# EVALUATION OF THE EFFECT OF COLLECTION SITE, FIXATIVE & ESTRUS CYCLE ON EQUINE UTERINE BIOPSY KENNEY GRADE

By

CYRUS SEPOY

Bachelor of Veterinary Science and Animal Husbandry
Bombay Veterinary College
Bombay, Maharashtra, India
1998

Master of Veterinary Science
Bombay Veterinary College
Bombay, Maharashtra, India
2002

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May, 2008

EVALUATION OF THE EFFECT OF COLLECTION

SITE, FIXATIVE & ESTRUS CYCLE ON

EQUINE UTERINE BIOPSY

KENNEY GRADE

Thesis Approved:

Dr. Gilbert Reed Holyoak

Thesis Advisor

Dr. Timothy Snider

Dr. Mark Payton

Dr. A. Gordon Emslie

Dean of the Graduate College

ACKNOWLEDGEMENTS

First and foremost I would like to thank my thesis advisor, Dr. Reed Holyoak, for his guidance, advice and above all encouragement and support throughout the study. His professional conduct, enthusiasm and approach to work are something that inspires me to improve my own persona. I would like to acknowledge my other committee members Dr. Mark Payton and Dr. Tim Snider as well, for their time, advice, effort and expertise that have allowed me to culminate this project. A special thanks to Dr. Terry Lehenbauer for helping in the analysis of the data.

My sincere appreciation extends to Dr. Charlie Broadus, our theriogenology resident and A.C. Box and all the other staff from the Oklahoma State University Veterinary Medicine Ranch for their time and assistance throughout this project. My colleague Sharla Birch also deserves a special mention for her help during the formulation of this project.

I would like to extend my gratitude to Dr. Grant Rezabek for his patience and advice as well as Jeanenne Ely and all the staff of the histopathology laboratory of the Oklahoma Animal Disease Diagnostic Laboratory for their help in procuring the fixatives and extending their resources for processing the entire sample.

Lastly I would like to extend my thanks to my family and friends, who bore all my petulance during the tough times and kept me focused. I would not be here today if not for their love, support and understanding.

TABLE OF CONTENT

## LIST OF TABLES



## LIST OF FIGURES

# Chapter I

## Introduction

Equine breeding is a multimillion dollar industry requiring timely and prompt breeding of mares to make it economically viable. Along with changing science and technology, the veterinarian has had to constantly update and upgrade his knowledge to keep pace with the expectations of the industry. Infertility of mares is a major concern for the equine breeding industry as it causes economic loss by reducing yearly foal production.

When it comes to diagnosing fertility problems in mares the veterinarian has come to trust several diagnostic procedures like rectal palpation, ultrasound, uterine biopsy, cervical and uterine cytology and/or culture, fiber optic examination and endocrine assays. Although ultrasound is now routinely used as a tool for assessment of uterine pathology, it is limited due to resolution capabilities, technical expertise required, as well as economic factors in some demographics. Although cytology can be used to determine endometrial inflammation, it has limited value if not used in tandem with uterine culture (86). Along with clinical and laboratory evidence of inflammation, uterine cultures are essential to establish etiological diagnosis of bacterial endometritis.

Despite its diagnostic potential, a fiber optic examination is limited because it is relatively invasive, cannot be routinely done under field conditions, and is limited to university hospitals and specialized clinics. Hence, transvaginal uterine biopsy is often the preferred tool not only to diagnose endometritis, both infectious and noninfectious,

but also to determine the ability of the mare to become pregnant and maintain that pregnancy to term.

Ideally, physical, microbiological, and clinical findings can be correlated to histomorphological changes found in the biopsy sample to provide a basis for development of a therapeutic plan and prognosis for fertility. Hence, endometrial biopsy aids in minimizing economic loss to the equine breeder from decreased foal production. The uterine biopsy should not be the sole criteria used to determine the therapeutic protocol of the problematic broodmare. It should be interpreted in conjunction with an accurate reproductive history, thorough rectal and vaginal examination, endometrial culture and cytology and ultrasonographic examination. Other ancillaries may involve hysteroscopy and an endocrinologic evaluation.

The histopathological grading system recommended for interpreting the biopsy and assigning breeding prognosis is the Kenney-Doig (52) four category (I, IIa, IIb and III) system. The expected foaling rate for Category I is 80-90 percent, Category IIa is 50-80 per cent, Category IIb is 10-50 percent and Category III is 10 percent. Although there is some controversy with regards to assigning the percentage foaling rate as compared to the histopathological grades, it is universally accepted that there is reduction in foaling rate as the grades increase. With so much importance being placed on the histopathological grading the obvious questions that arise are:

1) Is histopathological grading affected by the location of the biopsy or number of biopsy samples collected?
2) Is histopathological grading affected by the fixative used?

3) Is histopathological grading affected by the stage of estrus when the sample is collected?

Although there have been published reports over the last three decades which addressed various aspects of these questions, there is no uniformity in their results. As per the guidelines published by the Society for Theriogenology (82) uterine biopsy samples are to be submitted in Bouin's fixative because it produces a firmer specimen than formalin, with less tissue distortion and artifact on sectioning. Still other authors state that 10% buffered neutral formalin is a satisfactory fixative (97). However, there appears to be paucity of publications addressing effects of Bouin's fixative on uterine histopathological grade as compared to formalin. Bouin's has been the preferred fixative for uterine tissue since the 70's. The use of Bouin's as a fixative for uterine tissue seems to have been extrapolated from research carried out on fixation of testicular tissue (56). However, there exists a trend to move away from Bouin's, even in testicular tissue, as there are less hazardous alternatives available (21,56). Hence, we decided to test the hypothesis that Bouin's fixative is the best for uterine biopsy histopathological grading.

In 1975 Bergman and Kenney (7) concluded that a single biopsy is considered to be representative of the entire endometrium if no palpable abnormalities are noted. Although several scientists have established that this conclusion is valid (11,102), they expressed concern that future studies should take into account the location from which the biopsy was obtained.

The morphological changes of uterine endometrium of the mare during its annual reproductive cycle as well as through its estrus cycle have been well documented (17,50,53,77,83,89). However, the significance of these routine changes to

histopathological grading has not been studied. We therefore proposed to measure possible changes in the prognostic grading relative to the stage of the estrus cycle when the biopsy was collected. In view of this the following study was undertaken.

# Chapter II

## Literature Review

### 2.1. History of Biopsy

The use of endometrial histology for diagnosis of the health status of a mare's uterus is not a new concept and has been a standard procedure for about four decades. There is literature dating back to 1925, when Seaborn described the gross and histologic appearance of the reproductive tract of 19 normal mares after slaughter (81). There are reports of similar publications in the following decades as well. During 1941 two groups of scientists studied endometrial changes associated with the estrus cycle. Hammond and Wodzicki (37)studied the endometrial changes associated with stage of estrus cycle in ponies utilizing samples collected at necropsy (37). Similarly, Andrews and McKenzie also described histological changes of the endometrium and reproductive tract of horses throughout their estrus cycle (3). The latter, however, was the first to use consecutive endometrial biopsies, besides several other techniques, to describe the changes of the endometrium and other reproductive tract tissues.

The uterine biopsy, as well as culture techniques, continued to evolve over the next couple of decades and, in 1969, Brandt and Manning managed to obtain biopsy samples utilizing a gastrocamera fiberscope (16). They discussed changes associated with the equine endometrium in a normal cyclic mare and a mare suffering from endometritis. The next year Brandt found that a mare's clinical status could be more accurately

diagnosed by endometrial biopsy rather than uterine/cervical swab culture (15). Thus he restated the significance of endometrial biopsy to the equine practitioner. It was during this decade that biopsy was first used clinically. Besides recording the description of primary endometrial lesions, several new instruments and techniques were also developed for the collection of the uterine biopsy sample.

Tobler used an instrument capable of not only obtaining fluid from the uterus but also uterine biopsies (92) while Samuel, et al. utilized scanning electron microscopy to examine uterine endometrium in different stages of estrus cycle, anestrus, and various abnormal conditions and described the secretory and ciliary activity of the equine endometrium as compared to other large domestic mammals and laboratory animals (77).

The 70's saw further improvements in techniques and instrumentation as well as comparisons between cytology and biopsy. It was during this decade that questions were asked with regards to the advisability of using uterine biopsy or culture alone as a means to diagnose uterine health (108). The uterine biopsy was studied with great intensity over the next couple of years wherein histologic, cytologic and microbiological findings of normal and infertile mares were studied by several scientists. No history would be complete without mentioning the contributions made by Ricketts. He collected biopsy samples of 134 mares using Yoeman's basket punch forceps (60 cm in length) and described findings of the different histological descriptions between acute and chronic fibrotic endometritis, endometrial atrophy of aged mares, and endometrial hypoplasia and hyperplasia seen in mares which are chromosomal mosaics and following abortion, respectively (70,71). Ricketts was one of the few scientists who advocated the use of Bouin's solution for his biopsy samples in 1975. He also had a preference of collecting

the samples during diestrus as he felt that normal estrus cycle changes would make interpretation of the sample difficult.

Robert Kenney not only studied the prognostic value of endometrial biopsy of the mare but also developed a rating system for periglandular fibrosis by degree as well as extent and described its relationship with fertility and prognosis for pregnancy (51). In 1975, Kenney and Ganjam found that lymphatic lacunae were quite common in older mares and may occasionally lead to large endometrial cysts but generally caused widespread change throughout the uterine horns (53).

In a retrospective study carried out in 1982 by Concha-Bermejillo and Kennedy, where 79 biopsies were examined and graded using Kenney's three category system, the findings corroborated the work done by earlier scientists, in that, foaling rates were found to decrease as the biopsy grade increased (20). These scientists found that although some inconsistencies were to be expected in assignment of Kenney's grading system due to the fact that the prognostic grades are arbitrary, the magnitude of the discrepancy in their study denoted that even experienced pathologists who do not routinely evaluate endometrial biopsies would be likely to "misapply Kenney's prognostic criteria".

Slusher and associates in 1985 demonstrated that endometrial biopsy was an invaluable tool to determine the degree of fibrosis, however endometrial cytology specimen evaluation was superior in the assessment of response to intrauterine therapy (86). Their findings also corroborated the notion that cytologic examination may be superior to culture for the diagnosis of infection.

By the mid '80's, with the development of proper techniques for uterine culture (18), cytology (75,85,86) and immunological methods (103) and ultrasound examination

of the uterus (2), there were a number of options becoming available to the clinician for evaluation and diagnosis of the infertile mare. However, uterine biopsy not only competed with the newer diagnostic tools but held its own importance. In fact, a paper published by Waelchi and associates, who compared the histologic, cytology and bacteriological findings in 161 mares, showed that diagnosis of uterine pathology was more accurate when multiple diagnostic modalities were utilized (100). It was apparent from the study that culture results could be better interpreted in the presence of cytologic and histologic findings.

A review published in 1988 by Steven Van Camp gave a detailed account of progress of the uterine biopsy up to the 80's (97). In the same year Katila studied the histology of the post partum equine uterus and found the occurrence of neutrophils and lymphocytes in the endometrium (47). He cautioned scientists that neutrophils and lymphocytes were connected with the normal restoration of the endometrium post partum and should not always be interpreted as a sign of infection. Similarly Williamson and associates (1989) cautioned clinicians that biopsy should be utilized to assess the extent of degenerative changes of the endometrium and whether these changes could affect the mare's ability to carry the foal to term (107). He concluded that it was not justifiable to utilize uterine biopsy to predict whether a mare was resistant or susceptible to endometritis.

In the early 90's with the increasing understanding of immunology much research was done to differentiate acute and chronic uterine infection and also study the effects of certain lavage fluids and solutions (like DMSO) on the histological features of the endometrium (59). In 1993, Troedsson et al., summarized that humoral, cellular and

physical defense mechanisms are involved in the resistance against persistent uterine inflammation in mares (95). They found that in mares showing persistent endometritis, reduced myometrial contractility at peak inflammatory response stage caused decreased clearance of inflammatory products in the uterus and consequently a detrimental effect on phagocytosis by the polymorphonuclear cells.

By the 90's the use of biopsy was so firmly entrenched that the focus shifted from studying about uterine biopsies to utilizing biopsies to study other parameters. In 1992 Darenius reported the histological, bacteriological and cytological findings of the uterus after early fetal death in the mare (22). He found that acute as well as chronic degenerative endometritis appeared to be the most common cause of early fetal death.

The following year, Heuer and associates studied the uterine fluid obtained from 15 diestrus mares and correlated their findings with their endometrial biopsy classifications (42). They cultured 2-cell mouse embryos in each of the samples obtained and concluded that the uterine secretions from different classifications affect the viability of early murine embryos. In 1993, Reiswig and associates concluded that cytology, when used with uterine aerobic culture, was as advantageous as biopsy in diagnosing bacterial endometritis (69). However, uterine cytology was unable to detect the chronic inflammatory/fibrotic changes revealed by histopathology.

In contrast to Williamson's findings, Troedsson and colleagues found a significant correlation between histology biopsy grade and susceptibility to chronic uterine inflammation (93). Their study found that although moderate endometrial lesions did not correlate consistently with resistance to chronic uterine infection, a histologically

normal endometrium was associated with resistance to chronic uterine infection and severe histologic lesions were associated with susceptibility to chronic uterine infection.

Ferreira-Dias and colleagues studied the morphological characteristics of equine endometrium classified as per the Kenney 3-category scale, using light and scanning electron microscopy (30). They concluded that the degree of surface damage observed by scanning electron microscopy paralleled the number of fibrotic nests that were seen by light microscopy. They correlated the amount of surface swelling seen under electron microscope to the amount of inflammatory infiltration observed under light microscopy.

In 1994, Held and Schneider compared endometrial cultures and histopathology in assessing uterine infection in the mare (40). Their findings led them to believe that when a uterine infection was suspected, the decision for treatment should be based on the biopsy rather than the culture results. However, the culture would help in deciding the appropriate anti-microbial treatment.

Gomez-Cuetara and associates (1995) utilized endometrial biopsy samples to map the histological changes occurring in Andalusian, Thoroughbred and mixed breed horses postpartum (33). The following year, Threlfall summarized the importance of endometrial biopsy (90). He felt that the use of the biopsy should be mandatory for any mare that has been bred and failed to deliver a viable fetus. He concluded that although the biopsy could give prognostic information about inflammatory disease like endometritis or endometrosis, it would be of no help in deciding the type of antibiotic required and hence both diagnostics should be utilized simultaneously.

In 1996, the utilization of ultrasound as an alternative or an addition to other diagnostic procedures to diagnose endometritis in the mare was published (67). Dascanio

and associates also described the different diagnostic options in mare reproduction (23). They reiterated the prognostic importance of equine biopsy for predicting ability of a mare to maintain pregnancy, as well as stressed the importance of the practitioner to examine the slide himself so as to gain a better understanding of the extent of the lesions. They reported that on the basis of presence of neutrophils or lymphocytes one can differentiate between acute or a more chronic lesion of the endometrium. Reilas and associates (68) utilized endometrial biopsy to compare endometrial environment in estrus mares with intrauterine fluid accumulation were. They detected that anechogenic fluid accumulations during estrus were associated with fibrosis.

In the same year, Rickets and Barrelet published a retrospective review of histopathological features seen in 4241 endometrial samples of Thoroughbred mares over 25 years which had some invaluable insights in the study of endometrial biopsy (74). They concluded that endometrial hypoplasia is most commonly seen in younger mares with delayed endometrial maturity while endometrial hyperplasia, most commonly seen in younger mares, persists during delayed post-partum or post pregnancy failure of uterine involution. According to their perspective, mononuclear cell infiltrations develop in the endometrial stroma in relation to local immune responses to challenge by seminal proteins, micro-organisms, environmental debris and the products of pregnancy during a brood mare's life. They felt that glandular degenerative changes and stromal fibrosis are an inevitable change of aging and eventually lead to endometrial atrophy which is a reflection of gynecological senility.

Welle et al. in 1997 used endometrial biopsy of 44 broodmares 3,6 and 9 days postpartum to support his hypothesis that sufficient number of mast cells are necessary to

ensure a normal post-natal period and that the equine endometrium presents a mast cell subtype that is tryptase and chymase negative (106). With the growing importance of mapping the immune cell component of the equine endometrium, the following year Summerfield and Watson documented the distribution and number of endometrial macrophages during the estrus cycle of both normal mares and mares susceptible to persistent endometritis (89). They concluded that stage of the estrus cycle did not influence the number of macrophage-like cells in the lamina propria and that endometritis causes an increase in neutrophils in the endometrium.

To overcome the subjectivity and semi-quantitative nature of diagnosis of periglandular fibrosis, Evans et al. tried to develop an objective, quantifiable assay of endometrial periglandular fibrosis and correlate the findings with histologic and ultra-structural changes (28). They concluded that morphometry could be used to objectively assay equine endometrium as the results correlated well with morphological changes in endometrial biopsy samples.

Ferreira-Dias and associates morphologically compared equine endometrium categories (Kenney 3-category system) using light and electron microscopy and concluded severe ultra-structural changes may be one of the key factors in decreasing fertility of mares in category III as compared to the other two categories (31).

Betsch in 2000 completed a retrospective study of the endometrial biopsy in 485 mares (8). He graded the endometrial biopsies according to the modified Kenney and Doig (52) four category system and found that there is a significant statistical relationship between the severity of the lesion and the probability of the mare conceiving and carrying

the foal to term. He calculated foaling rates of 68%, 47%, 28%, 9% in category I, IIa, IIb and III, respectively.

The following year Ricketts and Barrelet utilized uterine biopsy to study the ability of mares to respond to uterine abnormalities (73). The same year Steiger and associates utilized endometrial biopsies of puerperal involution in mares to compare the clinical signs and correlate them with the pathology to understand the alterations leading to reduced fertility rate following peripartum disturbances (88).

Following the realization that the mare usually has an inflammatory response to semen, the focus of many scientists was directed at mapping the inflammatory response and understanding it. Tunon et al. were involved in the mapping of T-cell distribution in the equine endometrium post insemination (96). Although they found no variation in numbers of CD8+ cells either 6 or 48 hrs post insemination, there was an early (6h) recruitment of CD4+ cells which the authors felt was related to the activation of the inflammatory reaction seen during estrus. Similarly Troedsson and associates in 2001 studied the inflammatory response of the equine endometrium to semen (94). They concluded that breeding related endometritis is both up and down regulated by seminal components.

Due to the invasive nature of endometrial biopsy there were efforts to substitute examination of endometrial biopsy samples with less invasive techniques that could assess quantitative endometrial echotextural characteristics (60). The scientists found that although their ultrasound equipment and technology could detect some associations between mean endometrial numerical pixel value and biopsy grade there was no

association detected with endometrial fibrosis. They felt that further studies with larger number of mares would be required to confirm their findings.

In 2003 Schoon and Schoon wrote a critical review on the Kenney and Doig (52) system of endometrial categorization and expressed their concerns about its inadequacy due to its neglect of important histological findings like angioses and endometrial maldifferentiation. They felt that with the current progress in equine gynecopathology this system was "unacceptable from both medical and forensic aspects" (79). Another scientist, Klug expressed the need to expand the conventional Kenney and Doig system of classification as well (54). He argued that besides endometritis, endometrosis and endometrial lymphstasis findings, endometrial angiopathies and glandular maldifferentiation should be included together with conventional categorization and "epicrisis" to achieve a fertility prognosis of the mare.

Mansour et al. in 2004 analyzed the epithelial structures of the mare's endometrium through histomorphometry of the luminal and glandular epithelial cell heights, glandular lumen diameter, glandular density, percentage of glands with apparent lumen and intraluminal secretion (61). Their study concluded that histomorphometry could improve the consistency in biopsy evaluation by furnishing objective data.

In 2005 Nielsen conducted a study on endometritis and concluded that bacteriological culture and cytology obtained from an endometrial biopsy would provide the practitioner with most accurate result regarding both sensitivity and positive predictive value (63). The following year Abd-Elnaeim and associates, while studying the structural and hemovascular aspects of placental growth throughout gestation of five mares, reinforced previous findings which showed the disadvantage of age-related

endometrial degenerative changes (1). These changes have a deleterious impact not only on the microplacentome development but on both the extent and intimacy of physical and hematological contact at the fetomaternal interface and thus consequently on fetal growth.

Falomo et al. in 2006 showed that interpretation of bacteriological, cytological and histological examination findings are not always easy and "univocal" (29). They showed the importance of performing and comparing all the three in order to express a correct diagnosis especially in old mares, mares with an unknown history or those with a history of infertility and endometrial pathologies.

Scientists have known for some time that "blind" biopsies do not always accurately reflect the true condition of the endometrium. Studies of biopsies of 40 mare by Hecker and Hospes proved that that there was a distinct advantage of utilizing endoscopically obtained biopsy specimens in mares with local irregularities of the endometrium as a more definitive diagnosis could be made (38).

To reiterate Katkiewicks and colleagues in 2007 evaluated the structural changes in 107 mares and recommended utilizing endometrial biopsy as a method to get the exact diagnosis of the health status of the uterus (48). It should be noted that in addition to normal hematoxylin-eosin staining they also utilized other staining protocols as well as immunoperoxidase methods.

Also in 2007, Schlafer in a review article, concluded that "Although classic histopathology will likely remain a central tool, using additional histochemical and immunohistochemical procedures would provide valuable information." He further stated that the introduction of molecular techniques like FISH, RT-PCR and genetic

arrays analysis was inevitable and would lead to detailed information about the health status of the uterus. Furthermore, the introduction of quantitative testing would minimize the subjective analysis of the endometrial biopsy by providing greater accuracy and thus the value of endometrial biopsy would be enhanced due to its expanded use (78).

### 2.2. Biopsy Categorization

In 1978 Kenney categorized equine endometrial biopsies into 3 grades (I, II, III) depending on their inflammation and fibrosis with grade I exhibiting insignificant changes and grade III having a high degree of pathologic change (50). This was one of the early protocols utilized by pathologists to classify endometrial biopsies. Kenney used a custom built biopsy forceps (70 cm in length) with punch consisting of alligator jaws and basket for collection of uterine biopsy samples. This instrument is the forerunner of the modern uterine biopsy forceps. Kenney was of the opinion that the biopsy sample could be collected at any stage of the estrus cycle including during estrus after breeding without interfering with the pregnancy.

The true genius of Kenney was evident in the fact that this categorization, although dependent on the pattern, type and degree of extensiveness and severity of the inflammation and fibrosis seen in the biopsy sample, also took into account the stage of estrus cycle, the physical, gross, microbial and endocrinologic findings made by the clinician before interpreting the histologic findings. He called this “epicrisis” and he believed that while the category applied to the endometrium alone, the epicrisis would enable all the facts to be put together to predict the ability of the mare to become pregnant and carry a foal to term. He described the normal histologic changes that occur

during the estrus cycle and the standard procedure for obtaining a biopsy in the mare. He defined the candidates to undergo uterine biopsy as well as correct procurement and processing of the sample. Kenney advocated that a biopsy less than 1cm in length was inadequate which is a rule upheld to this date. Kenney preferred to fix the uterine tissue biopsy in Bouin's fixative for the 1$^{st}$ 24 hours before replacing it with 10% formalin or 70% ethanol because according to him "formalin fixative produced more tissue shrinkage and loss of cytological detail". Kenney attempted to correlate the pathological findings to their effect on fertility and prognosis for pregnancy and stated that a mare's uterus could improve and be reassigned a new category.

Kenney was not the only person to invent a grading system for uterine biopsy samples based on the histological findings. In 1978 Shideler presented prognostic grading categories for the uterine biopsy (83). Both systems were similar in that they used a three-tier rating scale. Mares belonging to the first category were normal to mildly affected, while those belonging to second category were moderately abnormal causing a decrease in fertility but could be reversed with proper care and management. The third category of biopsy had a poor prognosis and showed marked pathological changes both in degree and extent of the histologic lesions. Based on the biopsies of 86 mares Shideler found that foaling rate for category 1, 2 and 3 were 73.8%, 42.9% and 18.8%, respectively. Similarly, Kenney studied the uteri of 285 mares and he too determined that there was reduction in fertility rates as the endometrial category increased and he reported foaling rates of 70-92% for category I, 50-67% for category II and 4-10% for category III (49).

Both these systems had hardly been accepted when scientists began to realize that a majority of the mares' problems fell in the second category of histopathological

grading. Around that time, Gordon and Sartin, found that decreased reproductive performance was most commonly correlated with advancing age, poor perineal conformation, increased number of years barren, and a history of abortion, as well as moderate to severe fibrosis of the endometrium (34). They proposed a four category system wherein they modified the Kenney system by subdividing Kenney category II into two categories depending on the degree of fibrosis (i.e. mild to moderate or moderate to severe). Group I consisted of normal mares, while group II consisted of mares that were fertile after appropriate therapy. Group III consisted of mares that had the potential to conceive but not carry the foal to term while group IV consisted of mares who would not conceive.

Similarly in 1981, Doig et al. (24) came up with 4 category system of grading for uterine biopsies which was a modification of Kenney's system. They separated moderate fibrosis from severe fibrosis into different categories and designated the categories from A to D. They determined that the effect of mild to moderate fibrosis could be overcome with good management. Another important deduction made by them was that number of years the mare was previously barren also affected the prognosis within each category except category A. In 1982, Asbury modified the Kenney category II into (IIi) depending on presence of inflammation or category (IIf) due to presence of fibrosis or category (IIif) if both were present (4). His conclusions corroborated the findings of other scientists as he found that fibrosis was more important than inflammation in the process of assigning a prognostic grade to the biopsy sample. He emphasized that a mare, despite being histologically positive for inflammation, should be mated rather than treated provided the mare's endometrial culture was negative and she showed no clinical signs.

There are several other systems of categorization of the uterus of the mare but the one most commonly used was developed in 1986 by Kenney and Doig (52) . They proposed a four category system which subdivided the category II of the original Kenney scale into IIa and IIb. The most discerning point of this system of categorization is that it consists of an additive scale where in a mare with more than one qualifying lesion in the endometrium is assigned to the lower prognostic category. Hence mares having minimal or no notable pathological changes in the architecture of the endometrium are included in Category I. Similarly Category II consists of samples have changes that decrease the mare's ability to conceive or carry foal to term. Depending on the nature, frequency and distribution of the histologic architectural changes the biopsy would be categorized as IIa or IIb. Factors like slight to moderate inflammatory cell infiltration of stratum compactum, which may be diffuse or scattered, scattered but frequent foci of inflammation and fibrosis, scattered periglandular fibrosis of gland branches or nesting of glands or widespread lymphatic lacunae whether occurring singly or in combination are considered while assigning the grade. Category III consists of mares having endometria with widespread and/or severe pathological changes that diminish or prevent the mare's ability to conceive or carry foal to term. These factors are generally considered irreversible, unless the grade was based entirely on severe inflammation.

There are at least two other notable systems of histopathological classification of endometrial samples in existence today, Shideler and associates system of classification (84) and Ricketts and Alonso's system of classification (72).

**2.3. Change of biopsy findings with season and year:**

There have been several articles published which deal with the effect of the normal estrus cycle changes, annual cyclic pattern and age of the mare on its uterine architecture. In 1941 Hammond and Wodzicki reported a histological difference in the uteri in cycling mares depending on the stage of the cycle in which the sample was collected and it was not as well defined as in other species (37). Some of the most significant work of describing the histological changes during the estrus cycle was done in the 70's (15,34,77). Amongst them, Kenney deserves a special mention as he meticulously charted the histological changes of the endometrium and combined them with physical and behavioral findings so that the changes associated with estrogen and progesterone and their target organs could be determined (50). Similarly Ricketts too mapped the histological variations associated with the estrus cycle in the mare and interpretation of its findings in relation to the reproductive evaluation (71).

In 1982, Britton monitored the endometrial changes associated with the annual reproductive cycle with the help of cytology and biopsy and found that there was no consistent pattern of cell change throughout the cycle (17). Gross and LeBlanc studied the effect of seasonal morphologic changes of the endometrium of 5 mares and its effect on the histologic interpretation of periglandular fibrosis (35). Although they used Kenney's three category system of histologic classification, they found that seasonal changes of the endometrium affected quantitative assessment of fibrosis and consequently resulted in occasional changes in the assigned prognostic grade of the endometrium. This is a very significant finding considering that they used the precaution of utilizing the services of a single pathologist to interpret the slides and thus avoid bias

in histologic interpretation. In fact, Kenney and Doig stated that although the uterine biopsy could be collected at any time due to the ease of dilatation of the cervix, the inexperienced practitioner may need to obtain biopsies only during diestrus to minimize the variability in the endometrial architecture during interpretation (52). They not only described the histological variations between the seasons of the year but also variations related to stages of the estrous cycle within the physiological breeding season.

In addition to the histological variation between different stages of the estrus cycle there are several other changes that take place. Equine uterine cytology and culture results also vary with the different stages of the estrus cycle (101). Waelchli and associates discovered that there was a trend to get more positive cytological samples during estrus as compared to diestrus or anestrus. Although the culture results did not differ significantly between the cycle stages, they determined that estrus should be the preferred time for uterine examination.

In 2000 Aupperle and colleagues studied the cyclical endometrial steroid hormone receptor expression and proliferation in the mare (5). They realized that in areas of endometrosis or fibrotic areas due to the asynchronous glandular differentiation, the epithelial hormone receptor expression is out of phase. Similarly in 2005, Hedberg and associates studied the behavioral differences during the estrus cycle of the mare by studying their response in a novel object test and isolation test (39). Recently Hirsburner and associates compared the myometrial motility of uterine smooth muscle depending on the phase of the estrus cycle. They observed that in the longitudinal layer and in diestrus more contractions were seen over time that in circular layer and estrus (43).

**2.4. Endometrial biopsy and relationship with age:**

There are several research articles which established a correlation between increasing age and infertility and increase in endometrial fibrosis. One of the first teams to discover that decreased fertility was associated with advanced age, number of years the mare was barren, history of abortion, perineal conformation as well as degree of fibrosis was Gordan and Sartin (34). In 1981, these findings were confirmed by Doig and associates who found that the age of the mares and the average number of years barren increased with the severity of fibrosis noted in the biopsy samples. They also concluded that fertility depended on number of years that the mare was barren and that increased incidence of fetal loss was directly related to increasing severity of endometrial fibrosis.

Leishman and associates found that the number of layers of fibrosis surrounding glands in the deep part of the lamina propria were directly correlated with the years of barrenness (58). They found significant atrophy of the uterine glands that in mares that were barren for more than 3 years. Waelchli showed that mares older than the age of 11 had, on average, a higher Kenny grade scores and significantly lower fertility than younger mares (99). He concluded that even under non-uniform breeding management conditions, an endometrial biopsy had considerable prognostic potential and that the age of the mare must be considered while estimating her fertility potential.

In 1992, Held and associates compared data for 850 mares and determined that early pregnancy loss was associated with the age and biopsy grade but not with parity. More importantly they discerned that since more severe histological changes associated

with age occur in both primi- or multiparous mares, age related changes occur independently of previous pregnancy (41).

Gruninger and colleagues evaluated the morphology of endometrial blood vessels in uterine biopsy specimens from mares of varying age and parity (36). They found inflammatory vascular alterations in 20.5 % of the specimens, while only maiden mares showed unaltered vessels. Vessels of older mares were frequently affected with sclerotic changes. They established that incidence and severity of the angiosis increased with the number of previous pregnancies as well as advancing age of the mare. They were able to correlate that infertility in older, multiparous mares might be linked to angiosis. Recently Walter and associates concluded that chronic degenerative disease of the mare's endometrium is often characterized by changes in the uterine glands, including cystic dilation, hyperplasia and periglandular fibrosis (104). Additionally, mineralization of the cystic glands occurs and it was identified as osteopontin and bone sialoprotein in the lumen while osteonectin was seen in the glandular epithelium.

Many other articles relating to inflammation (57,68) immunological response, hormonal receptors and pathology (5,31,35,39,43,72,93,98,106,109) of the equine endometrium have been published to date. However, those reviewed above provide the clearest overview and context.

**2.5. Effects of endometrial biopsy on the reproductive cycle:**

Since endometrial biopsy is a surgical procedure, however benign it may appear, it does cause disruption of the normal reproductive environment of the uterus. Slight bleeding from the biopsy site is the most common sequelae seen (82). However, in 1976

Nitschelm and Van der Horst showed that uterine stimulation by the act of collection of biopsies on a daily basis can result in occurrence of estrus symptoms with progesterone content in blood higher than 1 ng/ml (64).

In 1978 Hurtgen and Whitmore showed that collection of an endometrial biopsy or culture on day 4 after estrus caused lysis of the corpus luteum as evidenced by a sharp decline in progesterone level and shortening of the interestrus interval (46). They attributed this to the fact that both techniques required cervical manipulation. A year later Hurtgen and Ganjam postulated that these changes in the estrus cycle of mares following intracervical or intrauterine manipulations during the luteal phase may directly or indirectly stimulate release of endogenous prostaglandin resulting in corpus luteum regression (45).

On the other hand Saltiel concluded that uterine biopsy did not affect estrus cycle unless uterine infection is present or induced during the procedure (76). In 1981 Baker and associates refuted this finding (6). They found that length of diestrus in mares did not change when biopsies were collected during estrus; however, biopsies collected post-ovulation day four induced premature luteolysis and reduced length of diestrus. They found negative results on bacterial cultures pre and post collection and thus concluded that the change in the estrus cycle was not due to uterine infection. They also postulated that infusion of gentamicin solution after collection of biopsy on post-ovulation day 4 prevented premature luteolysis. Ellsworth-Swihart and colleagues found that parental administration of phenylbutazone could block the luteolysis induced by uterine biopsy on day four post-ovulation possibly as a result of inhibiting prostaglandin synthesis from the inflamed uterine tissue (27).

In 1989 Gilbert studied the effect of collection uterine biopsy samples at different phases of the cycle on the estrus cycle length (32). Gilbert found that ovulatory interval was shortened in mares where in the biopsy was collected on days four and eight, it was lengthened when samples were collected on days 16 and 20 while cycle length remained unchanged if sample collection was on days 0 and 12. He concluded that these findings were similar to those found in bovine.

In 1992 Watson and Sertich studied the effect of repeated collection of multiple endometrial biopsy specimens on subsequent pregnancy in mares and concluded that it did not adversely affect pregnancy rate in genitally normal mares (105).

**2.6. Representativeness of Site and location of endometrial sample:**

Prior to the late 1980's scientists and clinicians had come to a consensus about the number of biopsy samples to be collected to be representative of a mares uterus. Both Ricketts in 1975 (71) and Bergman along with Kenney in 1975 (7) suggested that in absence of any palpable abnormality of the uterus a single mid-horn biopsy sample would be representative of the histopathology of the entire endometrium. However, if any palpable abnormalities were detected more biopsies would be required. Kenney and Bergman further stated that abnormalities detected prior to the procedure should also be biopsied.

In 1987, Blanchard, Kenney and associates reinvestigated the representativeness of a single endometrial sample (11). Although they found a significant difference in occurrence and severity of inflammation among locations, there was no difference for other findings. Their findings supported Kenney and Bergman's findings and they

concluded that one biopsy was generally representative of the entire endometrium except when there was doubt or concern about a Category III biopsy. In these instances, a repeat biopsy should be done. They found that biopsies taken near the cervix may have fewer glandular elements and thus may affect the grading pattern. They did caution that future studies should take into account location from which the biopsy sample is attained.

A similar study was undertaken by Waelchli in 1989 who corroborated the finding by concluding that in most cases a single biopsy examination, in combination with a thorough clinical exam, should be adequate to assess the mare's endometrium (102). In 1991, Ricketts and Alonso studied the assessment of breeding prognosis on the basis of paired biopsy sample taken before and after treatment. Thus, this paired biopsy sample took into account the mare's ability to respond to treatment and was found to be more accurate in the predictions of live foal than a single biopsy sample.

**2.7. Endometrial Maldifferentiation:**

While endometrial histopathologic lesions have been described thoroughly in the literature, a relatively new classification of endometrial pathology has recently been described. In 1999, Schoon and colleagues described the functional morphology of endometrial maldifferentiation in mares in which the primary diagnosis was based on the investigation of routine hematoxylin-eosin stained formalin fixed biopsy samples supplemented by immunohistochemical techniques (80). They suggested that endometrial maldifferentiation should be considered in the assessment of biopsy specimens as an important parameter leading to fertility problems.

Ellengerger, along with Aupperle and other associates in 2002, studied endometrial maldifferentiation caused by ovarian disorders in the mare with the help of biopsy morphology, endocrinological levels and immunohistochemistry (25). The endometrial maldifferentiation varied in type and degree, depending on the type of ovarian abnormality. Following ovariectomy the biopsy samples revealed diminished presurgical morphological irregularities and diminished atypical expression patterns of all immunohistochemical parameters. Hence they concluded that in cases of clinically apparent megaovary of unknown origin, histopathology of the endometrial biopsies may aid in the characterization of the lesion as an hormonally active ovarian disorder.

Again in 2005, Ellenberger et al. studied the immunohistochemical characteristics of equine endometrial maldifferentiation and its relationship with uterine secretory proteins (26). They found that endometrial maldifferentiation was characterized by abnormal expression patterns of steroid hormone receptors, atypical stromal expression of desmin, discontinuous glandular basal lamina and disturbed secretory protein patterns.

**2.8. Choice of Fixative:**

Bouin's fluid was first described in 1897 and utilized for the study of spermatogenesis (14). The first article that the author can find when Bouin's fluid was utilized for female reproductive tissue was by Newman in 1912 for the study of ovum in nine banded Armadillo (62). Bouin's and several modifications of the original formula have been in use since the 1900's. In fact, it was used by entomologists, botanists, zoologists and marine biologists. The author, however, would like to note that although utilization of Bouin's solution for histopathology of the equine endometrium was

mentioned in 1975 by Ricketts (71), the author did not find any prior study comparing Bouin's with 10% neutral buffered formalin (NBF) for endometrial biopsy. However, a study in 1976 did compare the quantitative differences of fetal rat lenses fixed in Bouin's as compared to formalin (9). They concluded that Bouin's produced less shrinkage, artifact and better visualization of mitiotic figures than formalin.

Several authors mention their preference to use Bouin's solution for 2-24 hours of histopathological fixation of endometrial samples (24,35,49,58). In 1980, Kwittken's study concluded that Bouin's solution was superior to formalin in overall fixation for tissue pathology (55). It would be noteworthy to mention that he was primarily concerned with cutaneous histopathology. Still other authors continued to utilize NBF for endometrial samples (17,85,102).

Some authors mentioned that formalin caused tissue shrinkage and loss of cytological detail (50). Boonstra and colleagues in 1983 measured the cervical tissue shrinkage caused by formaldehyde fixation, paraffin wax embedding, section cutting and mounting and found that it was approximately 15% of the original dimensions (12).

In 1984 Chapin and associates compared the effect of testes from rats, mice and rabbits fixed in different fixatives and embedded in either paraffin or glycol methacrylate (19). They concluded that in paraffin, Bouin's or Helly's fixative produced the fewest artifacts when tissues were embedded in glycol methacrylate, NBF produced the greatest intracellular detail. Steven van Camp in his 1988 review stated that Bouin's solution tended to harden tissue and thus help maintain the architecture of the endometrium (97). He stated that the histology laboratory and the interpreter should be consulted as to the fixative they preferred.

The concerns about toxicity of formalin have resulted in the search for alcohol based fixatives for light microscopy. Bostwick and associates compared NBF to an alcoholic fixative and found that there was no significant difference in quality of fixation, and alcohol-polythylene glycol was a suitable substitute to formalin for routine diagnostic surgical pathology (13). Prento and Lyon in 1997 disagreed with this finding and concluded that none of the proposed commercial substitutes for formalin are adequate for critical histopathology (66).

In 1996 Sertich presented an article at the annual conference for the American Society of Theriogenology which recommended the use of Bouin's fixative for endometrial biopsy fixation (82). Although there are several articles available discussing the type of fixative for testicular tissue, ones for uterine tissue are rare. Currently researchers have found that although the world-wide regulatory body for testing effects of medicinal products and chemicals on reproduction has recommended Bouin's fixative instead of formalin for testicular tissue, there may be a third fixative that is better. Creasy and Jonassen suggested the use of modified Davidson's fixative (mDF) which contained 15% ethanol, 5% glacial acetic acid and 30% formalin (21). In the same context Latendress and associates in 2002 confirmed this finding for both testicular tissue as well as eyes (56). This finding was again corroborated in 2005 by Howroyd and colleagues when they compared mDF against NBF and Bouin's fixative (44). They found that the mDF performed better for fetal rat testis as well and they stressed the importance of establishing the correct fixation conditions for each immunostaining protocol. The most recent article to work on feline testis again showed the superiority of Bouin or Davidson's fixative over formalin for histological processing (87).

While Bouin's fixative has been recommended for use with testicular biopsies, it is not without drawbacks. Besides having the risk of over-hardening tissues Bouin's fixative is labor intensive, requiring multiple alcohol rinses to remove picric acid for optimum preservation and immunohistochemical detection of tissue antigens that may potentially be used to identify and quantify cells and functional proteins with critical roles. The picric acid in Bouin's fixative is a health and safety hazard, as well as a laboratory waste disposal problem (55). Furthermore, picric acid when allowed to crystallize is a dangerous explosive. In summary of this part of the review, NBF and Bouin's fixative have significant drawbacks, whereas mDF may be a superior fixative to use for fixation of reproductive tract tissue samples.

# Chapter III

## Material and Methods

### 3.1. Animals

Ten open adult, cycling mares, with ages varying from 8-15 years, in residence at the Oklahoma State University College of Veterinary Medicine (OSU CVM) Ranch were used in this study. All the selected animals were normal on physical examination as well as had a normal appearing reproductive tract on physical examination and ultrasonographic examination. The study was undertaken between April 15 and July 15 of 2006. All the guidelines of the Institutional Animal Care and Use Committee of Oklahoma State University were followed throughout the experiment.

### 3.2. Ultrasound

The machine used was an Aloka 500 ultrasound scanner with a transrectal 5 Mhz linear transducer. The mares were scanned on a three times per week basis to gather information on the length of their estrus cycles and note their date of ovulation. After this information was gathered the mares were followed closely to determine the phase of estrus. Mares were considered to be in estrus when they had a pre-ovulatory follicle larger than 35 mm in diameter. There was an associated increased heterogeneous echotexture of the endometrium due to increased edema of endometrial folds and a palpably softening of the cervix. This was when the estrus samples were collected. Similarly diestrus was defined as the period when no follicle was above 25mm and there

was a functioning corpus luteum in one of the ovaries, increased homogenous echotexture of the endometrium as well as palpably firm cervix. It is important to note that ultrasonic appearance of the mare uterine tract was not the sole criteria for determining estrus and diestrus and that these findings were correlated to the estrus cycle noted in each mare. A period of 45 days was allowed after the first collection (i.e. estrus) before the second sample set was collected (i.e. diestrus). This was done with an idea to minimize the effects of the first biopsy set on the second set.

### 3.3. Biopsy

Three sites, base of the right and left uterine horn and uterine body, were biopsied from each mare. The mares were restrained in stocks with their tails wrapped. Their rectum was emptied and the perineum was cleaned using chlorhexidine scrub 2% and rinsed with water. Jackson uterine biopsy forceps with an overall length of 60 cm with an alligator type sample basket of 20 X 43 mm (Jorgensen Laboratories, Inc., Loveland, CO) were used to collect the biopsy samples. The clinician used sterile shoulder length sleeves covered by a sterile surgical glove that was sufficiently lubricated with sterile water soluble lubricant. The closed sterile biopsy forceps were inserted with the sterile gloved hand into the caudal genital tract. Using the forefinger of the sterile gloved hand as a guide the biopsy forceps was passed through the cervix and into the uterine body. The closed biopsy forceps were stabilized within the uterus with the opposite hand while the gloved hand was withdrawn and placed rectally.

By manipulation per rectum the biopsy instrument was positioned on the ventral aspect of the base of one of the uterine horns. The jaws of the basket were then opened

while an endometrial fold was pushed inside the jaws per rectum. The forceps were then closed and withdrawn. Care was taken that the sample obtained was large enough for it to be divided into three > 5 mm linear sections with a surgical scalpel blade and placed in the three different fixatives. This procedure was repeated for the opposite horn as well as the body of the uterus. The whole procedure was repeated for collection of both estrus and diestrus sample sets.

### 3.4. Tissue handling and fixation

The three fixatives chosen were 10 % normal buffered Formalin (NBF), Bouin's fixative and modified Davidson's solution (mDF). All three fixatives were obtained from Oklahoma Animal Disease Diagnostic Laboratory (OADDL). The mDF was prepared as per formula obtained from Latendresse et al. (56) which included 30% of a 37–40% formaldehyde, 15% ethanol, 5% glacial acid, and 50% distilled water.

The uterine biopsies from each site of each mare (left horn, right horn and body) were divided and fixed in each of the above 3 fixatives. This procedure was repeated for the diestrus collection as well. For samples in NBF and mDF, the tissues were allowed to fix for 24 hours and then transferred to 70% ethanol and kept there until processed. Samples fixed in Bouin's fixative were removed after 8 hours of fixation and transferred to 70% ethyl alcohol (ETOH). Post fixation, the ETOH was changed 2-3 times over the course of the day before transferring the specimens to a saturated solution of 70% ETOH and lithium carbonate to neutralize the picric acid in Bouin's fluid. This solution was changed 2-3 times further until the yellow color of Bouin's fluid was almost completely depleted from the sample. The samples were then stored in 70% ETOH until processed.

After trimming the tissues were processed through graded alcohols and xylene, embedded in paraffin, sectioned at 5µm, mounted, and stained with hematoxylin and eosin before being cover slipped.

### 3.5. Histopathological Grading

All the samples were then coded so that the pathologist was blinded to biopsy site, fixative and stage of estrus. The slides were graded histopathologically according to the Kenney and Doig (52) four category system (I, IIa, IIb and III) by a board certified pathologist who is accustomed to reading endometrial slides. Once all the slides were graded the samples were decoded and statistical analysis was performed.

### 3.6. Statistical Analysis

The statistical analysis was performed using PC-SAS software (SAS Institute, Cary, N.C.). The significance of site, fixative and time of collection was compared using analysis of variance (ANOVA) procedures (PROC MIXED in SAS) assuming a randomized complete block design with split plot arrangement where the block was the mares, the site of collection was the main plot factor and the split unit factors were fixative and time.

Additionally the data were also subjected to regression analysis followed by forward step wise regression analysis with the biopsy score assigned as the dependent variable and the criterion for an independent variable to enter was $P \leq 0.1$.

# Chapter IV

## Results

Once the slides were given histopathological grades as per the Kenney and Doig (52) scale by a single pathologist blinded to site, fixative and stage of estrus, they were decoded. The decoded data were analyzed statistically using the analysis of variance procedure with a randomized complete block design with split plot model. No interaction was seen between the tree variables i.e. site of collection, fixative and stage of estrus cycle. The result of the histopathological grading for each horse is shown in Appendix 1.

In addition the data were also subjected to forward step wise regression analysis with the biopsy score assigned as the dependent variable and the criterion for independent variable to enter was $P \leq 0.1$. Even with such a liberal criterion, stage of estrus cycle was the only variable that could be included significantly.

### 4.1. Comparison among Sites:

Assigned histopathological grades of endometrial biopsy samples obtained from each respective site (i.e. left horn, right horn and uterine body) from individual mares were compared to assess potential intra-mare differences among grade and site of biopsy. There was no significant difference in the endometrial grades ($p = 0.5299$) among the sites of sample procurement. The means and standard errors intra-mare are shown in Table 1. Our findings indicate that there is no significant variation in histopathological

grading irrespective of the site of collection of the biopsy sample. So a sample collected from any of the three locations (i.e. left horn, right horn or body of the uterus) would be representative of the histopathological grade of the uterus provided no abnormalities were detected.

**Table 1. Result of analysis of intra-mare variance between grade and site of biopsy.**

| **Site of collection** | **Mean** | **Standard Error** |
|---|---|---|
| Body of uterus | 3.08333 | 0.079872 |
| Left horn of uterus | 3.08333 | 0.083333 |
| Right horn of uterus | 3.15245 | 0.089852 |

$p = 0.5299$

**4.2. Comparison between fixatives:**

The three fixatives, buffered neutral formalin 10%, Bouin's and modified Davidson's we used to collect the sample were compared to assess their effect on the grading pattern for each site and the results showed no significant difference regardless of which of the three fixatives was used ($p = 0.6373$). As per the low standard error we can discern that there is very little variation in histopathological grading for an endometrial sample collected from a particular site of the uterus regardless of the 3 different fixatives used (Table 2).

When we compared the results of the biopsy scores in formalin, which is taken as the gold standard for most histopathology, with samples collected in the other fixatives

(i.e Bouin's or mDF) we see a negative coefficient (-0.24). Although the estimate of this measure is not statistically significant it does point to an improvement in the biopsy score if fixatives other than formalin were used.

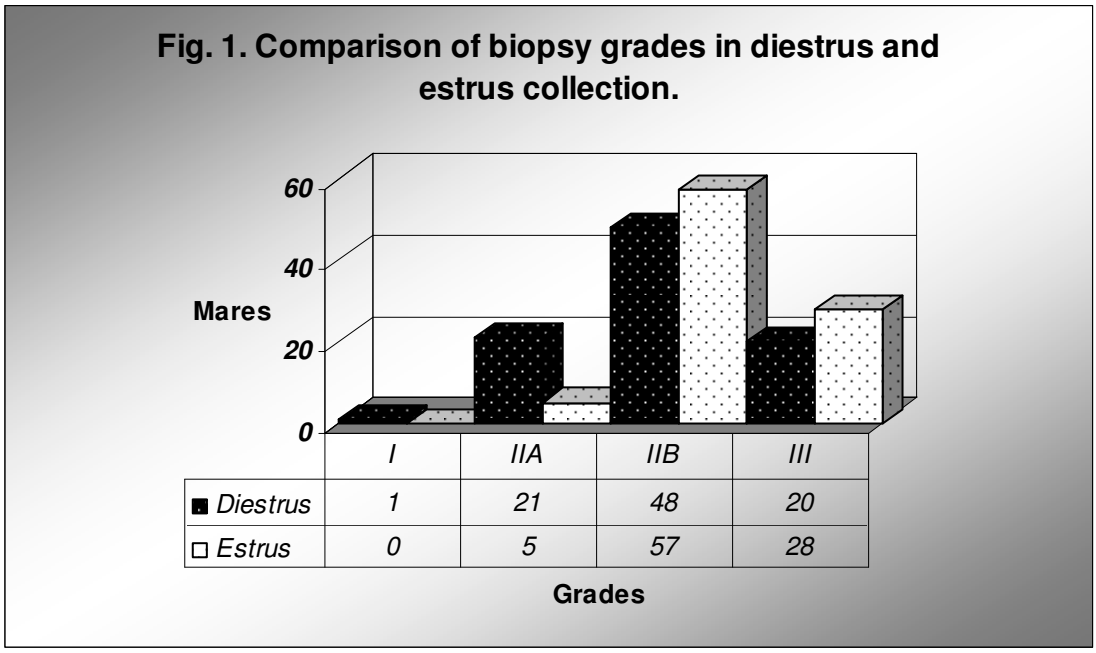
**Table 2. Result of analysis of intra-mare variance between histopathological grade and fixative used for sample collected at each site.**

| **Fixative** | **Mean** | **Standard Error** |
|---|---|---|
| Bouin's Fixative | 3.06667 | 0.081880 |
| 10% neutral buffered formalin | 3.13333 | 0.093478 |
| Modified Davidson's | 3.11864 | 0.076773 |

p = 0.6373

### 4.3. Comparison of time of collection:

We compared the time of collection of endometrial biopsy (i.e. estrus vs. diestrus). A graphical representation of the data at diestrus and estrus collections shows that the histological grades at estrus are consistently higher (Fig. 1). Analysis as per the regression model shows that with an R square of 0.050, stage of estrus cycle accounts for 5% of variation in the biopsy score.




We did find a significant difference in the histopathological grading scale depending on the time the sample was collected ($p = 0.0001$). Thus a paired biopsy sample taken from a mare at different stages of the estrus cycle may show variation in their histological grades despite using the same site of location or similar fixative. (Table 3)

**Table 3. Analysis of variance between histopathological grade in mares and the stage of estrus cycle the sample was collected:**

| **Time of Collection Estrus** | **Mean** | **Standard Error** |
|---|---|---|
| Diestrus | 2.95506 | 0.074800 |
| Estrus | 3.25556 | 0.058190 |

$p = 0.0001$

# Chapter V

## Discussion

Endometrial biopsy has proven to be a vital diagnostic tool in mare reproductive management. Although histopathology will continue to be the central basis for diagnosis of the health of the uterus, new emerging technologies and their application to the biopsy will not only expand the use of biopsy to predict the health status of mare's uterus but also minimize the subjective nature of histopathology (78).

It may be argued that the histopathological grading system as presented by Kenney and Doig (52) has outlived its efficacy and needs a long overdue upgrade to be consistent with the modern technologies available. Currently, the grading pattern does not take other pathological criteria like angioses and endometrial maldifferentiation into consideration, these should be included together with conventional categorization and "epicrisis" to achieve a more representative fertility prognosis of the mare (54,79).

Since the emphasis in the foreseeable future will be on predicting the health status of the uterus of the mare on the basis of tissue taken from an endometrial biopsy, we elected to reinvestigate the extent to which taking one sample was representative of the entire uterus and if choice of fixative has an impact on the biopsy grade. Our results corroborated the findings of Ricketts (71) and Bergman and Kenney (7) since there was no significant difference between the histopathological grades from tissue collected from the left horn, right horn or body of the uterus. Our results are from mares with no abnormalities of the uterus that could be detected either by palpation or transrectal

ultrasound. Kenney and Doig reiterated these findings in 1986 and noted that tissue sample "should be a minimum of 10 to 20 x 3 x 3 mm in size for proper histologic interpretation" (52). Blanchard and associates found significant differences in occurrence of inflammatory changes depending on site of biopsy collection; however, along with the fibrotic changes from samples at different locations, they too concluded that one biopsy was generally representative (11). However, they expressed their concerns on a few of their findings. For example, a grade III biopsy score, should be repeated. Also location of biopsy, if taken near the cervix, may exhibit a decreased number of glandular elements thereby affecting the mean number of fibrotic nests present. Thus, although they agreed with the consensus on this issue they did have a word of warning for practitioners and pathologists alike. Since our biopsy sites were not located near the cervix, there is little danger of this affecting our findings. Our results did not concur with the findings of Ricketts and Alonso, although our samples were paired as well. This may be due to the fact that no treatment was done between collections of the paired samples as in their case (72).

It would be pertinent to mention that in most cases pathologic changes occur uniformly throughout the surface of the endometrium, but occasionally they do tend to be incompletely uniform and thus confirmation of the pathologic changes may require a biopsy at a different site. Unless quantitative measurement techniques are applied to analyze routine endometrial tissue samples, histopathology reading will remain subjective. Hence, one must never condemn a mare on the basis of a single biopsy alone. Lastly, one should also consider that although the endometrial pathological changes diagnosed by histopathology point to a decreased ability of the mare to carry a foal to

term, these mare are not sterile. So one does have to consider the value of the mare's offspring as it may be worth the expense of maintaining and breeding the mare over several years and / or utilizing assisted reproductive techniques such as embryo transfer (52).

We hypothesized Bouin's to be a better fixative than NBF, and mDF to be the best of all the three fixatives used for the study. For years Bouin's has been touted as the best fixative for endometrial biopsy for its ability to preserve certain tissues and it's compatibility with trichrome stains. Kwittken did complete an objective study on cutaneous histopathology and concluded that Bouin's was better than formalin for several reasons (55). Bouin's fixative has been recommended for endometrial biopsy fixation by the Society for Theriogenology and the American College of Theriogenology in their symposia (82). However, depending on the preference of the histology laboratory and interpreter, NBF or Bouin's fixative has been used predominantly. Steven van Camp in his review stated that although most scientists preferred Bouin's because "the solution hardens the tissue and helps maintain the architecture of the endometrium", there were still other scientists who "found that putting the tissue directly in 10% buffered neutral formalin is satisfactory" (97). In this study we found no significant change in histopathological grading irrespective of the fixative used.

In 1987, the Formaldehyde Standard became law in the United States, alerting laboratory workers to the potential carcinogenicity of formaldehyde and laid down threshold levels to minimize exposure of employees (65). This led to a trend to move away from formaldehyde containing fixatives, or at least limit their quantities. Bouin's fixative is composed of the following constituents; buffered formaldehyde, acetic acid,

and picric acid. This solution is generally considered to be hazardous. Specifically, picric acid and formaldehyde are the most dangerous components of Bouin's Fixative. Picric acid is highly explosive when in crystal form and crystals often form as the solution evaporates along the edges of containers. Especially with the current growing concerns about bioterrorism Bouin's fixative is not only difficult to dispose of but also expensive for laboratories to deal with. In addition, Bouin's fixative is labor intensive as it requires multiple alcohol rinses to remove the picric acid. The formaldehyde is a known carcinogen and environmental hazard and although mDF does contain formaldehyde, the quantity is small as compared to NBF or Bouin's. With most human medicine and research laboratories recommending a switch from Bouin's to mDF as a means of eco-conservation, these results should be welcomed by pathologists and environmentalists alike as a less toxic fixative that could be used as a substitute for Bouin and NBF 10%. Our results are similar to the findings of Latendresse et al. in testicular tissue and eye tissue (56) that indeed mDF can be used to supplant Bouin's fixative. Although we did not find it superior to Bouin's or formalin as far as histological grading is concerned of the 10 mares used in the experiment, we did not find it to be inferior either.

Our results also seem to contradict Prento and Lyons study as well as Titford and Horenstein wherein they tested commercially available fixatives proposed to be formalin substitutes and found that none of the proposed fixatives performed as well as formalin for critical histopathology (66,91). It might be pertinent to mention, however, that neither Bouin's nor mDF was included in the trials.

A recent abstract presented at the 2006 conference of the Society of Theriogenolgy studied the effect of the three fixatives (NBF,Bouin's and mDF) on testicular and endometrial tissue collected at necropsy (10). For endometrial samples they concluded that formalin produced a higher number of artifacts as compared to the other two fixatives while the staining and contrast quality was best for mDF. Like our results they too found no difference between Bouin's fixative and mDF for morphological detail.

With new fixatives being developed for surgical diagnostic pathology (91), there should be systematic research done with these fixatives on endometrial biopsies as well. The goal of this research should be to find an environmentally friendly, nontoxic fixative that does not alter the histological prognostic grading pattern and is friendly to immunohistochemical staining as well.

Since 1975 as the endometrial biopsy was generally applied in practice, practitioners and pathologists have been aware of the variation in histological architectural of the endometrium not only throughout the estrus cycle but also the annual physiological breeding cycle. In fact, several authors, including Ricketts, felt endometrial biopsy should be collected during diestrus "since the changes of normal estrus may make interpretation difficult" (71). Other authors, including Kenney, were of the opinion that a biopsy could be collected at any stage of the estrus cycle (49) as long as stage of the cycle was duly noted. In 1986, Kenney and Doig (52), however, cautioned that although endometrial biopsy could be performed during any stage of the estrus cycle "to minimize the variability and avoid misinterpretation the inexperienced practitioner may prefer to obtain the biopsies only during diestrus when the endometrium is under maximum luteal influence." Yet others prefer estrus as the time of collection of biopsy sample (58). This

practice could also stem from the fact that Waelchli and associates discovered that there was a trend to get more positive cytological samples during estrus as compared to diestrus or anestrus (101) as well as the fact that a collection in estrus minimizes the chances of iatrogenic infection of the uterus as compared to diestrus.

In 1984 Gross and LeBlanc noted that seasonal changes reflected in endometrial glands and stroma influenced quantitative assessment of fibrosis, thus occasionally resulting in changes of the assigned prognostic category (35). Our results corroborate their findings as well as those of Blanchard and associates who felt that as a consequence of normal stromal edema during estrus as well as enlargement of nonfibrotic glands between gland nests, the number of nests per millimeter becomes less frequent which could affect the final biopsy interpretation (11). Despite these critical findings the scientific community has not yet acknowledged the need to fix the stage of the cycle for biopsy collection in the mare. To add to this confusion is a well established fact, that even, "experienced pathologists who do not regularly examine endometrial biopsies are apt to misapply Kenney's prognostic criteria." (20). All of these might result in misinterpretation of the prognostic grade and thus undermine the value of a diagnostic biopsy itself. Like Gross and Leblanc, we took the precaution of utilizing the services of only one pathologist to avoid bias in histologic interpretation. Even with selecting a pathologist who is conversant with histological grading of biopsies our results showed significant differences in histological grades allotted to each mare depending stage of cycle.

In summary, we found no significant changes in endometrial grading associated with any of the three fixatives used. However, in light of processing, carcinogenicity and

laboratory safety, mDF may be the fixative of choice. Our findings corroborated earlier findings that one biopsy sample is sufficiently representative of overall endometrial health. Finally, we found a significant difference in endometrial grades associated with stage of the estrus cycle, therefore, we recommend biopsies be obtained during diestrus when there are fewer edema-induced changes within the endometrium.

# Chapter VI

## Conclusion

As expected the results of this study indicate that the site of biopsy collection had no bearing on the histopathological grade. The results also show that one biopsy sample appears to be representative of the entire endometrium.

Our results showed that there was no significant difference between the histological grades despite fixing them in three different fixatives, i.e., 10% neutral buffered formalin, Bouin's fixative and modified Davidson's fixative.

Although our results did not indicate any significant improvement of using modified Davison as compared to the other two fixatives for endometrial samples, we recommend that practitioners consider switching to modified Davidson as a fixative for endometrial biopsy samples. Additionally, the fact that formalin and Bouin's have a large component of formaldehyde, which is a known carcinogen, should draw attention to our recommendation. Furthermore, Bouin's also contains Picric acid which is an explosive and a disposal biohazard by itself. While the shelf life of all three fixatives used in this study is the same, the cost of formalin is the lowest followed by Modified Davidson and then closely by Bouin's. Finally, almost all the literature indicates that fixing in Bouin's should not exceed 24 hours, after which the practitioner must transfer the sample to 70% ethanol or NBF before submission. All these factors lead to the conclusion that it would be more practical to collect samples in Modified Davidson as

compared to Bouin's or normal buffered formalin saline, although our results do not show any statistical difference amongst them.

Although several publications have conceded that the time of collection of endometrial biopsy could affect the endometrial grade, as far as the author knows, our's is the first study that found a significant difference in the histological Kenney and Doig (4 category) grading pattern depending on whether the sample was collected at estrus or diestrus. This is a crucial finding considering that although one pathologist was used for both estrus and diestrus sample grading for 180 endometrial samples, there was a variation in the histological grade.

We do have to concede that we blinded the pathologist with regards to the origin of the samples, as well as the physical and clinical findings and hence he was not able to form a proper "epicrisis" with regards to the samples. The fact that a multitude of endometrial biopsy samples, in practicality, are sent without proper history or clinical findings, can be attested to by any pathologist. In several situations the practitioner himself may be presented with a poor history and hence is unable to fix the stage of estrus when the biopsy is collected. This makes our findings significant as the prognostic value of the endometrial biopsy is undermined if there is no repeatability of the histological grade irrespective of the estrus cycle.

In certain cases paired biopsy samples are indicated and if we consider the variation in histology due the estrus cycle the reliability of these paired biopsies would be in question. Similar to the physiological estrus cycle the endometrium undergoes histological changes associated with the annual reproductive cycle as well. Considering

the significant manner in which histological grading is affected by the estrus cycle, it makes us wonder how the histological grading would be affected by the annual reproductive cycle as well.

Although our study only encompasses ten mares, we postulate that collection of endometrial biopsy be limited to a fixed stage of the estrus cycle of mare depending on the pathologist/laboratory concerned where the sample is to be read. This is indicated more so in cases where paired or multiple biopsies are proposed to be used.

## Bibliography


1. Abd-Elnaeim MM, Leiser R, Wilsher S, Allen WR. Structural and haemovascular aspects of placental growth throughout gestation in young and aged mares. Placenta 2006;27: 1103-1113.
2. Adams GP, Kastelic JP, Bergfelt DR, Ginther OJ. Effect of uterine inflammation and ultrasonically-detected uterine pathology on fertility in the mare. J Reprod Fertil Suppl 1987;35: 445-454.
3. Andrews FN, McKenzie FF. Estrus, Ovulation and Related Phenomena in the Mare. Missouri Agricultural Research Bulletin 329 1941.
4. Asbury AC. Some observations on the relationship of histologic inflammation in the endometrium of mares to fertility. Proceedings of the Annual Convention of the American Association of Equine Practitioners 1982;28: 401-404.
5. Aupperle H, Ozgen SHA, Schoon D, Hoppen HO, Sieme H, Tannapfel A. Cyclical endometrial steroid hormone receptor expression and proliferation intensity in the mare. Equine Vet J 2000;32: 228-232.
6. Baker CB, Newton DI, Mather EC, Oxender WD. Luteolysis in mares after endometrial biopsy. Am J Vet Res 1981;42: 1816-1818.
7. Bergman RV, Kenney RM. Representativeness of a uterine biopsy in the mare. Proceedings of the American Association of Equine Practitioners, 1975;355.

8. Betsch JM. Diagnostic and prognostic use of uterine biopsy in the infertile mare: retrospective study of 485 cases, 26e Journee de la recherche equine, 1 mars 2000., 2000;111-117.

9. Beverly A. Barash THS. Quantitative assessment of effects on fetal rat eyes of fixative and of time postmortem at fixation. Teratology 1976;13: 267-273.

10. Birch S, Snider T, Payton M, Holyoak G. Evaluation of Three Fixatives for Reproductive Biopsies in the Mare & Stallion. Proceedings Society for Theriogenology, Minneapolis, MN, 2006;66:672.

11. Blanchard TL, Garcia MC, Kintner LD, Kenney RM. Investigation of the representativeness of a single endometrial sample and the use of trichrome staining to aid in the detection of endometrial fibrosis in the mare. Theriogenology 1987;28: 445-450.

12. Boonstra H, Oosterhuis JW, Oosterhuis AM, Fleuren GJ. Cervical tissue shrinkage by formaldehyde fixation, paraffin wax embedding, section cutting and mounting. Virchows Arch A Pathol Anat Histopathol 1983;402: 195-201.

13. Bostwick DG, al Annouf N, Choi C. Establishment of the formalin-free surgical pathology laboratory. Utility of an alcohol-based fixative. Arch Pathol Lab Med 1994;118: 298-302.

14. Bouin P. Études sur l'evolution normale et l'involution du tube séminifère, I et II. I Modifications régressives du processus spermatogénetique provoquess expérimentalement. Arch f d'anat mic 1897;1: 225-264.

15. Brandt GW. The significance and interpretation of uterine biopsy in the mare., Proceedings of the 16th Annual Convention of the American Association of Equine Practitioners. p 355-362, 1970.

16. Brandt GW, Manning JP. Improved uterine biopsy techniques for diagnosing infertility in the mare. Vet Med Small Anim Clin 1969;64: 977-983.

17. Britton BA. Endometrial change in the annual reproductive cycle of the mare. J Reprod Fertil Suppl 1982;32: 175-180.

18. Brook D. Uterine culture in mares. Mod Vet Pract 1984;65: A3-8.

19. Chapin RE, Ross MD, Lamb JC. Immersion fixation methods for glycol methacrylate-embedded testes. Toxicol Pathol 1984;12: 221-227.

20. Concha-Bermejillo A, Kennedy PC. Prognostic value of endometrial biopsy in the mare: a retrospective analysis. J Am Vet Med Assoc 1982;181: 680-681.

21. Creasy DM, Jonassen H. Histological Fixation of Testis:Use of Davidson's fluid as an alternative to Bouin's fluid. Veterinary Pathology 1999;36: 518.

22. Darenius K. Early foetal death in the mare. Histological, bacteriological and cytological findings in the endometrium. Acta Veterinaria Scandinavica 1992;33: 147-160.

23. Dascanio JJ, Parker NA, Ley WB, Bailey TL, Purswell BJ, Bowen JM, Digrassie WA. Diagnostic procedures in mare reproduction: uterine evaluation, hysteroscopy, oviductal patency, and scintigraphy. Compendium on Continuing Education for the Practicing Veterinarian 1997;19: 1069-1076.

24. Doig PA, McKnight JD, Miller RB. The use of endometrial biopsy in the infertile mare. Can Vet J 1981;22: 72-76.

25. Ellenberger C, Aupperle H, Bartmann CP, Hoppen HO, Schoon D, Schoon HA. Endometrial maldifferentiation caused by ovarian disorders in the mare - morphological and immunohistochemical studies. Theriogenology 2002;58: 499-502.

26. Ellenberger C, Bartmann CP, Klug E, Hoppen HO, Hoffmann C, Bazer FW, Klug J, Allen WR, Schoon D, Schoon HA. Immunohistochemical characterization of equine endometrial maldifferentiation with special emphasis on uterine secretory proteins. Havemeyer Foundation Monograph Series 2005: 13-15.

27. Ellsworth-Swihart M, Archbald LF, Ingraham RH, Godke RA. Effect of phenylbutazone (PBZ) on luteolysis in the mare induced by uterine biopsy. Theriogenology 1985;23: 381-387.

28. Evans TJ, Miller MA, Ganjam VK, Niswender KD, Ellersieck MR, Krause WJ, Youngquist RS. Morphometric analysis of endometrial periglandular fibrosis in mares. Am J Vet Res 1998;59: 1209-1214.

29. Falomo ME, Corro M, Cavicchioli L, Castagnaro M, Marzini B, Ventura S, Rota A. Evaluation of uterine culture, cytology and biopsy in the mare. Ippologia 2006;17: 27-32.

30. Ferreira-Dias G, Nequin LG, King SS. Morphologic characteristics of equine endometrium classified as Kenney categories I, II, and III, using light and scanning electron microscopy. Am J Vet Res 1994;55: 1060-1065.

31. Ferreira-Dias GM, Nequin LG, King SS. Morphologic comparisons among equine endometrium categories I, II, and III, using light and transmission electron microscopy. Am J Vet Res 1999;60: 49-55.

32. Gilbert RO. The effect of endometrial biopsy on ovulatory interval in mares. Theriogenology 1989;32: 451-454.

33. Gomez-Cuetara C, Flores JM, Sanchez J, Rodriguez A, Sanchez MA. Histological changes in the uterus during postpartum in the mare. Anat Histol Embryol 1995;24: 19-23.

34. Gordon LR, Sartin EM. Endometrial biopsy as an aid to diagnosis and prognosis in equine infertility. Journal of Equine Medicine and Surgery 1978;2: 328-336.

35. Gross TL, LeBlanc MM. Seasonal variation of histomorphologic features of equine endometrium. J Am Vet Med Assoc 1984;184: 1379-1382.

36. Gruninger B, Schoon HA, Schoon D, Menger S, Klug E. Incidence and morphology of endometrial angiopathies in mares in relationship to age and parity. Journal of Comparative Pathology 1998;119: 293-309.

37. Hammond J, Wodzicki K. Anatomical and Histological Changes during the Oestrous Cycle in the Mare. Proceedings of the Royal Society of London Series B, Biological Sciences 1941;130: 1-23.

38. Hecker C, Hospes R. Endometrial biopsy in the mare - "blind" sampling of tissue or under hysteroscopic control? Pferdeheilkunde 2006;22: 140-144.

39. Hedberg Y, Dalin AM, Ohagen P, Holm KR, Kindahl H. Effect of oestrous-cycle stage on the response of mares in a novel object test and isolation test. Reprod Domest Anim 2005;40: 480-488.

40. Held JP, Schneider JF. Value of endometrial cultures and histopathology in assessing uterine infection in the mare. Proceedings of the Annual Convention of the American Association of Equine Practitioners, 1994;50-52.

41. Held JPE, Rohrbach BW. Ability of endometrial biopsy results, parity, and age to predict subsequent pregnancy in the mare, Proceedings of the Thirty-Seventh Annual Convention of the American Association of Equine Practitioners, San Francisco, California, December 1-4, 1991., 1992;183-188.

42. Heuer JA, King SS, Gardiner CS, Ferreira-Dias G, Nequin LG. Uterine secretions from different classifications affect the viability of early murine embryos cultured in vitro. Journal of Equine Veterinary Science 1993;13: 494-497.

43. Hirsburner G, Reist M, Couto S, Steiner A, Snyder J, van Leeuven E, Liu I. An in vitro study on spontaneous myometrial contractility in the mare during estrus and diestrus. Theriogenology 2006: 517-527.

44. Howroyd P, Hoyle-Thacker R, Lyght O, Williams D, Kleymenova E. Morphology of the fetal rat testis preserved in different fixatives. Toxicol Pathol 2005;33: 300-304.

45. Hurtgen JP, Ganjam VK. The effect of intrauterine and cervical manipulation on the equine oestrous cycle and hormone profiles. J Reprod Fertil Suppl 1979: 191-197.

46. Hurtgen JP, Whitmore HL. Effects of endometrial biopsy, uterine culture, and cervical dilatation on the equine estrous cycle. J Am Vet Med Assoc 1978;173: 97-100.

47. Katila T. Histology of the post partum equine uterus as determined by endometrial biopsies. Acta Vet Scand 1988;29: 173-180.

48. Katkiewicz M, Witkowski M, Zajac S. Endometrial biopsy of mares: visualization of healthy and diseased structures. Medycyna Weterynaryjna 2007;63: 463-466.

49. Kenney RM. Clinical aspects of endometrial biopsy in fertility evaluation of the mare, Proceedings of the 23rd Annual Convention of the American Association of Equine Practitioners Vancouver, British Columbia, 1977.

50. Kenney RM. Cyclic and pathologic changes of the mare endometrium as detected by biopsy, with a note on early embryonic death. J Am Vet Med Assoc 1978;172: 241-262.

51. Kenney RM. Prognostic value of endometrial biopsy of the mare. J Reprod Fertil Suppl 1975: 347-348.

52. Kenney RM, Doig PA. Equine endometrial biopsy. In: Morrow D (ed), Current therapy in theriogenology, 2nd edition: Philadelphia: WB Saunders, 1986;723-729.

53. Kenney RM, Ganjam VK. Selected pathological changes of the mare uterus and ovary. J Reprod Fertil Suppl 1975: 335-339.

54. Klug E. New diagnosis, basis for therapy and technology in veterinary reproductive medicine. - Does the extent and intensity of conventional reproductive examination of the horse fit the recent demand of purchase exams? - Part 1: Mare. Pferdeheilkunde 2003;19: 693-697.

55. Kwittken J. Bouin's solution versus formalin fixation in cutaneous histopathology. Mt Sinai J Med 1980;47: 600-605.

56. Latendresse JR, Warbrittion AR, Jonassen H, Creasy DM. Fixation of testes and eyes using a modified Davidson's fluid: comparison with Bouin's fluid and conventional Davidson's fluid. Toxicol Pathol 2002;30: 524-533.

57. LeBlanc MM. The equine endometrium and pathophysiology of endometritis. Proc Reprod Pathol Society for Theriogenology., 1997;78-84.

58. Leishman D, Miller RB, Doig PA. A quantitative study of the histological morphology of the endometrium of normal and barren mares. Can J Comp Med 1982;46: 17-20.

59. Ley WB, Bowen JM, Sponenberg DP, Lessard PN. Dimethyl sulfoxide intrauterine therapy in the mare: Effects upon endometrial histological features and biopsy classification. Theriogenology 1989;32: 263-276.

60. Maloufi F, Pierson R, Ball K, Otto S, Card C. Comparison between endometrial echotexture and endometrial histopathological features in mares, Proceedings of Annual Conference and Symposium of American College of Theriogenologists and Society for Theriogenology, 2002.

61. Mansour GD, Ferreira AMR, Fernandes FT, Henry M. Histomorphometry of epithelial structures of the mare's endometrium. Revista Brasileira de Ciencia Veterinaria 2004;11: 44-48.

62. Newman HH. The Ovum of the Nine-Banded Armadillo. Growth of the Ovocytes, Maturation and Fertilization. Biological Bulletin 1912;23: 100-140.

63. Nielsen JM. Endometritis in the mare: a diagnostic study comparing cultures from swab and biopsy. Theriogenology 2005;64: 510-518.

64. Nitschelm D, Van der Horst CJ. The influence of taking uterine biopsies on the concentration of some steroids in the blood and in the uterus, on the ovarian activity, and on the sexual behaviour of the mare. Tijdschr Diergeneeskd 1976;101: 796-804.

65. OSHA. Directive No. CPL 02-02-052 - CPL 2-2.52 - Enforcement Procedure for Occupational Exposure to Formaldehyde In: Labor (ed), Standard: 1910.1048, 1990.

66. Prento P, Lyon H. Commercial formalin substitutes for histopathology. Biotech Histochem 1997;72: 273-282.

67. Pycock JF, Newcombe JR. The relationship between intraluminal uterine fluid, endometritis, and pregnancy rate in the mare. Equine Practice 1996;18: 19-22.

68. Reilas T, Katila T, Makela O, Huhtinen M, Koskinen E. Intrauterine fluid accumulation in oestrous mares. Acta Vet Scand 1997;38: 69-78.

69. Reiswig JD, Threlfall WR, Rosol TJ. A comparison of endometrial biopsy, culture and cytology during oestrus and dioestrus in the horse. Equine Vet J 1993;25: 240-241.

70. Ricketts SW. Endometrial biopsy as a guide to diagnosis of endometrial pathology in the mare. J Reprod Fertil Suppl 1975: 341-345.

71. Ricketts SW. The technique and clinical application of endometrial biopsy in the mare. Equine Vet J 1975;7: 102-108.

72. Ricketts SW, Alonso S. Assessment of the breeding prognosis of mares using paired endometrial biopsy techniques. Equine Vet J 1991;23: 185-188.

73. Ricketts SW, Barrelet A. The ability of mares to respond to treatment for uterine abnormalities diagnosed by endometrial biopsy and some causes for failure to respond - A review of 1099 cases. Pferdeheilkunde 2001;17: 644-649.

74. Ricketts SW, Barrelet A. A retrospective review of the histopathological features seen in a series of 4241 endometrial biopsy samples collected from UK Thoroughbred mares over a 25 year period. Pferdeheilkunde 1997;13: 525-530.

75. Roszel JF, Freeman KP. Equine endometrial cytology. Vet Clin North Am Equine Pract 1988;4: 247-262.

76. Saltiel A. Effect of endometrial biopsy on oestrous cycle of the mare, 9th International Congress on Animal Reproduction and Artificial Insemination, June 1980, Madrid, Spain. Volume III, 1980;196.

77. Samuel CA, Ricketts SW, Rossdale PD, Steven DH, Thurley KW. Scanning electron microscope studies of the endometrium of the cyclic mare. J Reprod Fertil Suppl 1979: 287-292.

78. Schlafer DH. Equine endometrial biopsy: Enhancement of clinical value by more extensive histopathology and application of new diagnostic techniques? Theriogenology 2007.

79. Schoon HA, Schoon D. The Category I mare (Kenney and Doig 1986): expected foaling rate 80-90% - fact or fiction? Pferdeheilkunde 2003;19: 698-701.

80. Schoon HA, Schoon D, Wiegandt I, Bartmann CP, Aupperle H. "Endometrial maldifferentiation" - a clinically significant diagnosis in equine reproduction? Pferdeheilkunde 1999;15: 555-559.

81. Seaborn E. The oestrus cycle in the mare and some associated phenomena. Anat Rec 1925;30: 277-287.

82. Sertich PL. Breeding soundness examination of the mare, Proceedings American College of Theriogenologists and Society for Theriogenology Mare Reproduction Symposium. Hastings, Neb, 1996;9-20.

83. Shideler RK, McChesney AE, Morrow GL, Voss JL, Nash JG. Endometrial biopsy in the mare, Proceedings of the 23rd Annual Convention of the American Association of Equine Practitioners, Vancouver, British Columbia, December 1977, 1978;97-103.

84. Shideler RK, McChesney, A.E., Morrow, G.L., Voss, J.L., Nash, J.G. . Relationship of endometrial biopsy and other management factors on fertility of the broodmares. J Equine Vet Sci 1982;2: 5-10.

85. Slusher SH, Freeman KP, Roszel JF. Eosinophils in equine uterine cytology and histology specimens. J Am Vet Med Assoc 1984;184: 665-670.

86. Slusher SH, Freeman KP, Roszel JF. Infertility Diagnosis in Mares uising endometrial biopsy, culture and aspirate cytology. Proceedings of the American Association of Equine Practitioners., Toronto, Canada, 1985;165.

87. Soares JM, Beletti ME, Machado EdR, Silva M. Histomorphometry of cat (Felis domestica) testis using three different fixative solutions. Bioscience Journal 2006;22: 175-181.

88. Steiger K, Kersten F, Aupperle H, Schoon D, Schoon HA. Puerperal involution in the mare - a comparison of clinical signs and pathology. Pferdeheilkunde 2001;17: 627-632.

89. Summerfield NJ, Watson ED. Endometrial macrophage populations in genitally normal mares at oestrus and dioestrus and in mares susceptible to endometritis. Equine Vet J 1998;30: 79-81.

90. Threlfall WR. Accurate diagnosis and appropriate therapy of uterine disease, Proceedings American College of Theriogenologists and Society for Theriogenology Mare Reproduction Symposium. Hastings, Neb,1996;51-69.

91. Titford ME, Horenstein MG. Histomorphologic assessment of formalin substitute fixatives for diagnostic surgical pathology. Arch Pathol Lab Med 2005;129: 502-506.

92. Tobler EE. Collection of uterine fluid and uterine biopsy. Veterinary Medicine, Small Animal Clinician: VM, SAC 1966;61: 779-788.

93. Troedsson MH, deMoraes MJ, Liu IK. Correlations between histologic endometrial lesions in mares and clinical response to intrauterine exposure with Streptococcus zooepidemicus. Am J Vet Res 1993;54: 570-572.

94. Troedsson MH, Loset K, Alghamdi AM, Dahms B, Crabo BG. Interaction between equine semen and the endometrium: the inflammatory response to semen. Anim Reprod Sci 2001;68: 273-278.

95. Troedsson MH, Scott MA, Liu IKM. Pathogenesis and treatment of chronic uterine infection. Proceedings of the Annual Convention of the American Association of Equine Practitioners, 1993;595-601.

96. Tunon AM, Katila T, Magnusson U, Nummijarvi A, Rodriguez-Martinez H. T-cell distribution in two different segments of the equine endometrium 6 and 48 hours after insemination. Theriogenology 2000;54: 835-841.

97. Van Camp SD. Endometrial biopsy of the mare. A review and update. Vet Clin North Am Equine Pract 1988;4: 229-245.

98. Wada R, Fukunaga Y, Kanemaru T, Kondo T. Histopathological and immunofluorescent studies on transplacental infection in experimentally induced abortion by equine arteritis virus. Zentralbl Veterinarmed B 1996;43: 65-74.

99. Waelchli RO. Endometrial biopsy in mares under nonuniform breeding management conditions: Prognostic value and relationship with age. Can Vet J 1990;31: 379-384.

100. Waelchli RO, Corboz L, Winder NC. Comparison of histological, cytological and bacteriological findings in the endometrium of the mare. Zentralbl Veterinarmed A 1988;35: 442-449.

101. Waelchli RO, Kanzig M, Gygax A, Corboz L, Rusch P. The relationship between cycle stage and results of uterine culture in the mare. Zentralbl Veterinarmed A 1993;40: 569-575.

102. Waelchli RO, Winder NC. Distribution of histological lesions in the equine endometrium. Vet Rec 1989;124: 271-273.

103. Waelchli RO, Winder NC. Immunohistochemical evaluation of the equine endometrium during the oestrous cycle. Equine Vet J 1987;19: 299-302.

104. Walter I, Helmreich M, Handler J, Aurich C. Mineralised deposits in the uterine glands of mares with chronic endometrial degeneration. Veterinary Record 2003;153: 708-710.

105. Watson ED, Sertich PL. Effect of repeated collection of multiple endometrial biopsy specimens on subsequent pregnancy in mares. J Am Vet Med Assoc 1992;201: 438-440.

106. Welle MM, Audige L, Belz JP. The equine endometrial mast cell during the puerperal period: evaluation of mast cell numbers and types in comparison to other inflammatory changes. Vet Pathol 1997;34: 23-30.

107. Williamson P, Munyua SJM, Penhale J. Endometritis in the mare: A comparison between reproductive history and uterine biopsy as techniques for predicting susceptibility of mares to uterine infection. Theriogenology 1989;32: 351-357.

108. Witherspoon DM, Goldston RT, Adsit ME. Uterine culture and biopsy in the mare. Journal Of The American Veterinary Medical Association 1972;161: 1365-1366.

109. Zerbe H, Engelke F, Klug E, Schoon HA, Leibold W. Degenerative endometrial changes do not change the functional capacity of immigrating uterine neutrophils in mares. Reproduction In Domestic Animals 2004;39: 94-98.

# Appendix

**Histological grades as per mare, site, fixative and stage of estrus cycle.**

| Mare | Site | Fixative | Diestrus Collection | Estrus Collection |
|---|---|---|---|---|
| | | | | |
| 892 | Left Horn | Formalin | 2A | 2B |
| | | Bouin | 2A | 2A |
| | | Mod Davidson | 2B | 2B |
| | Right Horn | Formalin | 1 | 2B |
| | | Bouin | 2A | 2B |
| | | Mod Davidson | 2A | 2B |
| | Body | Formalin | 2A | 2B |
| | | Bouin | 2A | 2B |
| | | Mod davidson | 2B | 2B |
| | | | | |
| 918 | Left Horn | Formalin | 2A | 2B |
| | | Bouin | 2B | 2B |
| | | Mod davidson | 2B | 2B |
| | Right Horn | Formalin | 2B | 2B |
| | | Bouin | 2B | 2B |
| | | Mod davidson | 2A | 2B |
| | Body | Formalin | 2A | 2B |
| | | Bouin | 2B | 2B |
| | | Mod davidson | 2B | 2B |
| | | | | |
| 919 | Left Horn | Formalin | 2A | 2B |
| | | Bouin | 2A | 2A |
| | | Mod davidson | 2A | 2A |
| | Right Horn | Formalin | 2A | 2B |
| | | Bouin | 2A | 2B |
| | | Mod davidson | 2A | 2B |
| | Body | Formalin | 2B | 2A |
| | | Bouin | 2A | 2B |
| | | Mod davidson | 2B | 2A |
| | | | | |
| 920 | Left Horn | Formalin | 3 | 2B |
| | | Bouin | 2B | 2B |

| Mare | Site | Fixative | Diestrus Collection | Estrus Collection |
|---|---|---|---|---|
| | | | | |
| | | Mod davidson | 2B | 3 |
| | Right Horn | Formalin | 2B | 2B |
| | | Bouin | 2B | 2B |
| | | Mod davidson | 2B | 3 |
| | Body | Formalin | 2B | 2B |
| | | Bouin | 2B | 2B |
| | | Mod davidson | 2B | 2B |
| | | | | |
| 921 | Left Horn | Formalin | 2B | 3 |
| | | Bouin | 2B | 2B |
| | | Mod davidson | 2B | 2B |
| | Right Horn | Formalin | 2B | 2B |
| | | Bouin | 2B | 2B |
| | | Mod davidson | 2B | 3 |
| | Body | Formalin | 2B | 3 |
| | | Bouin | 2A | 2B |
| | | Mod davidson | 2B | 2B |
| | | | | |
| 924 | Left Horn | Formalin | 3 | 3 |
| | | Bouin | 2B | 3 |
| | | Mod davidson | 2B | 3 |
| | Right Horn | Formalin | 3 | 3 |
| | | Bouin | 3 | 3 |
| | | Mod davidson | 3 | 3 |
| | Body | Formalin | 3 | 2B |
| | | Bouin | 2B | 3 |
| | | Mod davidson | 2B | 3 |
| | | | | |
| 925 | Left Horn | Formalin | 2B | 2B |
| | | Bouin | 3 | 2B |
| | | Mod davidson | 2B | 2B |
| | Right Horn | Formalin | 2B | 3 |
| | | Bouin | 3 | 2B |
| | | Mod davidson | 3 | 3 |
| | Body | Formalin | 2B | 3 |
| | | Bouin | 3 | 3 |
| | | Mod davidson | 2B | 2B |
| | | | | |
| 926 | Left Horn | Formalin | 3 | 2B |
| | | Bouin | 2B | 2B |
| | | Mod davidson | 2B | 2B |
| | Right Horn | Formalin | 2B | 3 |
| | | Bouin | 2B | 2B |
| | | Mod davidson | 2B | 2B |
| | Body | Formalin | 2B | 3 |

| Mare | Site | Fixative | Diestrus Collection | Estrus Collection |
|---|---|---|---|---|
| | | | | |
| | | Bouin | 2B | 2B |
| | | Mod davidson | 2B | 2B |
| | | | | |
| 927 | Left Horn | Formalin | 2B | 2B |
| | | Bouin | 2A | 2B |
| | | Mod davidson | 2B | 2B |
| | | | | |
| 927 | Right Horn | Formalin | 2A | 2B |
| | | Bouin | 2B | 2B |
| | | Mod davidson | 2B | 2B |
| | Body | Formalin | 2A | 2B |
| | | Bouin | 2B | 2B |
| | | Mod davidson | 2A | 2B |
| | | | | |
| 930 | Left Horn | Formalin | 3 | 3 |
| | | Bouin | 3 | 3 |
| | | Mod davidson | 3 | 3 |
| | Right Horn | Formalin | 3 | 3 |
| | | Bouin | 3 | 3 |
| | | Mod davidson | 3 | 3 |
| | Body | Formalin | 3 | 3 |
| | | Bouin | 3 | 3 |
| | | Mod davidson | 3 | 3 |

**VITA**

**Cyrus Sepoy**

**Candidate for the degree of**

**Master of Science**

Thesis: EVALUATION OF THE EFFECT OF SITE OF COLLECTION, FIXATIVE AND STAGE OF ESTRUS CYCLE ON UTERINE BIOPSY GRADE

Major Field: Veterinary Biomedical Sciences

Bibliographical:

Personal Data: Born on 17th August, 1975, son of Behli and Ashish Sepoy.

Education: Graduated from Christ Church High School, Bombay, India in March 1991; Graduated with Pre-Degree from KC College, Bombay, India in June 1993; Received Bachelor of Veterinary Science and Animal Husbandry degree from Bombay Veterinary College, Bombay, Maharashtra, India in February 1998; Received Master of Veterinary Science degree from Bombay Veterinary College, Bombay Maharashtra, India in March 2001. Completed the requirements for the Master of Science degree in Veterinary Biomedical Sciences at Oklahoma State University in May, 2008.

Experience: Worked part time as Assistant Veterinary Officer, Amateur Riders Club, Bombay, India, March 1998 to May 2001; Also worked as a Research fellow, Veterinary Medicine Department, Bombay Veterinary College, India, October 1999 to May 2001; Assistant Veterinary Officer, R.W.I.T.C., Bombay, India, May 2001 to November 2004, Teaching Assistant, Veterinary Anatomy, Department of Physiological Sciences, College of Veterinary Health Sciences, Oklahoma State University, January 2005 to May 2006,

Professional Memberships: Indian Veterinary Association (Maharashtra Chapter). Indian Association of Equine practitioners.