—Original Article—

Equine Endometrial Gland Density and Endometrial Thickness Vary among Sampling Sites in Thoroughbred Mares

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The secretions of the equine endometrial glands are essential for the survival, growth, and development of the conceptus in early pregnancy, and endometrial gland density is directly related to successful pregnancy outcome. Endometrial biopsy is routinely used to assess the reproductive potential of broodmares. Some previous studies have shown that equine endometrial glands are uniformly distributed throughout the uterus; however, other work has shown variation of the endometrial architecture between biopsy sites, suggesting that a single biopsy is not representative of the entire endometrium. The aims of this study were to assess and compare the endometrial gland density and thickness at four sampling sites in the uterus (the central segment of each uterine horn, the uterine horn-body junction, and the caudal portion of the uterine body). Endometrial samples from five nulliparous Thoroughbred mares in diestrus were obtained at necropsy and used for subsequent histomorphometric analysis. The caudal uterine body had a significantly lower endometrial gland density and endometrial thickness than the other sites. This may result in nutrient deprivation and reduced survival of embryos or fetuses in this region of the uterus. The endometrial gland density and endometrial thickness did not significantly differ between the other regions sampled, indicating that they are similarly suitable for embryonic implantation and fetal development. Our results suggest that the endometrial structure of the caudal uterine body of the mare is not representative of the endometrial morphology at other sites. Thus, the caudal uterine body is not a suitable site for routine endometrial biopsy.

Key words: *endometrial biopsy, endometrial gland density, endometrial gland thickness, histomorphometry, nulliparous Thoroughbred mares*

The secretions of the equine endometrial glands, known as histotrophe or uterine milk, contain a multitude of proteins that are essential for the survival, growth and development of the conceptus in the early stages of pregnancy [21]. These secretions are particularly important for the nourishment of the conceptus in the peri-implantation period, before establishment of hemotrophic nutrition by the allantochorionic placenta [22]. In sheep, the endometrial gland density has been shown to be directly related to the survival and development of the conceptus [12]. Defects in conceptus elongation and survival in the uterine gland of knockout ewes are caused by the absence of endometrial glands and their secretions, rather than alterations in the expression of anti-adhesive or adhesive molecules on the luminal epithelium of the endometrium, or changes in the responsiveness of the endometrium to pregnancy recognition signals from the conceptus [11]. Although a knockout model of horses has not been developed, clinical experience suggests that endometrial glands are similarly essential for successful reproduction in the mare.

Endometrial biopsies are routinely used to assess the reproductive potential of broodmares. Some studies have compared the endometrial gland distribution at multiple endometrial biopsy sites in the mare, and they report that the endometrial glands are uniformly distributed throughout the uterus [4, 16, 19]. Based on these results, it has been concluded that differences in endometrial gland density of clinical samples are unlikely to be derived from

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the location of the biopsy site; however, other factors such as pregnancy, and seasonal and cyclical endocrine variations, can affect the endometrial architecture [4, 15]. These findings were based on 2-dimensional microscopic images of histological sections, which can be used to evaluate both the endometrial gland density and endometrial thickness [4, 15]. Recently, Lefranc and Allen used a computer-assisted morphometric method to measure the endometrial gland surface density in 3-dimensional microscopic images of 4 Welsh Pony mares that were necropsied during estrus [16]. Their results also support the clinical reliability of a single endometrial biopsy taken from any site in the uterus of the mare to assess the endometrial gland surface density [16, 17]. However, this technique is not able to estimate the endometerial thickness. This is a significant shortcoming as gland density is influenced by the endometrial thickness which varies with the estrous cycle and season [14–17]. In addition, the breeding histories of the mares were not reported [16, 17]. Although these studies suggest that the uterine biopsy site is unimportant for clinical interpretation, other studies have shown variations in endometrial pathology between biopsy sites in the same mare [6, 7], and a single biopsy is not representative of the entire endometrium in mares with endometrosis [6, 7, 23]. Endometrosis, also known as chronic degenerative endometritis (CDE) [2], or chronic endometrial degenerative disease [20], is associated with reduced density of the endometrial glands in Thoroughbred broodmares. CDE increases with parity and the age of the mare, and has been reported to progressively increase the risk of infertility due to early embryonic and fetal death [5]. In affected animals, assessment of the endometrial gland density from a single biopsy could result in an inaccurate diagnosis and prognosis.

The purpose of our study was to re-evaluate the effect of biopsy site on endometrial gland density and endometrial thickness in the equine uterus using a quantitative histometric method (two-dimensions) in a uniform population. The significance of the results, with respect to how representative a single biopsy sample can be, is limited to this particular subset of mares (young maiden mares) and shouldn't necessarily be extrapolated to older maiden mares over the age of 10 years [20].

Materials and Methods

Sample collection

Uteri were obtained from five nulliparous Thoroughbred mares, ranging in age from 3 years and 10 months to 4 years and 9 months that were presented for euthanasia because of musculoskeletal injury (catastrophic fractures and tendon ruptures) and routine necropsy in May and June of 2009. None of the mares had ever been bred or had or any history of reproductive pathology, and no gross or microscopic evidence of endometrosis were present in their uteri [2, 5, 20]. All of the mares were in diestrus (luteal phase) at the time of necropsy, as confirmed by the existence of a mature corpus luteum in observations of the surface and mid-sagittal sections of the ovaries. The study protocol was approved by the Animal Experimentation and Ethics Committee of the School of Veterinary Medicine, Kitasato University.

Endometrial samples (approximately 4 cm^2) were taken as transverse sections from each of four sites: the mid-portion of the left and right uterine horns (sites A and B, respectively), the uterine horn-body junction (site C), and the caudal uterine body (6 cm cranial to the internal opening of the cervix, site D). Samples were fixed in 10% (vol:vol) neutral-buffered formaldehyde for 3–7 days. After fixation the tissue samples were dehydrated and embedded in paraffin. 5-µm-thick sections from each sampling site were then routinely prepared and stained with hematoxylin and eosin (HE).

Endometrial morphology

The HE-stained sections were examined using light microscopy (100 × magnification). The endometrial gland density and endometrial thickness were determined in five randomly selected microfields in each sample.

Determination of endometrial gland density

The density of the endometrial glands was estimated by counting the number of endometrial gland ducts in each microfield; this included the endometrial gland ducts in the endometrial subepithelium, stratum compactum and stratum spongiosum. Endometrial gland density was expressed as the total number of endometrial gland ducts per 1 mm^2 endometrium (ducts/mm^2 endometrium). It is not possible to determine if the sections of ducts seen histologically are from the same or different endometrial glands; however, the glands themselves present a similar problem as they may be tortuous or non-tortuous. The ducts themselves are shorter than the glands and are less likely to appear multiple times in a histologic section, for this reason we refer to gland ducts rather than glands as the parameter of endometrial gland density. Endometrosis was not seen in any HE-stained specimen from any of the four different uterine sampling sites.

Determination of endometrial thickness

The endometrial thickness (distance between the endometrial epithelium and the stratum spongiosum) was determined by measuring the distance along a transect drawn perpendicular to the roughly parallel proximal and

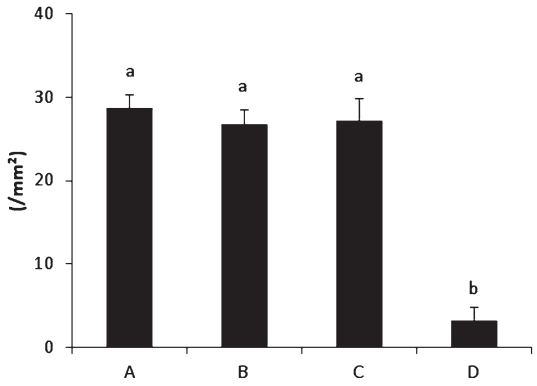

Fig. 1. Endometrial gland density at four regions of the uterus.
The error bars indicate standard error (S.E.) Significant between-site differences are indicated by different superscripts (a, b) ($p<0.05$). A= left uterine horn, B=right uterine horn, C=uterine horn/body junction, D=caudal uterine body.

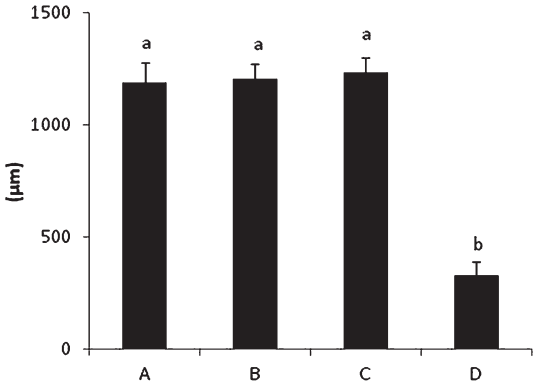

Fig. 2. Endometrial thickness. The error bars indicate standard error (S.E.)
Significant between-site differences are indicated by different superscripts (a, b) ($p<0.05$). A=left uterine horn, B=right uterine horn, C=uterine horn/body junction, D=caudal uterine body.

distal surfaces of the endometrium at an arbitrary single site visually considered to be thickest.

Statistical analysis

All data were analyzed using Microsoft Excel 2007® (Microsoft, Redmond, Washington, USA) with the add-in software Excel-Statistics 2010® (Social Survey Research Information, Tokyo, Japan). Data are presented as means ± standard error (S.E.). The significance of differences in endometrial gland density and endometrial thickness among the four sampling sites (A, B, C, D) was determined using the mean value of each site calculated from the values obtained from the five randomly selected microscopic fields. The data were analyzed by the Kruskal-Wallis test followed by the Steel-Dwass non-parametric multiple comparison test. The association between endometrial gland density and endometrial thickness was assessed using the Spearman non-parametric correlation test. Differences were considered significant at p values of < 0.05.

Results

Endometrial gland density

There were no significant differences in the endometrial gland density between sites A, B, and C; however, site D had significantly fewer endometrial glands/mm^2 than the other three sampling sites (Fig. 1).

Endometrial thickness

Comparison of endometrial thickness between sampling sites yielded results similar to those of the endometrial gland density. There were no significant differences in endometrial thickness among sites A, B, and C; however, site D was significantly thinner than the other sampling sites (Fig. 2).

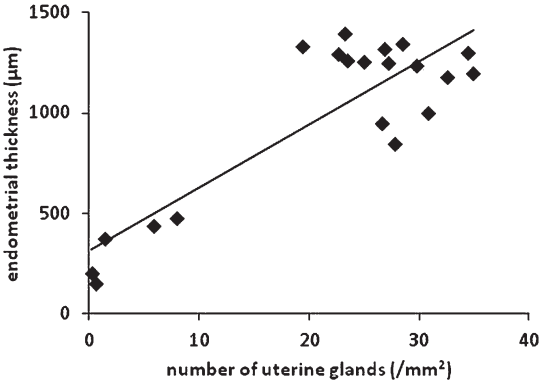

Fig. 3. Correlation between the endometrial gland density and endometrial thickness.
The correlation between endometrial gland density and thickness was almost statistically significant (Spearman's correlation coefficient (r)=0.394, p =0.0856, n=20).

Correlation between endometrial gland density and endometrial thickness

The correlation between endometrial gland density and endometrial thickness was almost statistically significant (Spearman's correlation coefficient (r)=0.394, p= 0.0856, n=20) (Fig. 3).

Discussion

In our study there was a significant difference in endometrial gland density between one sampling site and the others. This suggests that biopsy specimens should be evaluated relative to the site from which they were taken. Our findings partially agree with observations by several researchers who have reported that one endometrial biopsy is not sufficient for reliably diagnosing conditions such as endometrosis in a mare's uterus [6, 7, 23]. In addition, our finding that the endometrial gland density at site D was significantly thinner than at sites A, B, and C, suggests a markedly poor nutrient supply for the embryo in the caudal uterus relative to the uterine horns (sites A and B) and the horn-body junction (site C), which is the site of initial embryonic attachment, placental implantation and endometrial cup formation [13, 19, 25]. Accordingly, embryonic attachment to the caudal uterus may result in a decreased likelihood of successful implantation and maintenance of pregnancy. If an embryo implants in the caudal uterine body (site D), which has a markedly lower endometrial gland density, embryonal and fetal growth cannot be maintained until delivery [1, 9, 24]. This condition, known as a body pregnancy, is thought to result from improper mobility of the early conceptus [13] and typically results in early embryo loss [1, 3, 9, 13, 18, 24], or growth retardation resulting in spontaneous abortion in late gestation [1, 9, 24]. There are two types of body pregnancy: "cranial body pregnancy" in which the embryo implants in the mid-portion of uterine body, and "caudal body pregnancy" in which the embryo implants just cranial to the cervix. Jobert *et al*. reported that the pregnancy loss rate was 83% after vesicle fixation in the caudal uterine body, compared with 22% after vesicle fixation in the cranial uterine body, as determined by ultrasound [13]. The loss rate for embryos that fixed at the base of uterine horns was 5% [10]. The similarity in endometrial gland density between sites A, B and C suggests that the uterine horns and horn-body junctions are all similarly suitable for implantation and maintenance of pregnancy, and that the uterine body of the mare is significantly less able to support a pregnancy.

The values we obtained for endometrial thickness showed a similar trend to those for endometrial gland density, with only site D differing significantly from the other sampling sites. However, endometrial hypertrophy occurs during diestrus [14–17], and increases with implantation of the embryo in pregnancy. The correlation between endometrial thickness and gland density was almost statistically significant, suggesting that as the endometrium becomes thicker, the more its structure (sites A-C) becomes suitable for embryonic or fetal survival and development. Thus, site D, the caudal uterine body, appears to be less suitable for embryo implantation and pregnancy maintenance than the other sites examined.

In the absence of palpable abnormalities during rectal examination, endometrial biopsies are routinely used to assess the overall uterine status of potential broodmares. The ideal sites for endometrial biopsy are the central region of a uterine horn [19], or the uterine horn-body junction, because the conceptus normally implants at the base of one of the uterine horns [10]. Our results confirm that biopsies from these sites are most likely to reflect the status of the endometrium in the regions of likely fetal implantation.

Endometrial gland density in the mare has been described by several authors [10, 14–17], but only Keenan *et al*. have reported the actual number of endometrial gland tubules (tubules/0.5 cm^2) [14]. Although direct comparisons are difficult, the values for gland density obtained by Keenan (diestrus; 39–61 tubules/0.5 cm^2 and early pregnancy; 57–59 tubules/0.5 cm^2) were lower [14] than those obtained in our study (diestrus; 3–29 tubules/mm^2). This difference may be due to several factors. There were methodological differences between the studies, since Keenan *et al*. assessed gland density in biopsy samples [14], while we used histologic specimens obtained at necropsy. Biopsy may result in more superficial sampling of the endometrium, thus leading to the determination of endometrial gland surface density [14]. In our study, the endometrial gland density was determined in the combined endometrial subepithelium, stratum compactum and stratum spongiosum. Additionally, during diestrus the endometrial glands increase their tortuosity and branch into the deeper layers of the endometrium while under the influence of luteal progesterone, resulting in an increase in the number of endometrial glands seen on histologic sections [10, 14–16]. Also, fixation in formal saline, as used in our study, has been shown to cause less tissue shrinkage than other fixatives (such as Bouin's fixative [8]) which were used in other studies [14, 15]. The use of different fixatives may explain differences in the histomorphometric findings between our study and the work of other authors [14, 15]. Further work in this area is needed.

To our knowledge, this is the first study to use endometrial gland duct density to report site-related differences in endometrial gland density and endometrial thickness in healthy young mares. That we found differences where others didn't may be indicative of the greater accuracy of our technique of using endometrial gland ducts to estimate gland density. Although previous work has indicated that the biopsy site is important for the detection of endometrosis, our work suggests that it is also important for

routine reproductive assessments of healthy mares. Our results support the use of an endometrial biopsy technique using rectal palpation, endoscopy, or other means to identify the biopsy site rather than a blind sampling technique.

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