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The Endometrium of the Gynaecologically Healthy Mare during Oestrus

A clinical, morphological, chemical and immunological study

Ann-Marie Tunón

SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

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Betygsnämnd:	Professor Gunnar Alm, SLU Professor Hans Kindahl, SLU Professor Erkki Koskinen, Helsingfors universitet Docent Jan-Otto Lindell, Agria Docent Yvonne Ridderstråle, SLU

Abstract

Subfertility in mares increases with age. Among the common clinical findings in subfertile mares are an accumulation of intrauterine fluid, increased sensitivity to intrauterine infections and endometrial fibrosis, all of which are typical symptoms of persistent endometritis. It is therefore essential to study the gynaecologically healthy mare endometrium in order to gain a better understanding of its defence mechanisms. The aim of the research described in the present thesis was to study parameters of importance for the normal function of the equine endometrium during oestrus, in mares of various ages and parities. Special emphasis was placed on studying the formation of the intraluminal uterine fluid. In addition, the content of immunoglobulins in the intraluminal fluid and the occurrence and distribution of T-cells in the endometrium were determined.

A total of 92 mares, Warmblood, Standardbred and Finnish breeds, were examined once during oestrus. The gynaecological examination included palpation of the genital organs, ultrasonography, vaginoscopy, cytological and bacteriological sampling of the

endometrial surface, and the collection of undiluted uterine fluid. In addition, a videoendoscopical examination of the uterine mucosa was made, in connection with which small endometrial tissue samples were sampled for biopsy from visually selected areas. Based on the anamnesis together with the clinical examination and analyses of the collected samples, the mares that could be categorised as gynaecologically healthy were included in the studied population, 53 mares in total.

The fine structure of the secretory endometrium was studied at the light and electron microscopical levels. A distinct oedema of the lamina propria was evident. The glandular epithelium presented clear signs of secretory activity with pronounced secretory vesicles containing electron-dense granula in the ad-luminal cytoplasm and a well-developed supranuclear Golgi apparatus. Secretory products and cell debris were commonly found in the gland lumen. X-ray microanalysis was performed on frozen endometrial tissue samples and microdroplets of intraluminal fluid and serum. Concentrations of selected elements (Na, K, Cl, S, P and Ca) were analysed in secretory vesicles in endometrial gland epithelium and compared with concentrations in undiluted uterine fluid and in serum. The elemental concentrations in undiluted uterine fluid were similar to those measured in serum, whereas the concentrations of elements in the secretory vesicles differed sharply from those in both uterine fluid and serum. The protein profiles (SDS-PAGE) and concentrations of total protein, albumin and immunoglobulins A and G were determined in both undiluted uterine fluid and serum. There was a clear similarity between the protein profiles of uterine fluid and those of serum. Concentrations of total protein and albumin in uterine fluid were 52-56 % of those in serum. The concentration of immunoglobulin (Ig) A in undiluted uterine fluid was similar to its concentration in serum, whereas the concentration of IgG was approximately 45 % as high as its concentration in serum. The distribution and number of MHC class II, CD4 and CD8 expressing cells during oestrus in different segments of the endometrium were studied using immunohistochemistry. The three cell subsets were detected in all studied endometrial samples, with numbers of all three being higher in the uterine body than in the horn.

In conclusion, during oestrus, the gynaecologically healthy equine endometrium is oedematous, and the uterine glands show intense secretory activity. Both the elemental composition and the protein profile of undiluted uterine fluid resembled the corresponding composition/profile of serum, thereby leading to the conclusion that the cyclical accumulation of uterine fluid during oestrus to a great extent is a transudate which is likely to have a diluting effect upon the specific secretion of the glands. Age had no influence on any of the studied chemical or immunological parameters.

Kew words: normal endometrium, uterine fluid, morphology, x-ray microanalysis, protein profiles, T-cells, oestrus, mare.

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Ann-Marie Tunón
Department of Obstetrics and Gynaecology
and
Department of Anatomy and Histology
Uppsala

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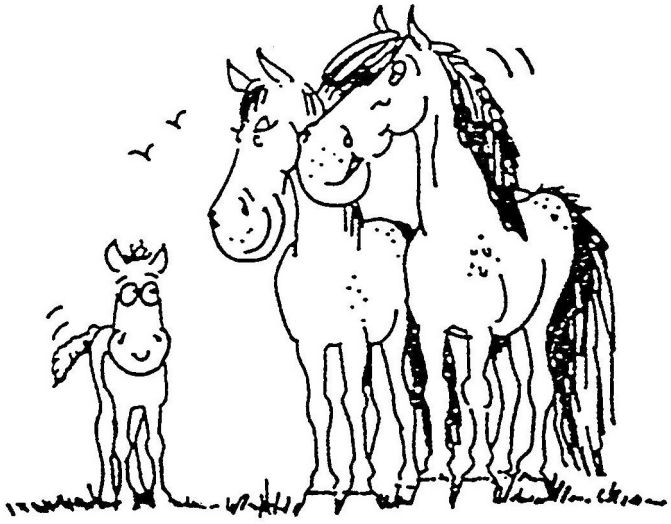

Abstract

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Author's address: Ann-Marie Tunón, Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, Centre of Reproductive Biology (CRB), Swedish University of Agricultural Sciences (SLU), P.O.Box 7039, SE-750 07, Uppsala, Sweden.

Contents

Appendix

Papers I-IV

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals **I-IV**:

I. Tunón, A-M., Rodríguez-Martínez, H., Haglund, A., Albihn, A., Magnusson, U. and Einarsson, S. 1995. Ultrastructure of the secretory endometrium during oestrus in young maiden and foaled mares. *Equine Vet J* 27 (5) 382-388.

II. Tunón, A-M., Ekwall, H., Nummijärvi, A. and Rodríguez-Martínez, H. 1999. X-ray microanalysis of the secretory epithelium of the endometrial glands and intraluminal uterine fluid in oestrus mares. *(Submitted).*

III. Tunón, A-M., Rodríguez-Martínez, H., Hultén, C., Nummijärvi, A. and Magnusson, U. 1998. Concentrations of total protein, albumin and immunoglobulins in undiluted uterine fluid from gynaecologically healthy mares. *Theriogenology* 50 (6) 821-831.

IV. Tunón, A-M., Rodríguez-Martínez, H., Nummijärvi, A. and Magnusson, U. 1999. Influence of age and parity on the distribution of MHC class II, CD4 and CD8 expressing cells in the equine endometrium during oestrus. *Am J Vet Res* (accepted for publication).

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Introduction

Pathological changes in the normal endometrium of the mare that lead to subfertility are common problems in equine reproduction. The pregnancy rate in mares after natural service is generally low, around 65-70 % (Rossdale and Ricketts, 1980). Selection of mares for breeding is based on good performances on the racing track etc., rather than on good reproductive performances. One problem in subfertile mares is the presence of persistent/recurrent endometritis. This condition, and the severity of histopathological findings, increase with age and multiparity (Ricketts and Alonso, 1991). Mononuclear cell infiltrations (chronic infiltrative endometritis) and glandular degenerative changes with peri-glandular and/or diffuse stromal fibrosis (chronic degenerative endometrial disease) are two of the most commonly recognised histopathologic features in endometrial biopsy specimens collected routinely from subfertile mares (Ricketts 1978).

Cyclical accumulation of uterine fluid during oestrus is often seen in excessive volume in subfertile mares (Knudsen, 1964a; Allen and Pycock, 1988). The anatomy of the equine uterus seems to favour this accumulation of fluid. The uterus consists of one uterine body and two uterine horns, with a short septum marking the internal bifurcation of the horns. On side view, the uterus may be V-shaped depending on the extent of suspension and on the relationship between the reproductive organs and other abdominal viscera (Ginther, 1992). The caudal part of the uterine horns is the lowest point at which fluid can accumulate. During oestrus, the uterus is flaccid, and the lumen is obliterated by the collapsed walls and the prominent endometrial folds (Ginther, 1992). Oedema is present in the *lamina propria* due to an increase in vascularity and congestion which causes movement of fluid from vessels into the interstitial area (Ginther, 1992). The surface epithelium of the endometrium is high columnar during oestrus, with vacuoles in the apical cytoplasm assuming a secretory role (Keenan et al., 1991). The branched endometrial glands extend through the entire *lamina propria*, and open on the endometrial surface (Kenney, 1978).

In a study based on slaughterhouse material from 1,476 mares, it was found that about 80% of the mares in oestrus had macroscopic and/or microscopic uterine oedema. No differences in plasma levels of progesterone or oestrogen were found between mares with or without oedema (Henry et al., 1981). In the same study, 6.4% of the mares had severe macroscopic oedema. Although these mares were infected slightly more often than those without oedema, there was no significant difference in infection frequency between these groups of mares. Nor did age influence the development of severe uterine oedema (Henry et al., 1981). The fluid

of the genital tract is important for sperm capacitation and fertilisation as well as for early embryonic development (Heuer et al., 1993). As mentioned above, excessive accumulation of intrauterine fluid during oestrus is an often reported clinical finding in subfertile mares which most probably impairs sperm transport, fertilisation and embryo development. However, small amounts of intrauterine fluid during oestrus do not seem to affect fertility rate (Reilas et al., 1997). Troedsson and Liu (1992) showed that following an intrauterine inoculation of bacteria, susceptible mares had an increased volume of uterine fluid compared with normal/resistant mares. LeBlanc et al. (1989) reported that after ovulation, mares susceptible to endometritis accumulated more fluid within the uterine lumen than did resistant mares. If the formation of free intraluminal fluid in both normal/resistant mares and subfertile/susceptible mares is associated with the mechanism which produces normal oestrous oedema remains to be determined. It is not established whether it is an increased secretory activity and/or a transudation effect. Ultrastructural studies of the secretory endometrium might help to answer this question. Only a few studies of the equine endometrium have included transmission electron microscopy (Helde, 1978; Keenan et al., 1991; Ferreira-Dias et al., 1999).

Many studies have been performed with the aim of examining uterine defence mechanisms in the mare and determining whether there is an impaired local immunity that is responsible for the increased susceptibility for uterine infections in some mares. In many of these studies, so-called susceptible mares have been studied and compared with so-called normal/resistant mares, often in a small number of animals, with unknown reproductive history, or of unspecified age (Blue et al., 1982; Asbury et al., 1984). Nor has a uniform set of criteria been established to define when a mare should be classified as normal/resistant or susceptible. The most commonly used is to term mares that eliminate infection within 72 h of intrauterine infusion of bacteria as resistant and thus term mares that remain infected as susceptible. Parameters that can be used to unequivocally define a gynaecologically healthy mare endometrium are needed to serve as reference points in studies of subfertile mares.

Coitus, parturition and genital examinations are three possible sources of intrauterine contamination. The cervical canal has no obstructing rings, and during oestrus, when coitus and most genital examinations occur, it becomes flaccid and easy to penetrate. Physical clearance, i.e. uterine secretion and myometrial contractions, may be important for the early elimination of infections, and it has been established that physical clearance is more efficient in young mares (Evans et al., 1987). The defence mechanisms of the uterus include both innate and adaptive immunity. The phagocytes of the innate immunity provide a first line of defence against bacterial infections (Janeway and Travers, 1998). The most important cells

of the adaptive immunity are the lymphocytes, T- and B-cells, which can recognise antigens and produce specific antibodies. There is evidence that uterine immunoglobulins are locally produced in the equine endometrium (Mitchell et al., 1982; Widders et al., 1984). When uterine flushings were analysed, Asbury et al (1980) and Mitchell et al. (1982) reported that concentrations of immunoglobulins in uterine fluid tend to be higher in mares with low resistance to endometritis than in more resistant mares. These findings were not confirmed by Troedsson et al. (1993a) when undiluted uterine fluid was analysed after intrauterine challenge of bacteria in resistant and susceptible mares. They did not find any significant differences between these groups of mares. It has yet to be established whether concentrations of immunoglobulins in the uterine fluid of healthy mares vary with age and parity.

The distribution of immunologically competent cells in the endometrium has been described in several species, e.g. in humans (Tabibzadeh et al., 1986), sheep (Lee et al., 1988), horses (Watson and Dixon, 1993), pigs (Bischof et al., 1994) and cattle (Cobb and Watson, 1995). The specific immune response is directed by T-helper cells which respond to and release different cytokines and thereby promote macrophage activation or favour antibody production (Roitt et al., 1998). Kamat and Isaacson (1987), who studied lymphocyte subsets in human endometrium, reported that cytotoxic T-cells were more frequent than helper T-cells. In the endometrium of the mare, helper T-cells were more numerous than cytotoxic T-cells, and in mares with endometritis, the densities of both subsets were significantly increased (Watson and Thomson, 1996). Although it has been stated that the morphology and function of the equine endometrium changes with age (Carnevale and Ginther, 1992), it remains to be established whether the distribution of T-cells is modified by these parameters, especially in gynaecologically healthy mares.

Aims of the study

The overall objective of the present work was to characterise various aspects of the oestrous endometrium of gynaecologically healthy mares differing in age and parity. Special emphasis was placed on studying the formation of the intraluminal uterine fluid. In addition, the content of immunoglobulins in the intraluminal fluid and the occurrence and distribution of T-cells in the endometrium were determined. All variables were investigated in relation to age and parity in order to establish reference values for pathological studies. The particular aims were to:

- describe the ultrastructure of the endometrium, especially the secretory epithelia,
- compare the elemental composition (using a selection of elements) of secretory vesicles with that of the undiluted intraluminal uterine fluid,
- determine the protein profiles in undiluted uterine fluid, as well as its concentrations of total protein, albumin, and immunoglobulins A and G,
- determine the distribution and numbers of MHC class II, CD4 and CD8 expressing cells in the equine endometrium and identify any differences in the distribution of these cell subsets between uterine segments.

Materials and Methods

Animals

A total of 92 mares, 3 to 19 years of age, were examined with the aim of recruiting some of them for the various studies. The mares were of Warmblood, Standardbred or Finnish breeds. Forty-five were privately owned; 35 were owned by the Agricultural Research Centre, Equine Research, Ypäjä, Finland, and 12 were owned by the Department of Obstetrics and Gynaecology, Swedish University of Agricultural Sciences (SLU), Uppsala.

Clinical examinations

The examinations were carried out during the physiological breeding seasons of 1991-1997 at the Dept. of Obstetrics and Gynaecology, SLU, Uppsala and at the Agricultural Research Centre, Equine Research, Ypäjä, Finland (1997). Prior to any intrauterine examination and/or sampling, the tail of the mare was bandaged and the perineal area was thoroughly cleaned and dried. The genital examination included palpation and ultrasonography of the internal genitalia per rectum, vaginoscopy, retrieval of bacteriological and cytological smears, collection of undiluted uterine fluid and videoendoscopy. Ultrasonography was used to evaluate ovarian status and to document the presence and extent of uterine oedema and intraluminal fluid. Videoendoscopy was performed in order to examine the endometrial surface and collect endometrial tissue for biopsy. Blood samples were collected for determination of estradiol-17β and progesterone concentrations in plasma. Each mare was sampled –as described above– once during oestrus.

Bacteriological and cytological examinations

Samples from the endometrial surface for bacteriological and cytological examinations were collected with long, double-guarded swabs. For bacteriological examination, the samples were placed into Amies' charcoal transport medium immediately after collection and were plated out on blood agar within 8 hours. Aerobic cultures were performed at 37°C for 48 hours. Colony growth was categorised as no growth, sparse- (1-10 colonies), moderate- (10-100 colonies) and heavy growth (>100 colonies). For cytological examination, the samples were immediately rolled onto slides, air-dried and fixed in 99.8% methanol and stained with May-Grünwald-Giemsa stain. Cytological smears were evaluated according to Knudsen (1964b).

Blood samples and hormone assays

Blood samples were taken by jugular venipuncture using evacuated tubes (Becton Dickinson Europe, Erembodegem, Belgium). Serum was collected in plain tubes and plasma in heparinised tubes. After coagulation and centrifugation the serum was stored at -70°C until used in the assays (total protein, albumin, IgA, IgG, SDS-PAGE and X-ray microanalysis, **Papers II and III**) described below. After centrifugation, blood plasma was stored at -20°C until analysed. Progesterone was determined using an enhanced luminescence immunoassay technique (Amerlite, Kodak Clinical Diagnostics Ltd., England). Serial dilutions of equine plasma with high concentrations of progesterone were parallel to the standard curve. The intra-assay coefficient of variation calculated from the precision profiles was below 7% for concentrations between 2 and 160 nmol/l. The inter-assay coefficients of variation for three quality-control samples were below 10% (2, 18 and 54 nmol/l). The lowest amount of progesterone detectable (defined as the intercept of maximal binding - 2 SD) was 0.2 nmol/l. Oestradiol-17ß was determined using an RIA kit (Diagnostic Products Corporation, Los Angeles, CA, USA), developed for use with bovine plasma (Sirois and Fortune, 1990). Serial dilutions of equine plasma with high concentrations of progesterone were parallel to the standard curve. The intra-assay coefficients of variation, calculated from the precision profiles, were 13% at 6 pmol/l and below 10% from 19 to 180 pmol/l. The inter-assay coefficients of variation for three control samples were 25% (13 pmol/l), 6% (39 pmol/l) and 10% (84 pmol/l). The lowest amount of oestradiol-17ß detectable (defined as the intercept of maximal binding - 2 SD) was 5 pmol/l. The hormone assays were carried out at the Department of Clinical Chemistry, Swedish University of Agricultural Sciences (**Papers I-IV**).

Collection of intraluminal uterine fluid

Uterine fluid was collected with a sterile intrauterine tampon for human use (Tampax® Regular, Tambrands Inc., Palmer, MA, USA, or OB, Mölnlycke Ltd. Sweden). Before use, the tampon was placed in an aluminium applicator and sterilised. The applicator was inserted into the cervical canal before the tampon was inserted into the uterine cavity where it was allowed to absorb uterine fluid for 30 minutes (Katila et al., 1990). The tampon was inserted directly after the collection of bacteriological and cytological swabs. After removal of the tampon from the uterine lumen, the fluid was squeezed out using a manual press. The retrieved fluid was kept on a bed of crushed ice until centrifuged, after which the supernatant was harvested and stored at –70°C until analysed.

Uterine endoscopy

Videoendoscopic examination of the endometrial surface and collection of endometrial tissue for biopsy were performed immediately after the uterine fluid had been collected, in accordance with Håkansson et al. (1992; 1993), with some modifications. Prior to the examination, each mare was sedated with detomidine hydrochloride i.v., 10 μg/kg bwt (Domosedan, Orion Pharma AB, Animal Health, Finland). The endoscope (Fujinon EC7-HM2, Omiya City, Japan, 150-cm-long with an external diameter of 14 mm) was inserted into and guided through the vagina and cervix by the operator. When the flexible tip of the endoscope entered the uterine body the advancement of the endoscope was stopped in order to insufflate CO_2 (CO_2-PNEU, WISAP, Germany) into the uterine cavity using manual flow control. Once the endometrial folds became visible on the monitor, the insufflation was allowed to run automatically. The uterine bifurcation was located and the endoscope was passed forward into one horn until the utero-tubal papilla became visible. After inspection of the endometrial surface, endometrial tissue samples for biopsy (1 mm^3 size) were collected with a flexible biopsy forceps (TW ll/13 b, MTW Endoscopie, Wessel, Germany) which was passed through the working channel of the endoscope. After the inspection and sampling of both horns, endometrial tissue specimens were collected from the body/horn junction. From each uterine segment, 3-5 tissue samples were collected and fixed as described below. The endoscope was used in combination with a videoprocessor (Fujinon EXP-302), and images were continuously monitored (Sony PVM-2043MD) and recorded (JVC BR-S605 E). Videoprints (Sony UP-5000P) were taken from selected areas. After the videoendoscopic examination, uterine lavage was performed using sterile saline solution at room temperature (20-22°C).

Endometrial specimen fixation and further processing

Chemical fixation for morphological studies

Endometrial tissue samples were fixed in a solution of 3% glutaraldehyde in 0.0067 M cacodylate buffer (500 mOsm, pH 7.2) overnight, post fixed in osmium tetroxide, dehydrated and embedded in Agar^{100} plastic resin (Agar Scientific Ltd, Stansted, UK) and, finally, sectioned for histological analysis (**Papers I-IV**).

Ultra-rapid cryofixation for X-ray microanalysis

Another complementary set of endometrial tissue samples retrieved from the body/horn junction were ultra-rapidly impact-frozen against a highly polished solid

copper surface chilled with liquid nitrogen (LN_2) to -196° C using a Reichert-Jung MM80® (Reichert-Jung Optische Werke AG, Wien, Austria) automatic metal-mirror slam-freezer. The samples were kept in cryo-vials and stored in LN_2 prior to X-ray microanalysis (**Paper II**).

Cryofixation for immunohistochemistry

Endometrial tissue specimens were placed in Optimal Cutting Temperature compound (Tissue-Tek®, Sakura Finetek USA, Inc., Torrance, CA, USA) and immersion-frozen in melting 2-Propanol (Merck, Darmstadt, Germany) followed by immersion in LN_2. The tissues were then stored at −70°C until further processing for immunohistochemical analysis (**Paper IV**).

Histological examination

Semi-thin (≅1μm thick) sections, were cut with an ultramicrotome (LKB 2088 Ultrotome®V, Bromma, Sweden), stained with buffered toluidine blue and examined under a light microscope.

Classification of mares

Out of the 92 originally examined mares, 53 passed the strict clinical, uterine bacteriological, cytological, endoscopic and histological criteria imposed. These criteria were: no history of fertility problems, normal oestrus behaviour, no gynaecological abnormalities at clinical examination, no abnormal accumulation of fluid/uterine oedema, less than 5% of leukocytes in cytological samples, no growth of pathogenic bacteria, normal endoscopic aspect of the mucosa and normal histology of the biopsied endometrium, as seen using light microscopy. Of the 53 mares declared gynaecologically healthy and included in the studies, 50 mares had less than 0.5 % of polymorphonuclear leukocytes (PMNs) in their swab sample and three mares had 0.5%-5% of PMNs. All but 12 were bacteriologically negative. Of these, seven had sparse (5) to moderate (2) growth of mixed cultures, four had sparse (2) to moderate (2) growth of Coryneform rods, and one had moderate growth of Klebsiella pneumoniae, all mares that had any bacteriological growth had less than 0.5% of PMNs. The sample of the mare with growth of Klebsiella pneumoniae was considered to have been contaminated during collection since no other sign of pathology was found. These 53 mares were, subsequently, included in the various studies (**Papers I-IV**). The age distribution was 3-16 years (mean 8 years), and the parity distribution was as follows: 14 were maiden mares,

18 had been embryo donors, and 21 had produced 1-6 foals. The mares were categorised by age and parity.

Transmission electron microscopy (TEM, Paper I)

Areas for further examination were selected in the light microscopy slides for subsequent processing. Ultrathin sections were cut with a diamond knife on a Reichert ultramicrotome (Reichert-Jung Optische Werke AG, Wien, Austria), counterstained with uranyl acetate and lead citrate and examined in a Philips 420 TEM or a Philips 201 TEM electron microscope at 60-80 kV.

X-ray microanalysis (Paper II)

Instrumentation

The analysis was performed with a JEOL 6320F scanning electron microscope (SEM, JEOL LTD, Tokyo, Japan) equipped with a cryo-stage (Oxford CT1500 HF, Oxford Instruments, Oxon, England) and an energy-dispersive X-ray spectrometer (LINK® Pentafet 30mm^2 UTW, Oxford Instruments, High Wycombe, England). The retrieved spectra were analysed on an Isis 300 (LINK® Isis, Oxford Instruments, High Wycombe, England) computer, using the software PBQuant computer programme.

Sample preparation

Endometrial glands: Each flat, frozen tissue sample (uterine body) was mounted vertically on a specimen holder under LN_2 and brought into the cryo-stage (–130°C), where it was fractured with a scalpel blade to get an unexposed, tissue surface. A slight water sublimation (30 seconds at –90° C) was performed in order to better visualise the tissue structures. Thereafter, the sample was again cooled down to –140° C and, still in the cryo-stage, the surface was sputtered with a 3-nm-thick layer of chromium to avoid charging effects during the analysis. The sample was transferred into the SEM and analysed at –120° to –130°C. Endometrial glands located close to the best frozen side (e.g. showing the smallest ice crystals) were located and chosen for analysis. Four spectra per gland were obtained from a group of adjacent epithelial cells including, in all cases, the apical cytoplasm (i.e. where the secretory vesicles were localised). An area of 180 μm^2

per gland was analysed at a magnification of x 4,500. A total of four glands from each horse were analysed.

Uterine intraluminal fluid – Ten to 15 micro-droplets (0.05 µl volume/drop) of frozen-thawed intraluminal fluid were quickly placed onto a carbon disc using a Hamilton Microliter® Syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) and frozen in LN_2 vapours (≅ −170°C). The disc was then transferred into the cryo-stage (see above) where it was sputtered with a 3-nm-thick layer of chromium as described earlier. No sublimation of the sample was performed. The entire surface area of ten droplets of intrauterine fluid from each horse was analysed at −120° to −130°C under x 25 magnification.

Quantitative X-ray microanalysis

The individual elemental spectra for sodium (Na), potassium (K), chlorine (Cl), sulphur (S), phosphorous (P) and calcium (Ca) were collected at a count rate between 800 and 1,500 counts per second. The analysis was performed at 12kV accelerating voltage and a beam current of 6×10^{-10}A. The spectra collection time was 100 seconds (live time) for all samples. The quantitative analysis was based on the ratio of the characteristic intensity to the background intensity in the same energy region peak-to-background (P/B ratio). The P/B ratios and the peak-to-continuum ratios obtained from the specimens were compared to those obtained from gelatine-based standards containing known amounts of mineral salts (Roomans 1988). Water was chosen as sample matrix, to resemble the main substance of the specimen. The value of each individual spectrum was expressed as mmol per kg wet weight.

Determination of proteins (Paper III)

Total protein concentration in serum and intraluminal uterine fluid

Concentrations of total protein were measured using the Biuret method (Peters et al., 1982) on an automated multi-channel analyser (Cobas Mira, Hoffman-La Roche AG, Basel, Switzerland) with a commercially available reagent kit (Unimate 7 TP, Hoffman-La Roche AG). Commercially available animal standard and control sera (Seronorm and Pathonorm, Nycomed, Lidingö, Sweden) were analysed on each assay occasion. The inter-assay coefficient of variation (CV) was 1.2%. The protein assays were carried out at the Department of Clinical Chemistry, Swedish University of Agricultural Sciences.

Protein separation

Proteins were separated on a sodium dodecylsulphate-polyacrylamid electrophoresis (SDS-PAGE) gel according to Laemmli (1970). Samples of uterine fluid, serum and marker proteins (LMW Electrophoresis Calibration Kit, Pharmacia Biotech) were dissolved in sample buffer containing 0.01 M Tris / 0.001 M EDTA / 1% SDS / 5% 2-mercaptoethanol (pH 8.0), heated for 5 minutes at 100° C and run under dissociating conditions in 8-25% polyacrylamide gradient gels (PhastGel Gradient 8-25, Pharmacia Biotech, Uppsala, Sweden) for 70-80 Vh. Protein bands were stained with Coomassie brilliant blue. The protein profiles were scanned and evaluated using an Ultroscan XL laser densitometer with GelScan XL 2.0 software (Pharmacia Biotech). The protein concentration of each band was calculated based on the relative area under the curve and the known total protein concentration of the sample. Molecular mass was determined from the migration distance of the protein bands using the marker proteins as standard.

Albumin in serum and uterine fluid

Concentrations of albumin in serum and uterine fluid were analysed using the bromocresol-green (BCG) method modified from Doumas et al. (1971) and validated by Keay et al (1983) with formation of an albumin/bromocresol-green complex at pH 4.2 and photometric measurement of absorbance at 600 nm after 25 seconds. The analyses were done on an automated multichannel analyser (Cobas Mira, Hoffman-La Roche AG, Basel, Switzerland) with a commercially available reagent kit (Albumin 1442201, Boehringer Mannheim, Germany). Commercially available animal standard and control sera (Seronorm and Pathonorm, Nycomed, Lidingö, Sweden) were analysed on each assay occasion. The inter-assay CV was 3.1%. The albumin assays were carried out at the Department of Clinical Chemistry, Swedish University of Agricultural Sciences.

Immunoglobulins A and G in serum and intraluminal uterine fluid

Concentrations of immunoglobulin (Ig) A and G in serum and uterine fluid were analysed using a single radial immunodiffusion test kit according to Mancini et al. (1964), specific for equine IgA and IgG, (RID-kit, The Binding Site, Birmingham, UK). In the IgA kit, the antibodies detected mono- as well as dimeric IgA, and the standard IgA was obtained from serum.

Immunohistochemistry (Paper IV)

Cryostat sections (6 µm thick) were placed on poly-L-lysine-coated slides (Polysine™ Microslides, Menzel-Gläser, Germany) and fixed in acetone for 10 minutes at room temperature (18-22°C). Endogenous peroxidase activity was blocked using 0.28% periodic acid for 25 seconds, and non-specific binding of the primary antibodies was blocked with 10% normal goat serum for 30 minutes at room temperature. Immunohistochemistry was performed using an avidin-biotin-peroxidase staining method. Mouse monoclonal antibodies (VMRD Inc., Pullman, WA, USA) were used as primary antibodies and applied overnight at +4°C. The antibodies used were equine (eq) MHC class II (H42A) at a concentration of 1 µg/ml, eq CD4-like (HB61A) at 0.02 mg/ml and eq CD8-like (HT14A) at 0.02 mg/ml. As secondary antibody, biotinylated goat anti-mouse immunoglobulins (Dakopatts A/S, Glostrup, Denmark) were applied at 1/300 dilution for 30 minutes. Horseradish-labelled avidin (avidin/HRP, Dakopatts A/S, Glostrup, Denmark) was added at 1/1000 dilution for 30 minutes. Diaminobenzidine was used as substrate. The sections were counterstained with Mayer's haematoxylin and mounted. Sections of an equine lymph node were used as positive controls. As negative controls, Mouse IgG1 (X 0931, Dakopatts A/S, Glostrup, Denmark) and Tris-buffered saline replaced the primary antibody. To quantify MHC class II, CD4 and CD8 expressing cells, all positively stained cells within five fields ($0.31 mm^2$) in the *stratum compactum*, a sample from the uterine body and another one, randomly selected sample from one horn, were counted using an ocular reticulum in a light microscope (Olympus BH2, Tokyo, Japan) at x 400 magnification. The reticulum was placed so that one side was parallel to the surface epithelium, whereupon five randomly picked-up areas were examined.

Statistical analyses

In **Paper I**, plasma levels of progesterone and oestradiol-17β were compared between groups of mares using a Student's t test for unpaired samples. In **Papers II-IV**, data were analysed using the SAS Statistical package (SAS version 6.12, SAS Institute Inc. Cary, NC, USA, 1996). Normality of distribution was assessed using the univariate procedure, and correlation coefficients were calculated using Spearman's method. Analyses of variance (GLM procedure) were used to evaluate the effects of *age* and *parity* **(Paper II-IV)** and *day of collection* **(Paper III)** on the traits. A Student's t-test was used to analyse the differences in elemental composition between endometrial glands, serum and uterine fluid **(Paper II),** the difference in protein concentration between serum and uterine fluid **(Paper III)** and the difference in numbers of labelled cells between the uterine body and horn **(Paper IV).** In a preliminary model in **Paper II**, the mares were categorised in

three different age groups (3-5, 6-8 and 9-16 years), and the effect of *breed* was also included. Neither *age* nor *breed* affected the elemental composition. *Breed* was thus omitted from the model, and it was concluded that the mares could be categorised in two age groups, 3-8 and 9-16 years. In **Paper IV,** values for CD4 expressing cells in the uterine horn sections were log transformed before analysis since they were not normally distributed. For clarity, these data were reconverted to anti-log values before presentation. In a preliminary model, the effects of *breed* and *day of collection* of the biopsy specimens during oestrus were included. *Day of collection* was categorised as coinciding with early (day 1 to 3) or late (day 4 to 6) oestrus. Neither *breed* nor *day of collection* of the biopsy specimens affected the number of cells expressing MHC class II, CD4 or CD8. Both effects were thus omitted from the final model. Data are reported as least-square mean (LSMean) values obtained from GLM unless indicated otherwise **(Paper IV)**. Differences were considered significant when $p<0.05$ **(Papers I-IV).**

Results

Ultrastructure of the equine endometrium (Paper I)

Light microscopy

The endometrium samples taken for biopsy consisted mainly of *stratum compactum*. The sections had a surface epithelium consisting of cuboidal to tall columnar cells, of which about half were ciliated. Some of the epithelial cells seemed to have basally located, empty hollow spaces, suggesting the presence of basal vacuoles. Secretory vesicles were located in the apical, supranulear area. Sometimes, the secretory cells presented blebs protruding towards the lumen. 'Macrophage-like' cells were often present, intermingled with the tall epithelial cells. A distinct oedema was present in the *lamina propria* where different cells e.g. mast cells, leukocytes and plasma cells, were seen in the vascularised connective tissue together with numerous tubular convoluted glands with a cuboidal/cylindric epithelium. In the gland ducts, there were ciliated and non-ciliated cells intermingled, similar to the situation in the surface epithelium. The deeper areas of the glands presented a higher cell density and a small or non-existing lumen. Cell debris was common in the lumen of the glands.

Transmission electron microscopy (TEM)

The TEM confirmed that the healthy mare endometrium during oestrus consists of a simple columnar surface epithelium with both ciliated and non-ciliated cells. Lateral cell-to-cell junctions consisting mostly of tight junctions and numerous desmosomes anchored the cells to each other. The basal plasmalemma was apposed to the basal lamina but rather often the cells were separated by large intercellular spaces which appeared in the light microscope as 'vacuoles'. The non-ciliated cells presented a cytoplasm rich in organelles, particularly mitochondria, rough endoplasmatic reticulum, Golgi apparatus and numerous secretory vesicles with a rather electron-dense amorphous content at the apical ad-luminal area. The apical membrane of the cells was covered with microvilli. The ciliated cells did not have as many secretory vesicles as the non-ciliated cells. The surface epithelium, particularly in the uterine body, often presented invasive cells, such as granulocytes and intraepithelial macrophages, intermingled with the epithelial cells.

The structure of the epithelium of the uterine glands varied with the depth of the section. The gland duct presented cells that were remarkably similar to those making up the surface epithelium, except that the non-ciliated cells did not present obvious secretory vesicles. The mid-deep glandular portion was, however, loaded

with apical secretory vesicles in the non-ciliated cells, while ciliated cells were less common here. In summary, the glandular epithelium presented clear signs of secretory activity with pronounced secretory vesicles containing electron-dense granula in the ad-luminal cytoplasm and a well-developed supranuclear Golgi apparatus. In the gland lumen, secretory products and cell debris were commonly found. The mechanism of secretion appeared to be merocrine, i.e. through exocytosis. No differences between maiden and foaled mares were observed.

Elemental composition of endometrial gland epithelium, uterine fluid and serum (Paper II)

All elements selected for analysis, i.e. sodium (Na), potassium (K), chlorine (Cl), sulphur (S), phosphorus (P), and calcium (Ca), were detected in endometrial glands, uterine fluid and serum, although the concentration of Ca in secretory epithelium and serum and the concentrations of P and K in intrauterine fluid and serum barely exceeded the detection limit (10 mmol/kg wet weight). There was a significant difference ($p=0.0001$) in concentrations of all analysed elements (except S) between the uterine secretory gland epithelium and the intraluminal fluid. Differences in concentrations between the uterine intraluminal fluid and serum were significant ($p<0.01$) for all analysed elements except K and P. The results revealed a resemblance in the relationship among the different elements in uterine intraluminal fluid and serum, with Na and Cl dominating. In the secretory vesicles, K and P were the major elements found, but only small amounts of Na and Cl were detected. Neither age nor parity affected the content of any of the analysed elements in the secretory glandular epithelium, uterine fluid, or serum.

Protein concentrations and protein profiles in undiluted uterine fluid and serum (Paper III)

Concentrations of total protein, albumin and immunoglobulins A and G in uterine fluid were 44-56% of those in serum, except for IgA, which had a similar concentration in both serum and uterine fluid. The protein profiles obtained showed six major peaks, with a clear similarity between the protein profile for serum and that for uterine fluid. The protein concentration was significantly higher in serum than in uterine fluid in peaks 2, 3, 4, and 6 ($p<0.001$). Neither age nor parity affected the concentration of total protein or albumin in uterine fluid. The concentration of the proteins corresponding to peak no. 3 in uterine fluid, with a molecular weight (mw) of 60-71 kDa, was significantly higher ($p= 0.04$) in young

mares (20.9 g/l) than in older ones (14.6 g/l). Parity had no effect on any of the uterine variables recorded. Day of sampling affected protein concentrations in uterine fluid, which were significantly higher on day two compared with day four of oestrus, ($p<0.05$). There was no correlation between uterine fluid and serum with respect to concentrations of total protein, albumin, IgA and IgG.

Distribution and number of MHC class II, CD4 and CD8 expressing cells in the equine endometrium (Paper IV)

The three subsets of cells (i.e. MHC class II-, CD4- and CD8-expressing cells) were found in all examined endometrial samples. The MHC class II expressing cells were prevalent in the *stratum compactum*, mainly as single 'monocyte-like' cells. MHC class II was also expressed by the surface epithelium and, to a lesser extent, by the gland duct epithelium and endothelial cells of minor vessels. Cells expressing CD4 and CD8 were predominantly seen lying near the surface epithelium. The mean ratio for CD4/CD8 expressing cells was 0.95 for the uterine body and 1.23 for the uterine horn. There were significantly more cells expressing CD4 and CD8 in the uterine body than in the horn ($p = 0.046$ and $p = 0.0001$, respectively). Age had no effect on any of the examined cell subsets. Parity significantly affected the number of MHC class II expressing cells in the uterine body as well as in the horn ($p = 0.02$ and $p = 0.04$, respectively). In the uterine body, the number of MHC class II expressing cells was significantly higher in maiden mares and foaled mares ($p = 0.008$ and $p = 0.036$, respectively) than in the ET-donor mare group. There were significantly ($p = 0.027$) more cells expressing MHC class II in the uterine horn of mares that had foaled compared with mares that had been ET-donors. Parity did not affect the number of CD4- or CD8-expressing cells. There were highly significant correlations ($p = 0.0001$) between the number of CD4- and CD8-expressing cells within the same uterine region, the correlation coefficients (r) being 0.68 for the uterine body and 0.81 for the uterine horn. There was no significant correlation in the number of labelled cells between the uterine body and horn, nor were there any corresponding correlations for CD4+ cells ($r=0.05$) or CD8+ cells ($r=0.24$). However, the number of cells expressing MHC class II in the uterine body was significantly correlated ($p = 0.03$, $r=0.48$) to the corresponding number in the uterine horn. There were no significant correlations between the concentrations of estrogen or progesterone in blood plasma and the numbers of cells expressing MHC class II, CD4 or CD8.

General discussion

Thorough knowledge of the gynaecologically healthy endometrium, morphologically as well as immunologically, is essential when studying the endometrium of subfertile mares. Development of sophisticated equipment for non-invasive imaging and intra-cavital observation, e.g. real-time ultrasonography and videoendoscopy, now offer the clinicians the opportunity to improve diagnostic accuracy when examining the female genital tract. Furthermore, analytical laboratory techniques such as scanning- and transmission electron microscopy, the use of cryobiological methods, advances in microspectrometry, improved biochemical protein determinations and the development of monoclonal antibodies for immunohistochemistry, etc., allow samples retrieved when examining the uterus to be more comprehensively analysed.

The overall objective of the present work was to characterise various aspects of the endometrium of gynaecologically healthy mares during oestrus. Oestrus is the particular stage of the oestrous cycle during which most clinical examinations are performed and is also the stage of natural mating or AI, all of which can contribute to the contamination of the lumen and the development of endometritis. Special emphasis was placed on studying the formation of the intraluminal uterine fluid and some uterine defence mechanisms.

The fine morphology of the endometrium was studied using both light microscopy and transmission electron microscopy (TEM **Paper I**). This work confirmed that the *lamina propria* appears oedematous during oestrus, as reported by Kenney (1978). Epithelial cells covered with microvilli, both on the surface and in the glands, especially in the deeper regions, were loaded with secretory vesicles, indicating a high level of secretory activity which was also reported by Helde (1978). Rough endoplasmatic reticulum, mitochondria and Golgi apparatus were abundant, indicating active protein synthesis. There were no clear signs of apocrine secretion, and it seemed that the mechanism of secretion was merocrine i.e. by exocytosis. TEM revealed that the basally located 'vacuoles' seen in the surface epithelium under the light microscope were actually large intercellular spaces. These spaces might have been formed by the pressure of the oedematous connective tissue of the *lamina propria*. In the study described in **Paper II,** X-ray microanalysis was used to compare concentrations of Na, K, Cl, S, P, and Ca in the intraluminal fluid and in the secretory vesicles of the epithelium of endometrial glands with those in blood serum. It was found that uterine fluid and serum resemble each other with regard to their proportional distributions of the analysed elements. Sodium and Cl were the dominant elements in uterine fluid and serum,

whereas K and P were the major elements in glandular epithelium. In addition, using SDS-PAGE (**Paper III**) for protein analysis it was shown that the protein profile of undiluted uterine fluid was similar to that of blood serum. The high albumin concentration in the uterine fluid, 56% of that in serum, is also interesting; indicating that the presence of this protein in the uterine cavity is probably the result of transudation. Moreover, the concentration of total protein in uterine fluid was 52% as high as its concentration in serum. Taken together, the above findings indicate that serum transudation strongly influences the composition of uterine fluid and most likely dilutes the specific secretion produced by the uterine glands.

The ultrasonographic examination did not disclose any free intraluminal fluid in any of the mares included in the different studies (**Paper I-IV**). The mechanism responsible for the excessive accumulation of intraluminal fluid during oestrus in some mares, preferably those classified as subfertile, has yet to be determined. It is unlikely that the accumulation of fluid could be due to increased secretory activity since there are no reports of increased glandular activity or a histological picture of an increase in the number of glands in these mares. It seems more likely that this accumulation of fluid is due to an increased transudation of intercellular fluid from the oedematous *lamina propria*. However, it is unclear whether the extent of fluid accumulation is directly related to the amount of oedema present. Henry et al. (1981) reported that of the genital tracts collected at slaughter from mares with severe macroscopic oedema, no accumulation of fluid was found in two-thirds of them while the other third had a small quantity of fluid in the uterine lumen. However, the reliability of these data might be questionable since the genital tracts were collected after slaughter, and thus some changes may have occurred post-mortem. Kotilainen et al. (1994) found that uterine fluid collection scores were positively correlated with the degree of endometrial oedema, although the correlation was low. Normally, the tissue fluid is resorbed from the intercellular compartment of the endometrium through the lymphatic and capillary vascular bed of the mucosa. This resorption might be suppressed or reduced in subfertile mares owing to lymphatic dysfunction as suggested by LeBlanc et al. (1995) and/or by impaired uterine activity as suggested by Troedsson et al. (1993b). Although the intraluminal fluid decreased spermatozoal motility in an *in vitro* test, small volumes of such fluid during oestrus do not seem to affect fertility rate (Reilas et al., 1997). In other studies it was found that the accumulation of intraluminal fluid after ovulation and in early pregnancy led to a lower pregnancy rate (McKinnon et al., 1987; Adams et al., 1987). Intraluminal fluid accumulation seems to play a negative role in uterine function. The extent to which this accumulation of fluid affects pregnancy rate has yet to be determined, although it appears that fertility is impaired when the volume of fluid exceeds a certain threshold level.

The ambition of the present work was to have a thorough selection of the mares finally included in the different studies (**Paper I-IV**). None of the mares had a history of uterine infections or fertility problems, and no abnormal clinical or histopathological signs were found in any of them. When mares considered gynaecologically healthy or resistant to endometritis are to be compared with mares considered to be subfertile or susceptible to endometritis, it is essential to have a firm set of criteria that can be used to assign mares to the different groups. Furthermore, one should be able to clearly define several parameters, such as reproductive history, age, parity, etc. since these parameters may be related to the susceptibility to the establishment of reproductive disorders. Williamson et al. (1989) reported that a history of recurrent endometritis provided a more sensitive and specific indication of susceptibility to uterine infection than a uterine biopsy with significant endometrial degeneration. In addition, the age of the mares is important, since it has been reported that the morphology and function of the uterus changes with age (Carnevale and Ginther, 1992), and there is a significant correlation between severity of chronic degenerative endometrial disease and age (Ricketts and Alonso, 1991). Moreover, when LeBlanc et al. (1989) compared resistant, young, nulliparous mares with susceptible, old, multiparous mares they did not find any significant difference in the retention of charcoal or microspheres between the different groups, but the susceptible mares accumulated larger volumes of intraluminal fluid after ovulation compared with the resistant mares. The extent to which this accumulation of fluid in older mares is due to chronic inflammatory changes in the endometrium, multiparity, or ageing is difficult to assess. Histopathological changes in the *lamina propria*, connected with a disorder in the capillary and lymphatic bed, might account for this accumulation (Ferreira-Dias et al., 1999).

Susceptibility to persistent/recurrent uterine infections in the mare is likely to be caused by a failure of the physical and/or immunological uterine defence systems. Conflicting results have been obtained in studies comparing normal and subfertile mares in terms of immunoglobulin concentrations in their genital tract. However, in all studies it was found that both IgA and IgG occurred in the genital tracts of both groups of mares. In gynaecologically healthy mares, the concentration of IgA in undiluted uterine fluid was similar to its concentration in blood serum, whereas the concentration of IgG was approximately 45% as high as its concentration in serum (**Paper III**). The results regarding subfertile mares are divergent considering whether there is an increase in the concentrations of immunoglobulins or not in this group of mares. Asbury et al (1980) and Mitchell et al (1982) reported that concentration of both IgG and IgA were elevated in susceptible mares whereas Williamsson et al (1983) reported higher concentrations of IgA, but not IgG, in mares with endometritis. In all these studies, uterine lavage was used which diluted the uterine fluid. Actual concentrations of uterine fluid in each lavage were

unknown, thereby decreasing the reliability of those results. In the study described in **Paper III** and the study by Troedsson et al. (1993a), undiluted uterine fluid was collected with a tampon using the technique developed by Katila et al. (1990). Elevated concentrations in subfertile mares compared with normal mares could not be found after inoculation of bacteria when Troedsson et al. (1993a) used undiluted uterine fluid. Thus, data obtained using these different methods for fluid retrieval cannot be meaningfully compared. There was no significant difference between young and older mares or between maiden mares and foaled mares regarding the concentration of IgA or IgG (**Paper III**). It can be concluded that immunoglobulins are released into the uterine lumen in gynaecologically healthy mares during oestrus and that this release is not influenced by age or parity. Moreover, the high concentration of IgA in the intraluminal fluid suggests that this immunoglobulin is locally produced, as has also been reported by Mitchell et al. (1982) and Widders et al. (1984). It has yet to be established whether the increased transudation that appears to occur in mares with an excessive accumulation of intrauterine fluid, just dilutes the concentration of immunoglobulins without a compensatory increase in immunoglobulin concentrations as can be interpreted from the study by Troedsson et al. (1993a) or if there is an increased secretion of immunoglobulins in these mares as suggested in earlier studies.

Immunoglobulins are produced by antibody-forming cells, i.e. activated B-cells that have differentiated and divided by an interaction with helper T-cells (Feldmann, 1998). The distribution and number of cells expressing MHC class II (antigen-presenting cells), CD4 (helper T-cells) and CD8 (cytotoxic T-cells) were described in the uterine body and horn in relation to age and parity (**Paper IV**). It was found that parity, but not age, influenced the number of these immunologically competent cells and that the distribution of T-cells in the gynaecologically healthy endometrium varied between the uterine body and horn. The finding that age did not affect the number of cells expressing MHC class II, CD4 or CD8 suggests that the decreased capacity to handle infectious organisms in older mares (Carnevale and Ginther, 1992) was not due to a reduced number of antigen-presenting cells or T-cells in the endometrium. The distribution of cells in the *stratum compactum* in the uterine body was basically in accordance with those obtained in earlier studies by Watson and Dixon (1993), Frayne and Stokes (1994) and Watson and Thomson (1996). In the uterine body, CD8-expressing cells were more abundant than CD4-expressing cells, which also has been reported in women (Bulmer et al., 1988) and sheep (Segerson et al., 1991). However, in the horse, Watson and Thomson (1996) reported that CD4-expressing cells were more numerous than CD8-expressing cells which, in the study presented in **Paper IV,** was seen only in the uterine horn. Numbers of CD4- and CD8-expressing cells were correlated within the same uterine segment, but there was no correlation between the uterine body and the horn for either cell subset. It was suggested that there is a regional recruitment of both

subsets into the equine endometrium which is not specific for each subset. The overall number of cells expressing MHC class II, CD4 and CD8 was greater in the uterine body than in the uterine horn. Since the uterine body is the region of the uterus where intraluminal fluid accumulates as well as the area where semen is deposited, it can be expected that the inflammatory response reported by Kotilainen et al. (1994) after insemination is reflected in this increased number of immunologically competent cells in the uterine body. Alternatively, this higher cell number might be interpreted as an elevated readiness against incoming microorganisms, semen etc.

During oestrus, the defence mechanisms should be able to eliminate micro-organisms introduced at coitus. When Williamson et al. (1987) challenged the uterus with bacteria, they found that the numbers of polymorphonuclear neutrophils (PMNs) rose dramatically within 2 hours of infusion of the bacteria, peaking at 6 hours with increased levels until at least 4 days after challenge. No difference in either the pattern of recovery or in concentrations of PMNs was seen between resistant and susceptible mares. The deposition of semen, especially of frozen thawed semen has been shown to provoke a transient endometritis in mares (Kotilainen et al., 1994), seen as an influx of PMNs into the uterus 6 hours after insemination. When Katila (1995) took serial samples of uterine fluid starting 0.5 hours after insemination with raw semen, it was concluded that the inflammatory response, measured as PMNs counts in uterine fluid, starts within 0.5 to 1 hour, peaks between 4 and 24 hours and is over within 48 hours. This early response to the introduction of semen into the uterine cavity was also seen as an increase in the number of helper T-cells (CD4+) as early as 6 hours post-insemination with raw semen (Tunón et al. 1999). However, increased numbers were not found 48 h after insemination. No significant difference was found between the initial number of CD8+ cells and the number registered 6 or 48 h after insemination This demonstrates that the early response of semen includes not only granulocytes but cells of the lymphoid lineage as well. This temporal activation of parts of the cell-mediated immune response must be studied in other time intervals before any reliable conclusions can be drawn. It remains to be determined whether frozen-thawed semen will provoke a stronger lymphoid response than fresh semen, as seen in the PMN response. Further studies, should be performed to determine how semen deposition affects the concentration of immunoglobulins in both normal and subfertile mares and whether the change in T-cell numbers seen after deposition of semen into the uterus differs between gynaecologically healthy and subfertile mares. Additional studies are also needed in mares susceptible to endometritis in order to establish whether the excessive accumulation of fluid often seen in these animals, which also strongly dilutes the secretory products, is the cause of the subfertility recorded either by reducing the survival of spermatozoa or the early

embryo or by suppressing the local defence mechanisms that normally protect the organ from infections.

General conclusions

The most important findings of the present work can be summarised as follows:

- The fine structure of the gynaecologically healthy mare endometrium during oestrus revealed evident signs of secretory activity as well as a conspicuous oedema of the *lamina propria*.

- Concentrations of the various elements studied in undiluted intraluminal uterine fluid were similar to those measured in serum, whereas the concentrations of elements in the secretory vesicles differed sharply from those in both uterine fluid and serum. Sodium and Cl were the dominant elements in uterine fluid, whereas K and P were the major elements in glandular epithelium.

- There was a clear similarity between the protein profiles of uterine fluid and serum. Concentrations of total protein and albumin in uterine fluid were slightly more than half as high (52-56 %) compared with those in serum. The concentration of immunoglobulin (Ig) A in undiluted uterine fluid was similar to its concentration in serum whereas the concentration of IgG was approximately 45 % as high as its concentration in serum.

- The three cell subsets, MHC class II expressing cells, CD4 and CD8 expressing T-cells, were detected in the mare endometrium, with numbers of all three being higher in the uterine body than in the uterine horn.

In conclusion:

The data indicate that serum contributes to a large extent to the composition of uterine fluid i.e. there is a considerable transudation to the uterine cavity, in gynaecologically healthy mares. This transudation is likely to have a diluting effect upon the specific secretion of the glands and, if substantial, it might be associated with subfertility.

There is no decrease in Ig A, Ig G, or cells expressing MHC class II (antigen presenting cells), CD4 (helper T-cells) and CD8 (cytotoxic T-cells) in the endometrium of older multiparous mares. This suggests that the increased susceptibility for endometritis usually seen in this category of mares could not be explained by a decrease in immunoglobulins or these immunologically competent cells.

The overall higher number of MHC class II, CD4 and CD8 expressing cells in the uterine body during oestrus, reflects either a locally expressed, higher readiness to act against micro-organisms or semen introduced during mating / AI, or a remnant response provoked by a previous exposure.

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