilli were visible in the mesosalpinx and infundibulo-cornual ligament of some specimens (fig. 11). The presence of clearly defined intercellular openings or "stomata" was only observed in the mesosalpinx of one cow (fig 12).

Although more evident in zones with low microvilli density, micropores 30 to 130 nm in diameter were visible both in the serosal surface of the uterus and

mesometrium and of the oviduct and adjacent structures (fig 13).

The distribution of spermatozoa was scored throughout the mesothelial surfaces. Numerous spermatozoa were observed over the peritoneum after intraperitoneal insemination. Attachment of sperm heads to mesothelial cells was sometimes noticeable (fig 14). After intrauterine insemination, no spermatozoa were found on the serosal surface of the five cows scored.

DISCUSSION

The peritoneal mesothelium of the genital area in the cow was shown as a single continuous layer of cells covered by microvilli. No morphological differences relating to the phase of the oestrus cycle were observed on mesothelial surfaces. Similarly, the presence of a dominant ovarian structure either follicular or luteal was not related to morphological characteristics of extra-ovarian mesothelial cells. However, morphological differences were observed in different areas. Microvilli density of the uterus and mesometrium was higher than of the oviduct and adjacent structures. Various reports (Andrews and Porter, 1973; Furubayashi et al., 1984) have described a greater presence of mesothelial microvilli of the organs which move about most actively in the body cavities. Andrews and Porter (1973) suggested that these dense microvilli protect the serosal surface from frictional damage arising from movement of internal organs over one another. Variations in density and length of microvilli may indicate differences in response to injuries. The practical advise based on of these results may be a need for great care when manipulating oviduct and adjacent structures by rectal palpation, ovum pick-up and surgery, because they have less protection than the uterine and mesometrial areas.

As the peritoneum is an important element in peritoneal dialysis, numerous studies have been undertaken to describe the structure and physiology of mesothelial cells (Odor, 1954; Baradi and Hope, 1964; Baradi and Rao, 1976; Slater et al., 1989). However, the mechanisms associated with absorption of fluid from the peritoneal

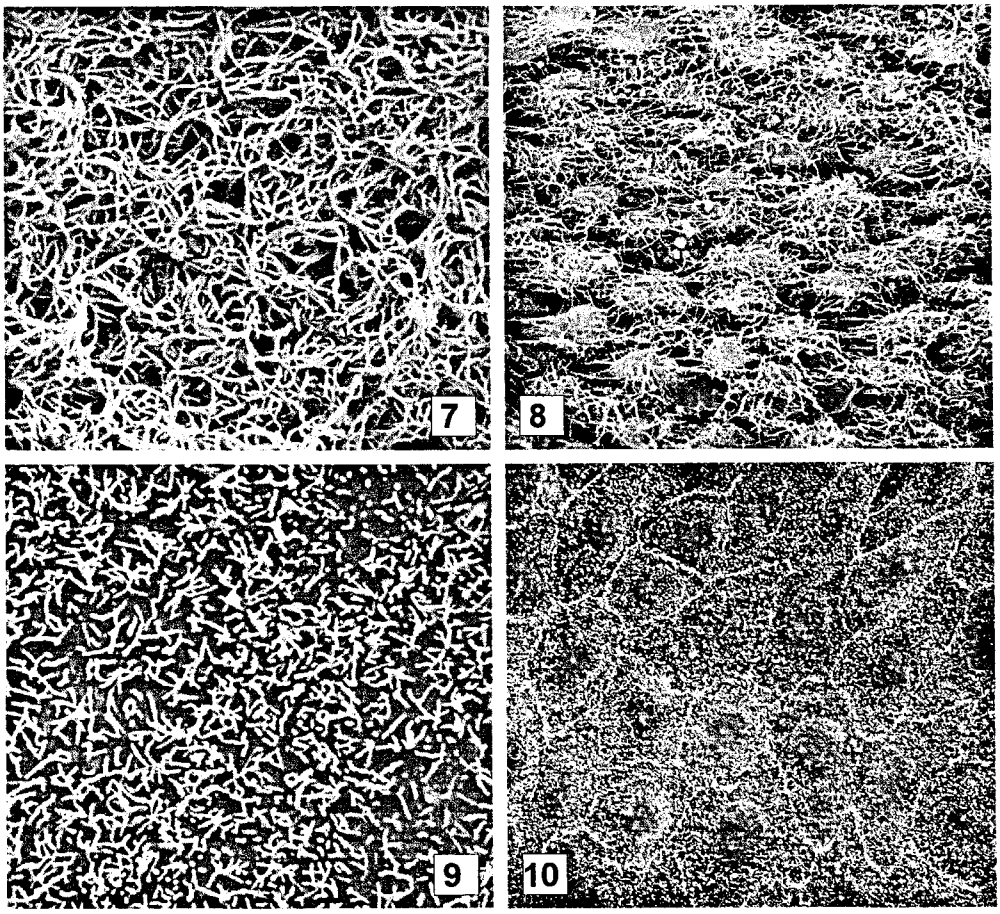

Fig. 7. A detailed view of mesometrial serosal surface. Numerous, long microvilli conceal the cell surface. x 3750

Fig. 8. This micrograph illustrates the surface features of the cells from the mesometrium. The central parts of cells protrude towards the peritoneal cavity and have a relatively small surface area. x 1500

Fig. 9. The serosal surface covering the mesosalpinx. Note the relatively lower microvilli density and length that allow appreciating of cell plasma membrane. x 3750

Fig. 10. Visceral mesothelium lining the external side of the infundibulum. This side view shows delicate, flat mesothelial cells with a relatively high surface area. x 750

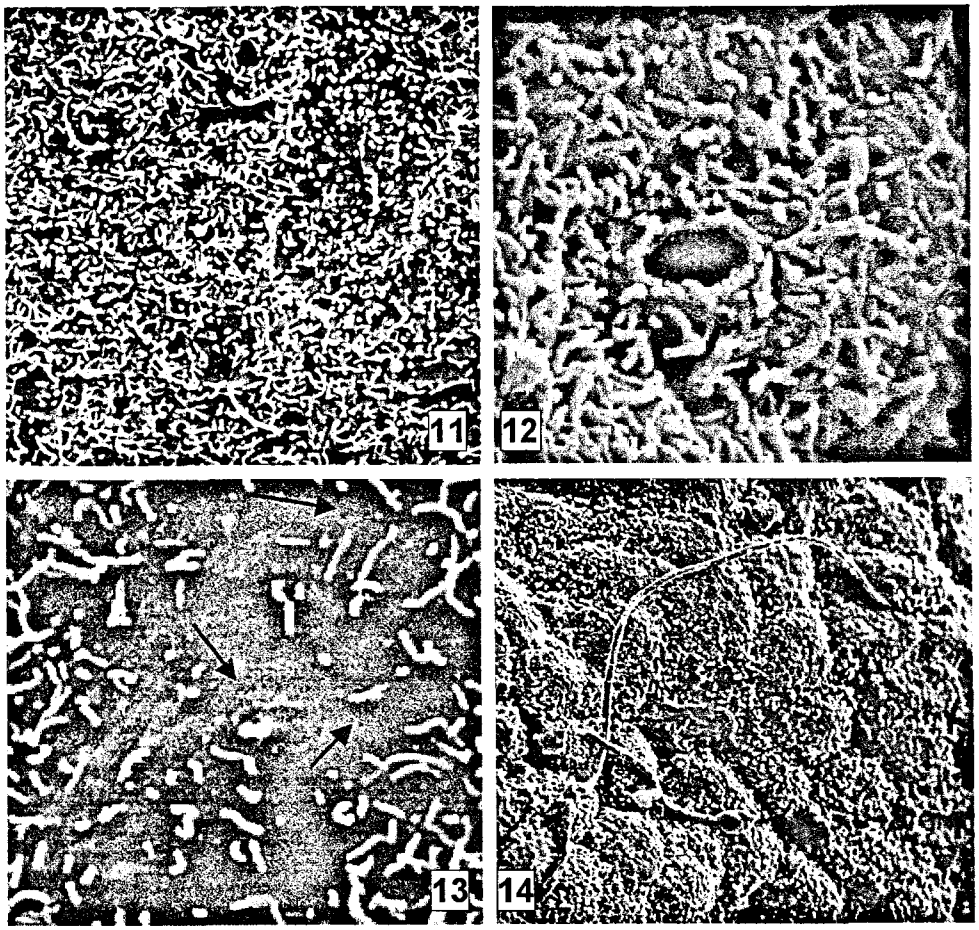

Fig. 11. In the mesosalpinx, the presence of single cilia or long microvilli was sometimes noticeable (arrows). x 2250

Fig. 12. In the mesosalpinx of one cow, clearly defined stomata were visible in the intercellular spaces. x 6000

Fig. 13. A higher magnification micrograph showing micropores visible in cell plasma membrane (arrows). x 7500

Fig. 14. This micrograph shows a spermatozoon with its head engaged in the surface of the mesothelium of the isthmic serosa after intraperitoneal insemination. x 1500

cavity are not completely understood. Intercellular mechanisms for absorption have been widely accepted as the major transport route (Casley-Smith, 1967; Cotran and Majno, 1967). Absorption of peritoneal fluid has been related to the presence of preformed openings or "stomata" between adjacent mesothelial cells (Allen, 1936; Tsilibary and Wissig, 1977). However, these stomata have also been interpreted as artifacts originating from preparation procedures (Odor, 1954). We only found clear intercellular openings, probably artifacts, in the mesosalpinx of one cow. Absorption of soluble material may furthermore be increased with higher microvilli densities due to an increase in cell surface area (Odor, 1954). On the other hand, the presence of invaginations in the cellular membrane, or micropores, can also indicate micropinocytotic activity. Therefore, availability for intracellular absorption could be higher in the uterine and mesometrial serosa than in the serosa of the oviduct and adjacent structures.

The presence of a surface coating over the microvilli and cell surface, associated with the mesothelium was observed in the rat using TEM (Andrews and Porter, 1973; Schwarz, 1974), and SEM procedures (Ettarh and Carr, 1996). This coating was considered to be the glycocalyx (Andrews and Porter, 1973). We found a layer of amorphous material, containing red blood cells, covering the mesothelial surface when the samples were processed without postfixation in osmium tetroxide. Probably, as pointed out by Ettarh and Carr (1996) in the mouse, this surface layer includes the glycocalyx and serous fluid fixed onto specimens.

Our findings show the existence of solitary cilia in the peritoneal surface of the cow. The occasional presence of solitary cilia on some mesothelial surfaces has been described in the peritoneum of both the rat (Andrews and Porter, 1973) and man (Slater et al., 1989). Little is known about their functional significance. Andrews and Porter (1973) suggested that they may represent the vestigial remnants of motile cilia reported in lower vertebrates.

During the follicular phase, the presence of a densely ciliated oviductal epithelium on the external side of the infundibulum, which forms a continuous band of between 25 and 100 mm, may be important in producing ciliary currents to ovum pick-up at ovulation.

The peritoneal cavity is accepted as being the destination of some spermatozoa following semen deposition into the reproductive tract (Mattner, 1963; Mattner and Braden, 1963; Hawk, 1982). However, although live spermatozoa have been found in the peritoneal cavity of several women (Horne and Thibault, 1962), there is no definite evidence of spermatozoa entering the abdominal cavity in animal species. We failed to demonstrate such presence using SEM procedures. This absence may be associated with either fixation procedures or loss of spermatozoa throughout the abdominal cavity.

Our results suggest the capacity of spermatozoa to bind with the mesothelial cell surface following intraperitoneal insemination. Sperm attachment to the oviductal epithelium appears to play a role in the regulation of sperm transport (Overstreet, 1983) and capacitation (Suarez, 1998). Motile, non-capacitated, acrosome-intact spermatozoa bind with oviductal epithelium with seemingly species-specific recognition (Lefebvre et al., 1997). However it is not fully understood whether spermatozoa become irreversibly engaged with the oviductal epithelium, or whether sperm-epithelial interactions result in capacitation changes and subsequent sperm release. Similarly is not known whether sperm motility within oviducts prevents sperm binding. More studies are needed to understand the biological implications of the peritoneal mesothelium on spermatozoa are similar to those of the oviductal epithelium.

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CAPÍTULO 6

Intraperitoneal Insemination and Retrograde Sperm Transport in Dairy Cows

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6 Intraperitoneal Insemination and Retrograde Sperm Transport in Dairy Cows

SUMMARY

To examine the efficiency of retrograde sperm transport following intraperitoneal insemination, live and dead spermatozoa were used at different concentrations, and sperm recovery from cervical mucus (0.5 ml) 2, 6, 12 and 24 hours following insemination was evaluated. Forty lactating Friesian cows, in their second to fourth lactation period, were used in this experiment. Thirty six cows received intraperitoneally either live or dead spermatozoa. Each group of 6 cows received one of three total sperm numbers of 30, 45 and 90 million. Four cows were inseminated with 90 million spermatozoa into the uterus and served as a control group. All cows were inseminated towards the end of oestrus. After intrauterine insemination sperm recovery declined, but motile and/or immotile spermatozoa were recovered from all cows at any time. In cows inseminated intraperitoneally, sperm was recovered from the cervix at 6 to 24 hours when 90 million were inseminated. A greater number of spermatozoa was recovered after dead than after live sperm inseminations. Only immotile, intact or broken spermatozoa and tailless heads were recovered after intraperitoneal insemination using either live or dead spermatozoa. No sperm was recovered for 30 and 45 million inseminations. Our results show that, following intraperitoneal insemination, there is passive sperm transport from the peritoneal cavity to the genital tract close to the time of ovulation, and suggest a higher sperm retention in the genital tract when live as opposed to dead spermatozoa are used.

INTRODUCTION

In mammalian species the usual site for fertilization is the ampullary-isthmic junction in the oviducts. Spermatozoa must move in ascending direction to reach the oocyte when semen is deposited into the vagina or into the uterus. Contractions of the smooth muscles in the uterine and oviductal wall, ciliary activity and sperm motion are mechanisms usually involved in sperm transport. Flow of uterine and tubal fluids could also participate in sperm passage to the cranial parts. However, spermatozoa can also reach the oviductal lumen from the peritoneal cavity. The feasibility of intraperitoneal insemination in several species, including the cow, leaves the way open for using the transperitoneal route is open (HUNTER, 1988). Intraperitoneal insemination is a method for assisted reproduction in humans (CROSIGNANI et al., 1991; KARLSTR™M et al., 1991; SERACCHIOLI et al., 1991), often giving better results than intrauterine insemination (EVANS et al., 1991; AJOSSA et al., 1997; MISAO et al., 1997). However, very few studies have been performed in cattle. The long history of success of the artificial insemination industry, which cannot be overemphasized in animal reproduction, has limited the development of other strategies than depositing semen at the traditional site, the uterine body. Nevertheless, new technologies such as sexing spermatozoa (JOHNSON et al., 1994), which application is restricted by the low number of sperm cells available for insemination, or the capsulation of semen (NEBEL et al., 1985), a process which appears to favor the slow release of spermatozoa, could have certain advantages by bypassing the barrier effect of the caudal segments of the genital tract. The peritoneal cavity could be an alternative. Our knowledge of intraperitoneal insemination is limited to 3 experiments in cattle (SKJERVEN, 1955; MCDONALD and SAMPSON, 1957; LOPEZ-GATIUS, 1995) which gave poor results, a low number of pregnancies. The objective of this study was to evaluate the efficacy of sperm transport into the genital tract following intraperitoneal insemination in dairy cows. Live and dead spermatozoa were used at different

concentrations, and sperm recovery from cervical mucus was monitored to record the access of spermatozoa from the peritoneal cavity to the genital tract.

MATERIALS AND METHODS

Animals

This study was conducted at a commercial dairy herd in Northeastern Spain. Forty lactating Friesian cows, in their second to fourth lactation period, were used in this experiment. Only cows not inseminated since last calving and with a period from calving (days open) longer than 60 d were selected. Cows with previous clinical disorders such as dystotic delivery, twining, retained fetal membranes, pathological uterine discharges and evident metabolic disturbances (milk fever, ketosis) were discarded. The cows were in excellent health and good body condition, cyclic and free of pathological abnormalities of the reproductive tract detectable by palpation per rectum.

Cows were synchronized when a corpus luteum was estimated by palpation per rectum. A luteolytic dose of cloprostenol (500 ug) and 250 IU of hCG and 1 mg of estradiol benzoate 12 hours after cloprostenol treatment were used for synchronization (LOPEZ-GATIUS, 1989). Standing to be mounted was the criterion used for determining oestrus. The cows were inseminated 12 to 15 hours after oestrous detection, and oestrus was confirmed by rectal examination at the time of insemination (LOPEZ-GATIUS and CAMON-URGEL, 1991). Animals were examined by palpation per rectum at 12-hours intervals for the presence of a follicle palpable on the ovary from insemination to ovulation. Ovulation was defined as the disappearance of the largest follicle (PIERSON and GINTHER, 1984).

Intraperitoneal insemination was applied in 36 cows randomly distributed in 2 groups, 18 cows received frozen-thawed (live) and 18 frozen-thawed and then

heat-killed (dead) spermatozoa. Each group of 6 cows received one of three total sperm numbers of 30, 45 and 90 million. In this way, semen was deposited intraperitoneally in 6 lots of 6 cows each. Four cows were inseminated into the uterus, with frozen-thawed semen using 90 million spermatozoa, and served as a control group.

Treatment of Semen and Inseminations

Frozen semen in 0.25 ml French straws from a single ejaculate, taken from a bull of proven high fertility housed at an artificial insemination center, was used in this experiment. The semen was diluted in Tryladyl (Minitub GmbH Abf□ll- und Labortechnik, Tiefenbach, BRD, Germany). Each unit of semen contained more than 65% postdilution and 40% postthaw progressive motility. Sperm volume was not modified by variations in the amount of spermatozoa supplied. Seminal doses were thawed for 20 sec at 35°C before use. For dead sperm inseminations, spermatozoa were heat-killed by immersion of the straws in water for 5 min at 50°C and then sperm immotility was assessed before insemination.

For intraperitoneal insemination, semen was injected into the peritoneal cavity through the vaginal wall at the dorsal fornix level, as described previously (LOPEZ-GATIUS, 1995). A sterile 19-gauge needle 4 cm long and a glass speculum 44 cm long and 10 mm in diameter and with a conic tip were used as additional accessories to standard AI equipment. The conic tip of the glass was carefully positioned by hand to the dorsal fornix. The AI catheter with the needle affixed to the end of the plastic sheath was then inserted into the glass speculum, and the vaginal wall was pierced in cranial direction by the needle. Semen was then slowly injected into the peritoneal cavity. Precautions were taken into account to eliminate the possibility of spermatozoa contamination into vagina and a sample of vaginal fluid was collected 10 minutes after insemination to assess absence of sperm. In the control cows, semen was deposited into the uterine body.

Sperm Recovery and Estimation of Number

Cervical fluid was collected with an insulin syringe, by hand introduced 2 to 3 cm into the cervical canal, at 2, 6, 12 and 24 hours after insemination. At least 0.5 ml of fluid was obtained each time. Sperm concentration was estimated using a Neubauer hemocytometer with 2 chambers. Spermatozoa, broken-spermatozoa and tailless heads were counted. Spermatozoa were classified as motile or nonmotile. If there was any evidence of motility, spermatozoa were considered to be motile.

Pregnancy Diagnosis

Cows returning to oestrus were registered as nonpregnant after confirmation of oestrus by rectal examination. In the remainder of the cows, pregnancy diagnosis was performed by palpation per rectum of 45 to 52 d after AI.

Statistical Analysis

Differences in sperm recovery from 2 to 24 hours after intraperitoneal insemination using either live or dead spermatozoa were checked using analysis of variance. All mean values are expressed as the mean ± standard error of the mean (SEM).

RESULTS

After intrauterine insemination the sperm recovery decreased from 2 to 24 hours, but motile and/or immotile spermatozoa were recovered from all cows in either time. Spermatozoa recovered from cervical mucus 2, 6, 12 and 24 hours after insemination were $32.4x10^4 \pm 14.2$; $8.4x10^4 \pm 4.13$; $4.3x10^4 \pm 1.3$; and $2.1x10^4 \pm 0.66$ spermatozoa /ml respectively.

In cows inseminated intraperitoneally, spermatozoa were not recovered from cervical mucus at any time using 30 million live or dead spermatozoa. Neither sperm recovery was possible when 45 million live spermatozoa were used and only 2 spermatozoa were recovered from a cow 12 hours after intraperitoneal insemination when 45 million dead spermatozoa were used. When a dose of 90 million live spermatozoa was deposited intraperitoneally, sperm recovery was not possible 2 hours later from any cow; spermatozoa were recovered from 3 cows 6 hours after insemination; and from 4 cows 12 and 24 hours after insemination. Two hours after intraperitoneal insemination with 90 million dead spermatozoa, no spermatozoa were recovered from any cow; by contrast sperm recovery was possible from all cows 6 and 12 hours after and from 4 cows 24 hours after insemination. Only immotile spermatozoa, intact or broken, and tailless heads were recovered following intraperitoneal insemination either using live or dead spermatozoa. A comparison of the concentration of spermatozoa recovered with reference to the type of semen used, 90 million dead or live spermatozoa, is shown in Table 1. Number of spermatozoa was higher (P=0.04) 6 hours after insemination with dead than with live spermatozoa, and 12 hours after insemination a tendency remained (P=0.09).

All cows ovulated by 12 to 24 hours after insemination.

Table 1. Sperm recovery from cervical mucus at different times following intraperitoneal insemination with a total of 90 million live or dead spermatozoa

Hours after insemination	Live sperm 10^4/ml (n=6)	Dead sperm 10^4/ml (n=6)	P
2	0	0	
6	0.3 ± 0.17 (0 to 1)	1.2 ± 0.35 (0 to 2.5)	0.04
12	0.5 ± 0.22 (0 to 1.5)	1.3 ± 0.4 (0.5 to 3)	0.09
24	0.8 ± 0.3 (0 to 1.5)	0.6 ± 0.23 (0 to 1.5)	0.67

Values are means + standard error of the mean with range in parentheses.

In this experiment, only 1 cow became pregnant, when an insemination dose of 45 million live spermatozoa was deposited intraperitoneally. Control cows did not become pregnant.

DISCUSSION

Having discovered motile spermatozoa in semen in 1677, Van Leewenhoek through a series of experiments established that spermatozoa enter the uterus after coitus and that the genital tract of the female is specifically shaped to ensure this event (FOURNIER, 1996). Our results show that the reproductive tract appears furthermore to be specially shaped to recover spermatozoa from the peritoneal cavity, even dead spermatozoa, and to transport them throughout the uterus to the vagina. Sperm motility is not necessary to access into the oviducts from the peritoneal cavity. In fact, oviducts can pick up starch (DECKER and DECKER, 1954) and carbon particles (WIMSATT and WALDO, 1945). Perhaps, the capturing of foreign particles from peritoneal cavity by the oviducts and the transport of part of this material to the exterior is a natural function of the female reproductive tract in mammalian species.

Our results agree with previous studies showing that a large proportion of the inseminate was discharged from the reproductive tract within 12 hours of intrauterine insemination (MITCHELL et al., 1985; NELSON et al., 1987). However, intraperitoneal inseminations using 30 and 45 million spermatozoa proved to be insufficient for assessing of retrograde sperm transport due to evidence of a lack of or very poor sperm recovery. By contrast, when a dosage of 90 million was used, spermatozoa were found in cervical mucus 6 and 12 hours after insemination in all cows. These results suggest that sperm concentration is an important factor to be considered in intraperitoneal insemination. However, caution should be taken when using high sperm concentrations. Risk of polyspermic fertilization could be higher following intraperitoneal insemination than deposition of semen into the uterus, as postulated by Adams (1969) with

reference to rabbit. In the human, there are reports of pregnancies after only 0.2 (LESEC et al., 1989) and 0.1 (TURHAN et al., 1992) million spermatozoa inseminated. Nevertheless, intraperitoneal insemination of the rhesus monkey was only effective when more than 50 million spermatozoa were used (VAN PELT, 1970) and, in guinea pig, the minimal number of spermatozoa required to produce conception was 30 million (ROWLANDS, 1957). In a previous experiment (LOPEZ-GATIUS, 1995), 9 repeat-breeder cows became pregnant after intraperitoneal insemination of 45 million spermatozoa. Only 1 pregnancy in the present study, also using 45 million spermatozoa, may be due to excessive manipulation of the cows and their genital tract during sample collection. More studies are needed to determine the optimum number and concentration of spermatozoa for intraperitoneal insemination in cattle.

In the present study, the collection of a 0.5-ml sample of cervical mucus at several times during a 24 hours period was not an attempt to quantify retrograde sperm transport from the peritoneal cavity. However, our results show a higher sperm recovery rate at 6 and 12 hours after insemination when dead spermatozoa were used. Sperm adherence to epithelial surfaces of the genital tract, mainly to oviductal epithelium, appears to play a role in regulating sperm transport (OVERSTREET, 1983; GOMENDIO et al., 1998). However, it is not fully understood whether spermatozoa become irreversibly engaged to oviductal epithelium, or whether sperm-epithelial interaction results in capacitation changes and subsequent sperm release. Similarly it is not well known whether sperm motility within oviducts prevents sperm binding, as occurs within the uterus (AUSTIN, 1964). Our results suggest a higher sperm retention within the genital tract when live spermatozoa are used. On the other hand, under our working conditions, only immotile spermatozoa were recovered following both live and dead sperm inseminations. As recently pointed out (LEFEBVRE et al., 1995; SUAREZ et al., 1997), epithelial sperm binding is probably the primary factor regulating the formation of the oviductal sperm reservoir in cattle, and the

elimination of dead spermatozoa and their transport to the exterior could just be a reflection of the enormous barrier effect of the female tract for spermatozoa.

In goats, Gonz lez (1972) found more spermatozoa within oviducts and uterus when applying intraperitoneal insemination during rather than outside oestrus. The optimal time for intraperitoneal insemination appears to be just prior to ovulation, from 8 to 16 hours before in rabbit (HADEK, 1958; MROUEH and MASTROIANNI, 1966) and from 0 to 12 hours in pig (HUNTER, 1978). The cows used in this study were all inseminated at the end of oestrus and before ovulation. The number of spermatozoa recovered from the cervical canal 6 hours after insemination suggests that entry of spermatozoa into the oviducts begins before ovulation. Sperm transport into oviducts could be similar to the pick up oocyte mechanism. The action of the infundibular fimbria appears to be sufficient to create a fluid flow towards the ostium of the oviduct at the time of ovulation (OVERSTREET, 1983). Subsequent contractions of the smooth muscles in the uterine and oviductal wall, which appear to propagate from oviducts to the cervix close the end of oestrus (RUCKEBUSCH and BAYARD, 1975), could transport sperm to the exterior.

Data reported in this experiment provide reasons for continuing the investigations on intraperitoneal insemination in cattle. Passive sperm transport occurs efficiently from the peritoneal cavity to the genital tract near to the time of ovulation. Questions relating to the behaviour of live spermatozoa and the capacitation process after intraperitoneal insemination are still to be answered.

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CAPÍTULO 7

Discusión general

7 Discusión General

El mesotelio peritoneal y el epitelio oviductal son dos superficies epiteliales situadas en espacios distintos que poseen una continuidad tanto anatómica, a nivel del infundíbulo, como funcional, respecto al transporte espermático tras la inseminación intrauterina o intraperitoneal. Sin embargo, la escasez de estudios en profundidad sobre las características anatómicas de la mucosa oviductal y la ausencia de estudios sobre las superficies serosas peritoneales en vacas adultas dificulta la comprensión de las interacciones que se establecen entre estos epitelios y los espermatozoides. Parece evidente que cuanto mejor se conozca el medio por el que deben pasar los espermatozoides se tendrán más posibilidades de aclarar algunos de los mecanismos no completamente comprendidos en el momento actual. A lo largo de esta discusión nos centraremos en los aspectos anatómicos y funcionales tratados en los capítulos 4, 5 y 6, con especial relevancia en el transporte de gametos tras la inseminación intrauterina e intraperitoneal.

El oviducto es una estructura anatómica en la que suceden acontecimientos tan relevantes como el transporte de gametos, fecundación y primeras fases del desarrollo embrionario. Los estudios sobre la anatomía y fisiología oviductales ponen de relieve su participación activa en dichos acontecimientos. En el Capítulo 4 realizamos una descripción detallada de la mucosa oviductal bovina a lo largo del ciclo sexual. En los trabajos desarrollados con anterioridad la mucosa del oviducto se describe como una amplia red de pliegues longitudinales, que disminuyen en altura y número hacia el útero. Estos pliegues muestran una importante ramificación y se extienden a lo largo de todo el oviducto de la vaca

(Hafez y Blandau, 1969; Johnson y Foley, 1974; Hunter, 1988). Sin embargo, a lo largo de este estudio hemos puesto de manifiesto una estructura tridimensional más compleja que la descrita con anterioridad.

El infundíbulo de la vaca está estructurado en dos lados diferentes, un lado amplio y un lado estrecho. Los pliegues mucosos más altos del lado estrecho se sitúan cerca del borde libre. Lombard et al.(1950) describió la misma disposición de los pliegues para toda la superficie infundibular. Sin embargo, nuestros resultados muestran como el endosalpinx próximo al borde libre del lado ancho forma una red de cordones interconectados, que convergen formando los pliegues primarios. Estos pliegues aumentan en altura antes del orificio abdominal. En ambos lados del infundíbulo, los pliegues primarios se fusionan o desaparecen en su curso descendente. Las saculaciones en el infundíbulo son amplias y superficiales y parecen formarse por extensiones de los pliegues laterales que proceden de las paredes de los pliegues primarios adyacentes. En el interior de estas saculaciones hay hileras de pliegues de escasa altura. La inclinación de los pliegues secundarios determina la formación de fondos de saco con su abertura orientada hacia el ovario.

Los cilios que recubren el infundíbulo y la ampolla se consideran directamente implicados en la captación y el transporte del ovocito recién ovulado. Odor y Blandau (1973) hallaron una relación directa entre el transporte de los ovocitos sobre el infundíbulo y el número de células ciliadas presentes en el epitelio. En nuestro estudio hemos observado un mayor número de células ciliadas en las paredes y ápices de los pliegues primarios que en los espacios entre pliegues. Hallazgos similares se han descrito en la mujer (Ferency *et al.*, 1972; Patek *et al.*, 1972). Probablemente, la disposición de pliegues altos, sinuosos y ricos en células ciliadas cerca del orificio abdominal facilita el transporte tanto de los ovocitos como de otras partículas o células presentes en la cavidad peritoneal, hacia la ampolla.

En la ampolla se observó una estructura arborescente, coincidiendo con los hallazgos de Gaddum-Rosse y Blandau,(1973). La divergencia de los pliegues es frecuente en la ampolla, pero, ocasionalmente, los pliegues se fusionan o desaparecen. Adicionalmente, nosotros distinguimos dos tipos de pliegues longitudinales en función de su altura, unos pliegues altos y otros medios o bajos. Estudios previos habían observado una amplia distribución de células ciliadas durante la fase folicular y células secretoras prominentes durante la fase luteal tardía (Abe y Oikawa, 1993). En este estudio se ha descrito además una clara disminución de las células ciliadas en los espacios entre pliegues durante la fase folicular, al igual que ocurría en el infundíbulo.

En la unión ámpulo-ístmica se observaron frecuentes divergencias e interconexiones de pliegues, en concordancia con Hunter *et al.*,(1991). Funcionalmente, el encuentro de los gametos masculino y femenino se asume que sucede en esta región. La presencia en esta zona de un gran número de espermatozoides viables tras la ovulación es una causa de polispermia (Austin, 1963), una de las principales anomalías de la fecundación (Hunter, 1996). La anatomía de la mucosa en esta área, con numerosas proyecciones luminales y criptas basales, puede constituir la última barrera anatómica para los espermatozoides. Adicionalmente, observamos un perfil irregular de los pliegues, formando proyecciones luminales redondeadas y la aparición de criptas estrechas en las bases de los saculaciones.

Los hallazgos al nivel del istmo y de la unión útero-tubárica fueron similares a los anteriormente descritos. La convergencia y divergencia de pliegues son poco frecuentes en el istmo, de acuerdo con Hunter *et al.* (1991). Los pliegues se aplanan, ensanchan, divergen y desaparecen en la unión útero-tubárica, coincidiendo con las descripciones de Wrobel *et al.*, (1993). Nuestras observaciones referentes al istmo y la unión útero-tubárica sostienen los hallazgos de Wrobel *et al.* (1993), que describen un sistema de saculaciones entre las proyecciones mucosas, con la parte ciega en dirección a la ampolla.

Adicionalmente, observamos un aumento caudal de la inclinación de los pliegues secundarios y de la orientación de las saculaciones a nivel del istmo. La arquitectura de los pliegues mucosos en el istmo caudal y unión útero-tubárica podría ser un obstáculo añadido para el ascenso de los espermatozoides y contribuir a al formación del reservorio espermático funcional (Hunter *et al.*, 1991). El aumento caudal y la desaparición craneal de la inclinación de los pliegues secundarios y la orientación de las saculaciones apoyan esta idea.

En inseminación artificial se utilizan dosis seminales con un número de espermatozoides considerablemente menor que en la monta natural. Esta reducción de la concentración espermática adquiere ciertos niveles de riesgo cuando se intentan aumentar rentabilidad y expansión comercial del material genético, se pretenden utilizar machos subfértiles (Hunter y Greve 1997) o cuando se realiza el sexaje de espermatozoides (Hunter y Greve, 1998). En estas circunstancias, los elementos barrera del tracto genital se convierten en factores muy limitantes del éxito. En este sentido, la inseminación intraperitoneal se presenta como una alternativa a la inseminación intrauterina convencional, ya que en ella se elimina o disminuye el efecto de las barreras físicas del tracto genital. En un estudio reciente realizado en vacas repetidoras, se conseguía la misma proporción de gestaciones utilizando inseminación intrauterina e intraperitoneal (López-Gatius, 1995). Dada la escasez de trabajos realizados sobre inseminación intraperitoneal, solamente tres en vacuno (Skjerven, 1955; McDonald y Sampson, 1957; López Gatius, 1995), diversos aspectos como los mecanismos de transporte espermático o el número de espermatozoides por dosis, no se conocen con precisión.

Tras la inseminación intraperitoneal, los espermatozoides contactan directamente con las células mesoteliales del peritoneo. Las características de estas células pueden influir en la eficiencia del transporte espermático hasta el oviducto. Las superficies serosas se han descrito con cierto detalle en diversas especies laboratoriales (Odor, 1954; Baradi y Hope, 1964; Baradi y Rao, 1976, Andrews y

Porter, 1973) y en la especie humana (Slater *et al.*, 1989; Tsilibary y Wissing, 1977). Sin embargo, existen numerosos factores como la especie, la región anatómica y la edad que influyen en las características morfológicas de estas células y hacen difícil la extrapolación de una especie a otra o incluso de una región anatómica a otra para un mismo individuo. La ausencia de descripciones morfológicas sobre el mesotelio peritoneal de la vaca motivó la realización del segundo trabajo en el que se realizó una descripción de la superficie del mesotelio que recubre el tracto genital de la vaca y los ligamentos asociados. Los resultados de este estudio se incluyeron en el capítulo 5.

La característica común de todas las regiones fue la presencia de microvellosidades, con mayor densidad y longitud en el útero y mesometrio que en el oviducto y estructuras adyacentes. Algunos trabajos (Andrews y Porter, 1973; Furubayashi et al., 1984) han relacionado la densidad de microvellosides con la motricidad de los órganos, estando más cubiertos los órganos más activos. Andrews y Porter (1973) propusieron que las microvellosidades protegen la superficie serosa de las lesiones producidas por el rozamiento. Variaciones en densidad y longitud indicarían diferencias de protección. Las implicaciones prácticas de estos resultados pueden ser la necesidad de una precaución especial cuando se manipulen el oviducto y las estructuras adyacentes durante la palpación rectal, "ovum pick-up" y cirugía, porque poseen una protección menor que el área uterina y mesometrio. Las implicaciones de las microvellosidades en el transporte de espermatozoides tras la inseminación intraperitoneal no son claras. Es posible que se produzca una retención entre las microvellosidades, especialmente en las zonas más pobladas. Sin embargo, en las muestras procesadas sin postfijación en tetraóxido de osmio se observó una capa de material amorfo cubriendo las superficies mesoteliales. Estructuras análogas se han descrito en la rata (Andrews y Porter, 1973; Schwarz, 1974) y ratón (Ettarh y Carr, 1996) y se han considerado como el glicocálix y material seroso fijado sobre las muestras. La existencia de esta superficie amorfa puede indicar la presencia in vivo de una superficie deslizante que cubre las microvellosidades impidiendo la retención espermática.

En la lado externo del infundíbulo se observa un epitelio oviductal densamente ciliado durante la fase folicular, formando una banda entre 2.5 y 10 mm a lo largo del borde libre. La presencia de este epitelio puede ser importante en la creación de corrientes ciliares para la captación del ovocito en la ovulación y de espermatozoides tras la inseminación intraperitoneal.

También se han observado cilios solitarios en la superficie peritoneal de la vaca. Su trascendencia en la creación de corrientes de líquido peritoneal y como consecuencia, en el transporte espermático, es bastante incierta. De hecho, no se ha podido aclarar si se trata de cilios o microvellosidades largas y su escaso número hace suponer que se trata de elementos vestigiales. Se han descrito cilios solitarios en las superficies mesoteliales de la rata (Andrews y Porter, 1973) y el hombre (Slater *et al.*, 1989). Se sabe poco de su papel fisiológico y Andrews y Porter (1973) propusieron que pueden representar elementos vestigiales de cilios mótiles de los vertebrados inferiores.

Los mecanismos de absorción del líquido peritoneal pueden estar relacionados con la absorción del líquido que acompaña a los espermatozoides tras la inseminación intraperitoneal. La absorción del líquido peritoneal se ha relacionado con la presencia de orificios entre células mesoteliales adyacentes (Allen, 1936; Tsilibary y Wissig, 1977). Sin embargo, estos orificios se han interpretado también como artefactos (Odor, 1954). Nosotros solamente encontramos orificios intercelulares en el mesosalpinx de una vaca, y posiblemente se trataba de artefactos. En cambio, la absorción puede aumentarse también con el aumento de la densidad en microvellosidades debido a un aumento en el área superficial de las células (Odor, 1954). Por otro lado, la presencia de microporos en la membrana celular puede indicar también una actividad micropinocitócica. Por lo tanto, la absorción intracelular puede ser mayor en las regiones uterina y mesometrial que en la serosa del oviducto y estructuras adyacentes.

La cavidad peritoneal se asume que es el destino de algunos espermatozoides tras la monta o inseminación intrauterina (Mattner, 1963; Mattner y Braden, 1963; Hawk, 1983). Sin embargo, aunque se han observado espermatozoides vivos en la cavidad peritoneal de la mujer (Horne y Thibault, 1962), no existe una evidencia definitiva de la entrada de espermatozoides en la cavidad peritoneal de las especies animales. Nosotros no pudimos demostrar este hecho utilizando el microscopio electrónico de barrido. Esta ausencia de espermatozoides puede estar asociada tanto a los procedimientos de fijación como a la pérdida de espermatozoides en la cavidad peritoneal.

Nuestros resultados sugieren la capacidad de los espermatozoides para unirse a la superficie de las células mesoteliales tras la inseminación intraperitoneal. La unión de los espermatozoides al epitelio oviductal parece participar en la regulación del transporte espermático (Overstreet, 1983) y en la capacitación (Suarez, 1998), pero se necesitan más estudios para comprender las implicaciones biológicas del mesotelio peritoneal sobre los espermatozoides.

En el Capítulo 6 realizamos un estudio del flujo retrógrado de espermatozoides tras la inseminación intraperitoneal. Nuestros resultados muestran cómo el tracto reproductor femenino es capaz de capturar espermatozoides de la cavidad peritoneal, incluso espermatozoides muertos, y transportarlos a través del útero hasta la vagina en el moco cervical. Quizá, la captura por los oviductos de partículas extrañas desde la cavidad peritoneal y el transporte de parte de este material hasta el exterior es una función natural del tracto reproductor femenino en todas las especies de mamíferos.

Nuestros resultados coinciden con otros estudios previos en los que un gran número de espermatozoides se elimina desde el tracto reproductor en las 12 horas siguientes a la inseminación intrauterina (Mitchell *et al.*,1985; Nelson *et al.*,1987). Sin embargo, la inseminación intraperitoneal con 30 y 45 millones de espermatozoides parece insuficiente para la valoración del transporte retrógrado

debido a la escasa o nula recuperación de espermatozoides. En contraste, cuando se se utilizaron dosis de 90 millones, se encontraron espermatozoides a las 6 horas tras la inseminación en todas las vacas. Estos resultados sugieren que la concentración de espermatozoides es un factor muy importante a considerar en la inseminación intraperitoneal. Sin embargo, se debe tener precaución al usar altas concentraciones de espermatozoides. El riesgo de fecundación polispérmica puede ser mayor tras la inseminación intraperitoneal que cuando se deposita semen en el útero, como propuso Adams (1969) en la coneja. En la especie humana, se ha conseguido la gestación tras inseminar con 0.2 y 0.1 millones de espermatozoides. En contraste, la inseminación intraperitoneal en el mono rhesus resultó solo efectiva cuando se utilizaron más de 50 millones de espematozoides y en la cobaya el número mínimo de espermatozoides necesarios para la concepción fue de 30 millones. En un estudio en vacuno, 9 vacas repetidoras quedaron gestantes tras la inseminación intraperitoneal con 45 millones de espermatozoides. La obtención de una sola gestación en el estudio desarrollado en esta tesis, utilizando también 45 millones de espermatozoides, se puede deber a la excesiva manipulación de las vacas y su tracto genital durante la extracción de las muestras. En conclusión, se necesitan más estudios para determinar el número óptimo de espermatozoides para la inseminación intraperitoneal en vacuno.

Nuestros resultados muestran una mayor recuperación de espematozoides en moco a las 6 y 12 horas tras la inseminación cuando se utilizaron espermatozoides muertos. La adherencia de los espermatozoides a las superficies epiteliales del aparato genital, principalmente al epitelio oviductal, parece importante en la regulación del transporte espermático. Sin embargo, no se sabe com certeza si los espermatozoides permanecen irreversiblemente unidos al epitelio oviductal, o si las interacciones entre el espermatozoide y el epitelio conllevan a la capacitación de los espermatozoides y subsecuente liberación de los mismos. Tampoco se comprende muy bien si la motilidad de los espermatozoides dentro del oviducto previene la unión de los mismos al epitelio, como sucede en el útero. Nuestros resultados sugieren una mayor retención en el tracto genital cuando se insemina

con espematozoides vivos. Por otra parte, bajo nuestras condiciones de trabajo, solamente se encontraron espermatozoides muertos tras la inseminación tanto con espermatozoides vivos como muertos. Probablemente, la unión de los espermatozoides al epitelio es el factor primario que regula la formación del reservorio oviductal en vacuno, como se ha sugerido recientemente (Lefebvre *et al.*, 1995; Suarez *et al.*, 1997), y la eliminación de espermatozoides muertos al exterior podría ser un reflejo del enorme efecto barrera del tracto reproductor femenino para los espermatozoides.

Referente al momento de inseminación en el caso de la inseminación intraperitoneal, González (1972) encontró más espermatozoides en oviductos y útero de cabras inseminadas durante el estro que fuera del mismo. El momento óptimo para la inseminación intraperitoneal parece ser previo a la ovulación, entre 8 y 16 horas en la coneja (Hadek, 1958; Mroueh y Mastroianni, 1966), y entre 0 y 12 horas en la cerda (Hunter, 1978). Las vacas de este estudio se inseminaron durante la mitad del estro y antes de la ovulación en todos los casos. El número de espermatozoides recogidos en el canal cervical 6 horas tras la inseminación sugiere que la entrada de espermatozoides en el oviducto comienza antes de la ovulación y probablemente es mayor cercana al momento de ovulación. El mecanismo de transporte de espermatozoides hasta la luz oviductal podría ser similar al mecanismo de captación del ovocito. La acción de las fimbrias oviductales parece ser suficiente para crear un flujo de fluido hacia el ostium del oviducto en el momento de la ovulación (Overestreet, 1983). Además, las contracciones de la pared útero-tubárica, que parecen propagarse en ese momento desde los oviductos hacia el cuello uterino cerca del final del estro (Ruckebusch y Bayard, 1975), podrían transportar los espermatozoides hasta el exterior.

Los resultados obtenidos en esta experiencia proporcionan un incentivo para continuar el estudio de la inseminación intraperitoneal en vacuno. Existe un transporte pasivo de espermatozoides desde la cavidad peritoneal hacia el tracto genital, próximo al momento de la ovulación. Algunas cuestiones como el

comportamiento de los espermatozoides vivos y el proceso de capacitación de espermatozoides tras la inseminación intraperitoneal permanecen sin responder.

En resumen, en el desarrollo de este trabajo hemos intentado realizar una descripción anatómica de la mucosa oviductal y mesotelio peritoneal y analizar las implicaciones en el transporte espermático realizando estudios funcionales. Las dos superficies epiteliales poseen peculiaridades anatómicas regionales que podrían tener implicaciones en el transporte espermático. Respecto a la inseminación intraperitoneal, se necesitan más estudios para el conocimiento de los aspectos biológicos implicados en la misma.

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CAPÍTULO 8

Conclusiones

8 Conclusiones

1. La mucosa oviductal bovina presenta peculiaridades regionales de manera que:

a. La estructura de los pliegues mucosos es típica para cada segmento. En el infundíbulo se distinguen dos lados, un lado amplio y otro estrecho. La evolución de los pliegues mucosos hacia el borde libre es diferente en el lado amplio, con una disminución de altura hacia el borde libre y en el lado estrecho, en el que no se produce este hecho. En la ampolla se intercalan pliegues longitudinales altos con otros más bajos. En la unión ámpulo-istmica se observan numerosas ramificaciones de los pliegues, formando un entramado complejo y en el istmo se observan entre 4 y 8 pliegues longitudinales de escasa altura. La orientación de los pliegues mucosos secundarios en la pared de los pliegues primarios es opuesta en los segmentos craneales y caudales del oviducto.

b. Las áreas entre los pliegues de la mucosa del oviducto de la vaca muestran un alto grado de organización a pesar de su complejidad. En todas las regiones se observan oquedades de manera que en el infundíbulo y la ampolla adquieren una forma oval con hileras de pequeños pliegues en sus bases; en la unión ámpulo-istmica son depresiones saculares en las que desaparecen las hileras de pliegues en las bases y aparecen criptas estrechas; y en el istmo y unión útero tubárica se observa una progresiva orientación caudal de las saculaciones con la apertura dirigida hacia el útero y criptas estrechas en las paredes y bases de las mismas.

2. El epitelio oviductal bovino presenta variaciones morfológicas no sólo entre las diferentes fases del ciclo sexual y entre los diferentes segmentos sino también entre las áreas basales y apicales de los pliegues primarios longitudinales.

3. En el mesotelio peritoneal de la vaca se observa una mayor longitud y densidad de microvellosidades y una menor área superficial de las células en la serosa úterina y mesométrica que en la serosa oviductal y de los ligamentos asociados. También se observan peculiaridades regionales como la presencia de una banda de epitelio oviductal en la cara peritoneal de infundíbulo adyacente al borde libre.

4. Tras la inseminación intraperitoneal con 45 millones de espermatozoides se observó la unión de algunos de los mismos a la superficie de las células mesoteliales. En cambio, no se pudo demostrar la presencia de espermatozoides en la cavidad peritoneal tras la inseminación intrauterina con 180 millones de espermatozoides.

5. El tracto reproductor de la vaca es capaz de capturar espermatozoides de la cavidad peritoneal, incluso espermatozoides muertos, y transportarlos a través del útero hasta la vagina en el moco cervical. En este transporte influyen la concentración espermática y la calidad de los espermatozoides, de manera que los espermatozoides muertos son mejor transportados que los vivos.

CAPITULO 9

Perspectivas

9 Perspectivas

El trabajo desarrollado en esta tesis se incluye dentro de una línea de investigación que estudia los aspectos básicos y aplicados del transporte espermático en la vaca tras la inseminación intrauterina e intraperitoneal. Los resultados obtenidos nos han permitido plantear nuevas cuestiones sobre las que incidir en el futuro. En el caso de la inseminación intrauterina estudiaremos aspectos tales como la lateralidad en el transporte espermático oviductal y uterino en relación con las estructuras ováricas y la anatomía y fisiología de la mucosa uterina en relación con el transporte espermático. También profundizaremos en el estudio de la inseminación intraperitoneal, desarrollando trabajos sobre el tratamiento previo de los espermatozoides, la sincronización de la ovulación, la estimulación de la motricidad oviductal o el lugar de deposición. Estos estudios nos permitirán buscar alternativas a la inseminación intrauterina convencional y comprender aspectos básicos de la fisiología de la reproducción.

Resum
Resumen
Summary

Resum

La inseminació artificial és la tècnica reproductiva amb més repercussió econòmica de quantes s'han posat en pràctica en ramat vacu. Tot i això s'han desenvolupat pocs estudis anatòmics en profunditat sobre la mucosa de l'oviducte i el mesoteli peritonial, malgrat la seva participació en el transport espermàtic. Per tant, l'objectiu general d'aquesta tesi fou realitzar un estudi anatòmic de la mucosa de l'oviducte i el mesoteli peritonial en la vaca, i avaluar la seva repercussió en el transport espermàtic després la inseminació intrauterina i intraperitonial.

Es van utilitzar els oviductes de 36 vaques cícliques per estudiar l'anatomia de la mucosa oviductal bovina al llarg del cicle sexual. Es van dividir els oviductes en 12 segments iguals per les seves anàlisis. L'infundíbul oviductal es va observar com una estructura amb forma d'embut que rodeja l'orifici abdominal. Presenta un cantó ample i un cantó estret. La mucosa del cantó ample consta d'un sistema de cordons interconnectats de poca altura que convergeixen distalment formant els plecs primaris. Els plecs del cantó estret emergeixen sobtadament des de l'extrem lliure i es fusionen cap a l'orifici abdominal Hi ha plecs al llarg de la llum de l'oviducte. Les àrees que hi ha entre els plecs mostren una organització molt complexa. Els espais que hi ha entre els plecs estan ocupats per plecs secundaris petits i interconnectats que s'uneixen per formar un sistema de saculacions. En l'infundíbul, aquests fons de sac s'obren cap a l'ovari, mentre que els fons de sac presents en l'istme caudal en la unió útero-tubàrica s'obren cap a l'úter. Es van observar canvis cíclics, regionals i inter-regionals marcats en l'epiteli dels oviductes estudiats. Durant la fase fol·licular, les cèl·lules secretores augmenten el nombre i protrusió cap a les àrees basals entre els plecs i en l'interior de les saculacions i fons de sac de l'infundíbul i ampolla. Malgrat que

aquestes diferències disminueixen en els segments caudals, l'epiteli que cobreix les àrees basals d'algunes saculacions de l'istme conté grups de cèl·lules secretores prominents. En el període pròxim a l'ovulació es van trobar nombrosos espermatozoides en la perifèria de l'istme caudal, dins les saculacions de les zones basals entre els plecs, i dins les saculacions i fons de sac de la unió útero-tubàrica. També es van observar alguns espermatozoides en les àrees perifèriques de la unió ampulo-istmica i l'ampolla. Referent al conjunct de la mucosa oviductal es van observar variacions importants entre els diferents segments, entre les àrees basal i apical dels plecs i entre les diferents fases del cicle éstric.

Les característiques superficials de les cèl·lules mesotelials que cobreixen el tracte reproductor i ligaments accessoris es van examinar en 25 vaques cícliques mitjançant microscòpia electrònica d'escombratge. En la cara externa de l'infundíbul, la mucosa oviductal sobrepassa el marge lliure, formant una banda contínua que amida entre 0.25 i 1 cm d'amplada. Aquest epiteli oviductal mostra variacions cícliques amb una predominança de cèl·lules durant la fase fol·licular. D'altra banda, les cèl·lules mesotelials es troben cobertes per micropilositats. L'àrea superficial de les cèl·lules i la densitat i la longitud de les micropilositats es van amidar mitjançant un analitzador d'imatges. La superfície peritonial en l'úter i mesometri tenen una major densitat i longitud de micropilositats i una menor àrea superficial cel·lular que l'oviducte i estructures adjacents. També es va observar: la presència de microporus en la membrana plasmàtica de cèl·lules; cilis solitaris en el mesosàlpinx i lligament infundíbul-cornual d'algunes mostres; i orificis intercel·lulars en el mesosàlpinx d'una vaca. Quan les mostres es van processar sense post fixació en tetraòxid d'osmi, una capa fosca de material amorf cobreix totes les superfícies. No es van observar diferències clares referides al cicle estric, la lateralitat o el cantó de la borsa ovàrica (intern vs extern). Després de la inseminació intrauterina de cinc vaques no es van trobar espermatozoides sobre el mesoteli peritonial. Després de la inseminació intraperitonial de dues vaques es van trobar nombrosos espermatozoides units a les superfícies mesotelials. Aquests resultats indiquen que hi ha variacions regionals però no

canvis cíclics en les superfícies mesotelials que cobreixen l'àrea genital de la vaca i els lligaments associats.

Finalment es descriviren els resultats d'un estudi sobre l'eficiència del transport retrògrad d'espermatozoides després la inseminació intraperitonial. Per això s'utilitzaren espermatozoides vius i morts a diferents concentracions i es va avaluar la quantitat d'espermatozoides en mostres de moc (0.5 ml.) a les 2, 6, 12 i 14 hores després la inseminació. Per aquest experiment es van utilitzar 40 vaques Frisones entre la segona i la quarta lactació. Trenta sis vaques es van inseminar intraperitonialment amb 30, 45 i 90 milions d'espermatozoides. Quatre vaques es van inseminar amb 90 milions d'espermatozoides en l'úter i varen servir de grup de control. Totes les vaques es van inseminar cap el final de l'estre. Després la inseminació intrauterina, el nombre d'espermatozoides va disminuir, però es varen recollir espermatozoides mòbils o immòbils en totes les vaques de qualsevol temps. En les vaques inseminades intraperitonialment amb 90 milions d'espermatozoides, es varen recuperar espermatozoides a la cèrvix entre les 6 i 24 hores. Es van recollir un major nombre d'espermatozoides quan es van inseminar amb espermatozoides morts que amb espermatozoides vius. Només es van recollir espermatozoides immòbils, intactes o trencats i caps sense cua després la inseminació intraperitonial tant amb espermatozoides vius com amb morts. No es va recuperar cap espermatozoide després d'inseminar intraperitonialment amb 30 i 45 milions d'espermatozoides. Els nostres resultats mostren que, després de la inseminació intraperitonial, hi ha un transport passiu d'espermatozoides prop de l'ovulació de la cavitat peritonial cap el tracte, i suggereixen una major retenció espermàtica dins del tracte genital quan s'utilitzaren espermatozoides vius que quan s'utilitzaren espermatozoides morts.

Resumen

La inseminación artificial es la técnica reproductiva con mayor repercusión económica de cuantas se han puesto en práctica en ganado vacuno. Sin embargo, se han desarrollado pocos estudios anatómicos en profundidad sobre la mucosa oviductal y el mesotelio peritoneal, a pesar de su participación en el transporte espermático. Por lo tanto, el objetivo general de esta tesis fue realizar un estudio anatómico de la mucosa oviductal y mesotelio peritoneal en la vaca, y evaluar su repercusión en el transporte espermático tras la inseminación intrauterina e intraperitoneal.

Se utilizaron los oviductos de 36 vacas cíclicas para estudiar la anatomía de la mucosa oviductal bovina a lo largo del ciclo sexual. Se dividieron los oviductos en 12 segmentos iguales para su análisis. El infundíbulo oviductal se observó como una estructura con forma de embudo que rodea el orificio abdominal. Presenta un lado amplio y un lado estrecho. La mucosa del lado amplio posee un sistema de cordones interconectados de escasa altura que convergen distalmente formando los pliegues primarios. Los pliegues de la lado estrecho emergen repentinamente desde el borde libre y se fusionan hacia el orificio abdominal. Hay pliegue a lo largo de la luz del oviducto. Las áreas entre los pliegues muestran una organización muy compleja a lo largo de la luz del oviducto bovino. Los espacios entre pliegues están ocupados por pliegues secundarios pequeños e interconectados que se unen para formar un sistema de saculaciones. En el infundíbulo, estos fondos de saco se abren hacia el ovario, mientras que los fondos de saco presentes en el istmo caudal en la unión útero-tubárica se abren hacia el útero. Se observaron cambios cíclicos, reginales e interregionales marcados en el epitelio de los oviductos estudiados. Durante la fase folicular, las

células secretoras aumentan en número y protusión hacia las áreas basales entre los pliegues y en el interior de las saculaciones y fondos de saco del infundíbulo y la ampolla. Aunque estas diferencias disminuyen en los segmentos caudales, el epitelio que cubre las áreas basales de algunas saculaciones del istmo contiene grupos de células secretoras prominentes. En el periodo próximo a la ovulación se encontraron numerosos espermatozoides en la periferia del istmo caudal, dentro de las saculaciones de las zonas basales entre los pliegues, y dentro de las saculaciones y fondos de saco de la unión utero-tubárica. También se observaron algunos espermatozoides en las áreas periféricas de la unión ampulo-istmica y la ampolla. Referente al conjunto de la mucosa oviductal, se observaron a su vez variaciones importantes en la mucosa oviductal entre los diferentes segmentos, entre las áreas basal y apical de los pliegues y entre las diferentes fases del ciclo éstrico.

Las características superficiales de las células mesoteliales que cubren el tracto reproductor y ligamentos accesorios se examinaron en 25 vacas cíclicas mediante microscopía electrónica de barrido. En la cara externa del infundíbulo, la mucosa oviductal sobrepasa el margen libre, formando una banda continua que mide entre 0.25 y 1 cm de anchura. Este epitelio oviductal muestra variaciones cíclicas con una predominancia de células ciliadas durante la fase folicular. Por otros lado, las células mesoteliales se encuentran cubiertas por microvellosidades. El área superficial de las células y la densidad y longitud de las microvellosidades se midieron mediante un analizador de imágenes. La superficie peritoneal en el útero y mesometrio tienen una mayor densidad y longitud de microvellosidades y una menor área celular superficial que el oviducto y estructuras adyacentes. También se observó la presencia de microporos en la membrana plasmática de las células, cilios solitarios en el mesosalpinx y ligamento infundibulo-cornual de algunas muestras, y orificios intercelulares en el mesosalpinx de una vaca. Cuando las muestras se procesaron sin postfijación en tetraóxido de osmio, una capa oscura de material amorfo cubre todas las superficies. No se observaron diferencias claras referidas al ciclo éstrico, la lateralidad o el lado de la bolsa ovárica (interno

vs. externo). Tras la inseminación intrauterina de cinco vacas no se encontraron espermatozoides sobre el mesotelio peritoneal. Tras la inseminación intraperitoneal de dos vacas se encontraron numerosos espermatozoides unidos a las superficies mesoteliales. Estos resultados indican que hay variaciones regionales pero no cambios cíclicos en las superficies mesoteliales que cubren el área genital de la vaca y los ligamentos asociados.

Finalmente se describen los resultados de un estudio sobre la eficiencia del transporte retrógrado de espermatozoides tras la inseminación intraperitoneal. Para ello utilizaron espermatozoides vivos y muertos a diferentes concentraciones y se evaluó la cantidad de espermatozoides en muestras de moco (0.5 ml.) a las 2, 6, 12 y 24 horas tras la inseminación. Para este experimento se utilizaron cuarenta vacas Frisona entre la segunda y cuarta lactaciones. Treinta y seis vacas se inseminaron intraperitonealmente con 30, 45 y 90 millones de espermatozoides. Cuatro vacas se inseminaron con 90 millones de espermatozoides en el útero y sirvieron de grupo control. Todas las vacas se inseminaron hacia el final del estro. Tras la inseminación intrauterina, el número de espermatozoides disminuyó, pero se recogieron espermatozoides mótiles o inmóviles en todas las vacas a cualquier tiempo. En las vacas inseminadas intraperitonealmente con 90 millones de espermatozoides, se recuperaron espermatozoides del cérvix entre las 6 y 24 horas. Se recogió un mayor número de espermatozoides cuando se inseminó con espermatozoides muertos que con espermatozoides vivos. Solamente se recogieron espermatozoides inmóviles, intactos o rotos y cabezas sin cola tras la inseminación intraperitoneal tanto con espermatozoides vivos como con muertos. No se recuperó ningún espermatozoide tras inseminar intraperitonealmente con 30 y 45 millones de espermatozoides. Nuestros resultados muestran que, tras la inseminación intraperitoneal, hay un transporte pasivo de espermatozoides cerca de la ovulación desde la cavidad peritoneal hacia el tracto genital, y sugieren una mayor retención espermática dentro del tracto genital cuando se utilizaron espermatozoides vivos que cuando de utilizaron espermatozoides muertos.

Summary

Artificial insemination is the reproductive technology with highest economical impact in cattle. However, anatomical studies of the oviductal mucosa and peritoneal mesothelium, structures that directly participle in sperm transport, are limited. Therefore, the general aim of this thesis was to get more insight in the anatomy of the bovine oviductal mucosa and peritoneal mesothelium evaluate implications in sperm transport after intrauterine or intraperitoneal insemination.

The oviducts of 36 cyclic cows were divided into 12 equal segments for analysis. The oviductal infundibulum is an asymetric funnel shaped structure surrounding the ostium. It is divided along the free boarder of the mesosalpinx and presents one wide side and one narrow side. The mucosa of the wide side posses a system of low interconnected cords that converge distally forming primary folds. The folds in the narrow side start sharply from the free margin and fuse toward the ostium abdominale. Areas between folds throughout the lumen of the bovine oviduct show a high degree of complex organization. Interfolds spaces are occupied by secondary and small interconnected folds which join to form a system of cul-de-sacs. In the infundibulum, these cul-de-sacs open toward the ovary, while cul-de-sacs present in the caudal isthmus and in the TUJ open toward the uterus. Marked cyclic, regional and interregional changes were observed in the epithelium of the oviducts studied. In the follicular phase, the secretory cells increase in number and prominence toward basal areas between folds, and within pockets and cul-de-sac areas of the infundibulum and ampulla. Although these

differences decrease in caudal segments, the epithelium covering basement areas of some isthmic pockets contain groups of bleb secretory cells. Near the time of ovulation, numerous spermatozoa were found in the periphery of the caudal isthmus within pockets of basal interfold areas, as well as within pockets and cul-de-sacs of the utero-tubaric junction. Individual spermatozoa were also observed in peripheral areas of the ampullary-isthmic junction and ampulla. Marked, variations were observed in the bovine oviductal mucosa depending on the oviductal segment, basal or apical areas of the folds, and phase of the oestrus cycle.

The surface features of mesothelial cells of the reproductive tract and adjacent ligaments from 25 cyclic cows were examined by scanning electron microscopy. In the external side of the infundibulum, the oviductal mucosa exceeds the free margin, forming a continuous band measuring 0.25 to 1 cm in width. This oviductal epithelium shows cyclical variations with a predominance of ciliated cells during the follicular phase. Mesothelial cells were covered by microvilli. The cell surface area and microvilli density and length were measured with an image analyser. Peritoneal surfaces in the uterus and mesometrium have a higher microvilli density and length and a smaller cell surface area than in the oviduct and adjacent structures. We also observed the presence of micropores in the cell plasma membrane, of solitary cilia in the mesosalpinx and infundibulo-cornual ligament of some specimens, and of stomata in the mesosalpinx of one cow. When samples were processed without postfixation in osmium tetroxide, a dark layer of amorphous material covered all surfaces. No clear differences were observed associated with oestrus cycle, laterality or the side of ovarian bursa (internal vs. external). After intrauterine insemination of five cows, no spermatozoa were found on their peritoneal mesothelium. Numerous spermatozoa were found after intraperitoneal insemination being attached throughout mesothelial surfaces. These results indicate that there are regional variations but no cyclic changes in the surface features of mesothelial cells covering the genital area in the cow.

To examine the efficiency of retrograde sperm transport following intraperitoneal insemination, live and dead spermatozoa were used at different concentrations, and sperm recovery from cervical mucus (0.5 ml) 2, 6, 12 and 24 hours following insemination was evaluated. Forty lactating Friesian cows, in their second to fourth lactation period, were used in this experiment. Thirty six cows received intraperitoneally either live or dead spermatozoa. Each group of 6 cows received one of three total sperm numbers of 30, 45 and 90 million. Four cows were inseminated with 90 million spermatozoa into the uterus and served as a control group. All cows were inseminated towards the end of oestrus. After intrauterine insemination sperm recovery declined, but motile and/or immotile spermatozoa were recovered from all cows at any time. In cows inseminated intraperitoneally, sperm was recovered from the cervix at 6 to 24 hours when 90 million were inseminated. A greater number of spermatozoa was recovered after dead than after live sperm inseminations. Only immotile, intact or broken spermatozoa and tailless heads were recovered after intraperitoneal insemination using either live or dead spermatozoa. No sperm was recovered for 30 and 45 million inseminations. Our results show that, following intraperitoneal insemination, there is passive sperm transport from the peritoneal cavity to the genital tract close to the time of ovulation, and suggest a higher sperm retention in the genital tract when live as opposed to dead spermatozoa are used.

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CURRICULUM VITAE

JESÚS YÁNIZ PÉREZ DE ALBÉNIZ, nacido en Logroño (La Rioja) en diciembre de 1969, inicia sus estudios de Veterinaria en la Universidad de Zaragoza en el curso 1988-89. Obtuvo el título de Licenciado en Veterinaria en Junio de 1993. Posteriormente, durante el curso 1993-94, realiza un Master en Desarrollo Rural y Empresas Agroalimentarias en Maeztu (Alava), becado por el Gobierno Vasco.

En 1994, inicia su actividad investigadora en el Servicio de Investigación Agraria de la Diputación General de Aragón con una beca del Instituto Nacional de Investigaciones Agraria (INIA), participando en varios proyectos relacionados con la caracterización de la mortalidad embrionaria ovina. Cursa el módulo de Reproducción Animal dentro del Curso de Postgrado de Producción Animal en el Instituto Agronómico Mediterráneo de Zaragoza

Durante el verano de 1996, se incorpora al Departamento de Producción Animal de la Universitat de Lleida. Desde ese momento, participa como becario en diversos contratos/convenios de investigación firmados con empresas del sector lácteo y lleva a cabo diversos programas de control reproductivo en explotaciones de vacuno lechero.

Actualmente, y desde Enero de 1997, disfruta de una Beca Predoctoral para la formación de Investigadores concedida por el Comissionat d'Universitats i Recerca de la Generalitat de Catalunya.

Durante la elaboración del presente trabajo, ha realizado dos estancias en otros centros de investigación. La primera, en Agosto-Septiembre de 1998, en el Department of Dairy Science, Virginia Polytechnic Institute and State University (E.E.U.U) bajo la supervisión del Dr. Saacke y el Dr. Nebel. La segunda estancia, durante Junio-Septiembre de 1999, en la Université Catholique de Louvaine (Bélgica), bajo la dirección del Dr. Massip.

1988-93	Degree of Veterinary at School of Veterinary. University of Saragossa.
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1998	Visiting worker in the Department of Dairy Science. Virginia Polytechnic Institute and State University (USA). From 9th August to 30th September Professors: R. Nebel and R. Saacky
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