

Preservation, handling, and common artifacts of the endometrial and testicular biopsy

Christopher Premanandan,^a Erin Runcan,^b Marco Coutinho Da Silva^b

^aDepartment of Veterinary Biosciences and ^bDepartment of Veterinary Clinical Sciences, College of Veterinary Medicine, The Ohio State University, Columbus, OH

Introduction

The value of histologic evaluation of tissues in theriogenology is two-fold: diagnostics utilized for autopsy evaluation (abortion cases) and assessment of changes in tissue structure through biopsy evaluation. Abortion cases involve the evaluation of fetal membranes and fetal organs in addition to any required ancillary diagnostic testing. Any tissue from the reproductive tract can be evaluated from the surgical biopsy perspective and these tissues are frequently evaluated at private diagnostic laboratories and state diagnostic laboratories in terms of general pathology. Specimens in this category include whole testicles after orchietomy and whole female tubular reproductive tracts cranial to the cervix, and ovaries. Additionally vaginal or vestibular lesions as well as penile and preputial lesions may be evaluated. However, in addition to these commonly submitted surgical pathology specimens, the theriogenologist often submits a unique subset of specimens required to evaluate the potential fertility of a breeding animal, namely endometrial and testicular biopsies. Equine and canine endometrial biopsies and canine testicular biopsies are the most common tissue submitted for evaluation to The Ohio State University Theriogenology and Veterinary Reproductive Pathology Service. Each of these tissues has unique characteristics in terms of preservation and evaluation that can impact the histopathologic evaluation and results received by the submitting clinician. This review will cover the ideal methods of tissue fixation and preservation and will focus on the common artifacts, idiosyncracies and difficulties unique to the interpretation of endometrial biopsies and testicular biopsies.

Fixation, storage and shipping

Tissue fixation prior to processing for histologic sectioning is generally categorized into two methods: physical (cryopreservation, heat fixation) and chemical. While cryopreservation has a place in diagnostic histopathology, chemical preservation is by far the most common method of fixation. Chemical fixation acts by inducing either protein denaturation (coagulation) or protein crosslinking. The most common cross-linking agent utilized for the fixation of surgical biopsy specimens from the reproductive system is 10% neutral buffered formalin (NBF). Fixatives such as Bouin's solution and modified Davidson's medium induce protein denaturation (coagulation) by disruption of hydrogen and electrostatic charges. Each of these fixatives has advantages and disadvantages (Table). In the authors' experience, there appears to be confusion regarding the best fixative to use for reproductive tissues. There is no ideal fixative that completely eliminates cellular preservation artifacts. Preparation of tissue prior to fixation is often far more important for optimal results. Ideal fixation takes place when a tissue is less than or equal to 0.5 cm in thickness in one dimension. The middle regions of excessively thick tissue can undergo autolysis while completely immersed in preservative. Temperature can affect the rate of tissue fixation as well. Environmental room temperature (20°C) is adequate for most purposes. Increases in temperature can hasten the fixation process, however, this can also increase the rate of autolysis in tissue. Conversely, refrigeration can impede the diffusion of fixative into the tissue. While variations in temperature have the clearest implications regarding tissue fixation in research, one must consider its implications for clinical samples when shipping tissues to a diagnostic facility.

Ten percent NBF is composed of 4% formaldehyde in addition to phosphate buffers that maintain pH around 7. The neutral pH prevents the formation of artifactual formalin pigments. Neutral buffered formalin preserves tissue by forming stable covalent cross linkages (methylene bridges) between amino acids.¹ There are several advantages to utilizing this fixative, the first being that the majority of pathologists are accustomed to evaluating tissues fixed in NBF. Also, NBF is the most readily available preservative and often provided by commercial diagnostic laboratories for biopsy submissions. If properly stored, small quantities of NBF can be kept for long periods of time before it is utilized. In addition, although not ideal for some diagnostic and research purposes (such as immunohistochemistry),

tissue can be stored in NBF for long periods of time. For a majority of specimens submitted for evaluation by a reproductive pathologist, NBF is a suitable fixative.

Utilizing NBF does have disadvantages. Immunoreactivity for immunohistochemical and immunofluorescence assays can be reduced with long periods of storage in NBF. In these situations, tissue should be transferred to 70% ethanol or phosphate buffered saline if additional storage time is necessary. Alternatively, processing and storing the tissue as paraffin embedded blocks soon after the minimum fixation period helps to retain immunoreactivity. The accumulation of formic acid in NBF can result in deposits of an artifactual pigment in tissue known as acid hematin.² It is commonly observed in autopsy specimens stored in NBF for extended periods of time, however, it is rarely an issue for biopsy specimens due to the rapid turnover of tissue for processing². For practitioners specializing in reproduction, this is more of a concern for the aborted or stillborn fetus submitted for evaluation. Formaldehyde does pose a health risk to personnel (both acute toxicity and as a carcinogen) but this risk can be easily minimized through proper storage and handling of the chemical. The quality of the histologic sections obtained with NBF fixation is adequate for most diagnostic purposes. However, tissue shrinkage can occur and, in some tissues, nuclear detail - and to a lesser degree, cytoplasmic detail - can be obscured. This tends particularly to be the case in thicker testicular biopsies in which separation of the spermatogenic epithelium from the underlying basement membrane can take place. In addition, the identification of the cells comprising the adluminal compartment can be difficult within the seminiferous tubules.

Bouin's fixative is a formaldehyde based fixative that contains a small percentage of acetic acid (5%) and picric acid (0.9%). Similar to NBF, Bouin's has advantages and disadvantages. Bouin's penetrates tissues rapidly and uniformly. Because this fixative induces both protein crosslinking and protein coagulation, this fixative has a reputation for excellent preservation of cellular detail.³ This is particularly useful in testicular biopsies but is of less value in endometrial, ovarian and vaginal biopsies. However, Bouin's has some unique properties that present some disadvantages. A distinguishing component of the fixative is presence of picric acid, a compound that imparts the coagulative action of this particular fixative. However, picric acid is flammable and explosive in its dried form. Excessive storage times in Bouin's can render tissues brittle and difficult to process, causing marked artifacts in histologic sections that can make interpretation of biopsies difficult (Figure 1). Tissues should be fixed in Bouin's for 24-48 hours and then transferred to 70% ethanol to prevent excessive hardening.^{4,7,8} Occasionally, repeated rinses in 70% ethanol or saline may be required depending on the application, such as immunohistochemistry.

Davidson's medium (also known as Hartmann's fixative and modified Davidson's fixative) is a formalin-based fixative that has additional components of glacial acetic acid (5%) and ethanol (14.25%). The distinction between Davidson's fixative and modified Davidson's fixative is unclear in histology references as the components of both are identical in most material safety data sheets. Similar to Bouin's, this solution induces both protein coagulation and protein crosslinking. Tissue fixation in this medium is rapid and tissues (particularly small biopsies) should be transferred to 70% ethanol in 24-48 hours. Davidson's medium has a good reputation regarding preservation of cellular detail, particularly of the nucleus and chromatin patterns⁵. While this may not be an advantage for endometrial biopsies, it has been demonstrated as an advantage for testicular biopsies. Rat testes fixed in modified Davidson medium demonstrated less shrinkage artifact than 4% paraformaldehyde or Bouin's fixative, particularly in the central region of the testes.⁶ In addition, nuclear detail was better when tissue was fixed in modified Davidson's medium.⁶ In a similar study, rat, rabbit, dog and cynomolgus macaque testes demonstrated less shrinkage artifact when fixed in modified Davidson's medium compared to Bouin's fixative.⁸ This study also compared acrosomal staining with periodic acid Schiff. Acrosomal staining in rat testes was judged to be subjectively more prominent when fixed in modified Davidson's medium.⁸ Overall clarity of detail was also better with modified Davidson's medium in addition to immunohistochemical staining of proliferating cell nuclear antigen, androgen receptor and protein gene product 9.5.⁸ The Society of Toxicologic Pathology (STP) has stated that modified Davidson's medium is recommended over Bouin's for regulatory studies involving fertility, and formalin should be avoided.⁹

Numerous other types of fixatives, processing and techniques are useful for reproductive tissue particularly for research purposes, but a detailed overview of these modalities is out of the scope of this discussion. Some of these are limited in their usage due to their availability, cost, or inherent safety concerns. For instance, Zenker's fixative is described as a fixative useful for testicular biopsies in particular,⁶ however, since the solution contains mercuric chloride, its usage in practice can be cumbersome in the event of spillage. Plastic embedding of testicular tissue for histologic sections provides excellent cellular detail and minimal processing artifact but is costly and requires more technical expertise.¹⁰ Other modalities of tissue processing and staining are available depending on the requirements of the tissue from the biopsy.

General concepts and artifacts

The female reproductive system and endometrial biopsies

In general, most tissues from the female reproductive system are adequately fixed by immersion in NBF as long as care is taken to insure an adequate NBF to tissue ratio (10:1). Normally sized canine and feline ovaries will typically fix well when immersed whole; however, if there is an excessive amount of adipose tissue in the mesosalpinx, mesovarium and ovarian bursa, exposing the ovary by opening the bursa (particularly in canines) is prudent. Equine and bovine ovaries of normal size should be partially sectioned prior to immersion. Enlarged ovaries (typically due to neoplastic disease) should be sectioned, but the practitioner who does not have the luxury of a pathology service directly attached to the clinical facility may be forced to obtain representative regions of the ovary rather than submitting the whole specimen. The canine and feline uterus can be submitted intact for evaluation; however, the practitioner must keep in mind that a relatively small proportion of the organ will be examined histologically. In our service, the standard protocol is to examine a cross section and longitudinal section of the both uterine horns and the uterine body.

Technical aspects regarding equine endometrial biopsy collection, fixation, processing and evaluation are well-described in the literature.¹¹ Endometrial biopsies from llamas and cattle are very similar to equine endometrial biopsies in terms of these factors.^{12,13} Descriptions regarding the canine endometrial tissue biopsy in regards to collection have been described as well as lesions associated with infertility in the bitch.^{14,15,16} Collection methods include punch biopsies, transcervical endometrial biopsies and wedge biopsies. Many histologic artifacts are shared between endometrial biopsies of any species. Numerous other general artifacts are present associated with fixation and tissue processing. Since these artifacts are common among all organ systems, the more clinically relevant artifacts pertaining to reproduction are presented here. Crush artifact is often present at the edges of a tissue section that manifests as nuclear stretching or "streaming" (Figure 2A and B). Hemorrhage is often present at the edge of a specimen associated with tissue sampling and care should be taken not to interpret this as a lesion. The presence of hemosiderin-laden macrophages can be helpful to determine if the hemorrhage was present in the tissue before the biopsy was taken. Another common artifact takes place with the transient increase in pressure on tissue that takes place during the biopsy procedure that causes invagination or "telescoping" of endometrial glands into the adjacent segment (Figure 2A). Storage during shipping and the manner in which the specimen is processed can have a significant impact on interpretation and results. The typical equine endometrial biopsy can be immersed in fixative directly following the procedure. Less discrimination regarding sectioning of the specimen is required as the typical biopsy does not include the myometrium. However, depending on the manner in which the canine endometrium is sampled, some care may need to be taken to insure adequate results are obtained. The transcervical biopsy typically results in the smallest tissue volume, similar to what is obtained in an endoscopic gastrointestinal biopsy. Because of the size of the specimen, care should be taken with fixation and transport of the specimen. Ideally, screw top containers of smaller volume (2-5 mls) should be utilized to minimize risk of tissue loss at the laboratory. Specimens can be placed in mesh cassettes (Figure 3) or, alternatively, placed in a small folded piece of paper towel prior to immersion. Due to the small size of the specimens, crush artifact tends to be significant and can often prevent adequate

evaluation of the tissue. The typical punch biopsy used in canines ranges from 2-6 mm and the amount of endometrial tissue available for evaluation depends on the size of the punch, the amount of crush artifact present and surgical experience with this procedure. Because of the disproportionate amount of endometrial tissue present compared to myometrial tissue present, orientation and embedding can cause issues with interpretation. These issues typically take place if the endometrial tissue is faced away from the cutting surface of the block and result in a section containing only myometrium. This can be easily remedied by requesting that the lab or pathologist melt the paraffin block and reorient the specimen (Figure 4). This, of course, excludes transcervical biopsies that do not contain a portion of myometrium at all. It is helpful in both transcervical and punch biopsies to sample from both horns and the uterine body to improve the correlation between overall changes of the endometrium from a small tissue sample and the entire uterus. Wedge biopsies provide the largest amount of endometrial tissue for evaluation and seldom present a problem with tissue orientation. However, this type of sample may present a problem in terms of tissue closure during surgery from a fully involuted uterus taken during anestrus. In all circumstances, care should be taken to minimize the amount of crush artifact in the tissue by avoiding manipulation of the tissue directly with fingers. Fine forceps or needles should be used to gently tease the tissue into the container from the biopsy instrument.

The male reproductive system and testicular biopsies

Similar to the female reproductive system, tissues from the male reproductive tract are adequately fixed in NBF, provided that the fixative to tissue ratio is optimal. Testicles from adult dogs, horses, and ruminants should be sectioned prior to fixation. Testicles from felines and young dogs can be immersed whole provided that the width of the tissue is less than 1 cm.

Testicular biopsy is an underutilized diagnostic tool for the assessment of infertility in domestic animals. As a consequence, it has been neglected by most veterinary pathologists in terms of understanding the nuances of the biology of the tissue reflected in the histology. In contrast, the role of the testicular biopsy is fairly well delineated in human medicine.^{17,18,19} Confirmation of obstructive azoospermia in infertile men with normal testicular size and hormonal profiles are one indication for a testicular biopsy. Another major indication is the therapeutic biopsy in which testicular spermatozoa extraction is utilized for intracytoplasmic sperm injection.^{17,18} In general, however, it is thought that the testicular biopsy has little value if a thorough clinical evaluation is performed as the underlying causes of abnormalities in spermatogenesis cannot be generally be determined histologically.¹⁹ Considering that the needs of a therioendocrinologist can be much different than those of a human fertility specialist, and testicular causes of infertility can be much more different between domestic animals and in humans, it is worth investigating the diagnostic value of the testicular biopsy in veterinary medicine.

The testicular biopsy has been established as safe in the dog, bull, and stallion and the procedure is covered in greater detail in many references.²⁰⁻²³ Methods of sampling testicular parenchyma include open incisional/wedge biopsies, Tru-cutTM needle biopsies and fine needle aspirates (FNA). Although wedge biopsy provides the most parenchyma for evaluation with the least amount of artifact, the potential complications (testicular hemorrhage, post-biopsy adhesions) generally outweigh its benefits. It is generally recommended that at least 100 seminiferous tubule cross sections are available for proper evaluation of spermatogenesis in a specimen.¹⁷ Many Tru-cutTM needle biopsies are incomplete to some degree and do not contain the recommended 100 cross sections. Therefore, at least two samples from the same testis may be required to provide an adequate sample. Crush artifacts tend to be the highest in this type of specimen (Figure 2B). In addition, significant variation can occur in regards to the degree of testicular degeneration or hypospermatogenesis observed in different regions of the testes. Therefore a single Tru-cutTM needle biopsy may not reflect the overall changes present.

Fine needle aspirates of testicular parenchyma can provide an overall assessment of spermatogenesis. While the use of FNA is well established for the diagnosis of testicular tumors,²⁴ the diagnosis of underlying causes of infertility in male animals is likely a more valuable use of this technique. Fine needles aspirates and large needle aspirates have been shown to be safe regarding future reproductive function of the testes.^{25,26} The cytologic characteristics of normal adult canine testicular

FNA as well as canines from four weeks old to 52 weeks old have been established. A Sertoli cell index and sperm cell index are used to provide a reference point and a basis to evaluate spermatogenesis.^{27,28} However, in a similar fashion to the needle biopsy, an aspirate of a single region of the testicular parenchyma may not be reflective of the overall state of spermatogenesis in the organ. Similar parameters have been studied in the stallion.^{29,30}

Conclusion

Endometrial biopsy remains an integral part of assessing fertility in domestic animals, particularly in the bitch and the mare. Testicular biopsy and testicular FNA are not yet as well established for assessing fertility in males. However, reference parameters exist to assist the clinician in investigating testicular causes of infertility. Multiple methods of fixation exist for both types of samples, but the clinician must be aware that each solution has drawbacks and care should be taken to insure that artifact that can impede interpretation is minimized. In terms of preservation prior to submission, endometrial biopsies can be fixed in NBF with little concern in terms of the quality of the histologic section obtained. Although NBF will work well for the fixation of testicular biopsies in most cases, consideration can be given to utilizing Bouin's or modified Davidson's medium. However, care should be taken to either process the tissue soon after the recommended fixation period or transfer the tissue to 70% ethanol for shipping for longer term storage.

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Table. Comparison between the most common fixation solutions for endometrial and testicular biopsies.

	10% NBF (neutral buffered formalin)	Bouin's fixative	Modified Davidson medium
Length of fixation	Indefinitely (unless immunohistochemistry is required). NBF penetrates tissue at a rate of 0.5-1 mm per hour	24-48 hours followed by transfer to 70% ethanol	24-48 hours followed by transfer to 70% ethanol
Safety issues	Carcinogen, ensure proper storage of solution	Similar to NBF. Dried picric acid is an explosive hazard	Similar to NBF
Artifact potential	Mostly shrinkage artifact. Acid hematin formation (artifactual pigment)	Can induce tissue hardening, erythrocyte lysis	Mostly shrinkage artifact

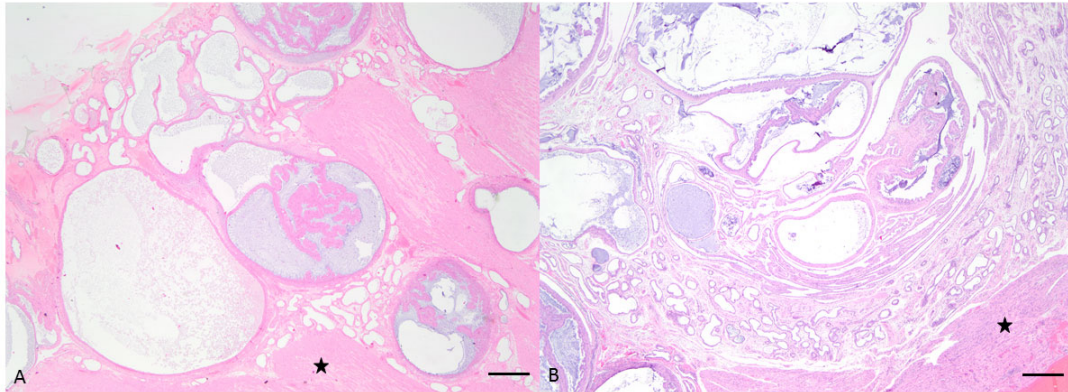


Figure 1. Endometrial biopsies fixed in Bouin's solution (A) and 10% NBF (B) for approximately 10 days (hematoxylin and eosin staining). Note the relative lack of differential staining in A as opposed to B. The star labels the myometrium in both images. Bar = 200 µm.

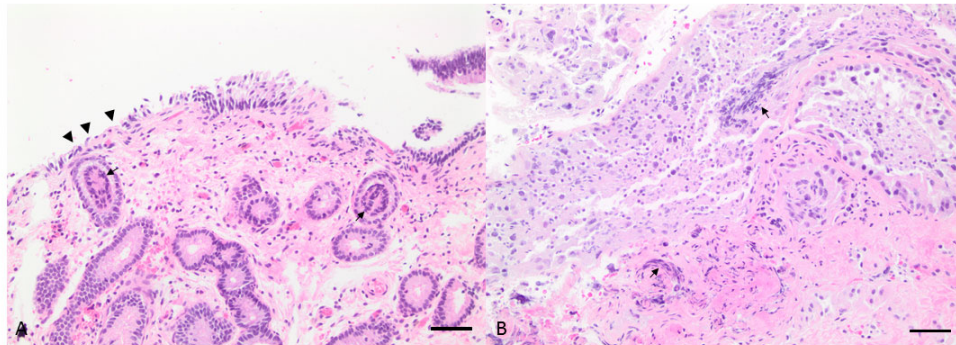


Figure 2. Examples of artifact in an endometrial biopsy and testicular biopsy. A. An equine endometrial biopsy exhibiting artifactual loss of surface epithelium (arrowheads) and invagination (telescoping) of endometrial glands (arrows). B. A canine testicular biopsy with marked crush artifact. Seminiferous tubules are difficult to visualize and nuclear streaming (arrows) is prominent. Bar = 50 µm.

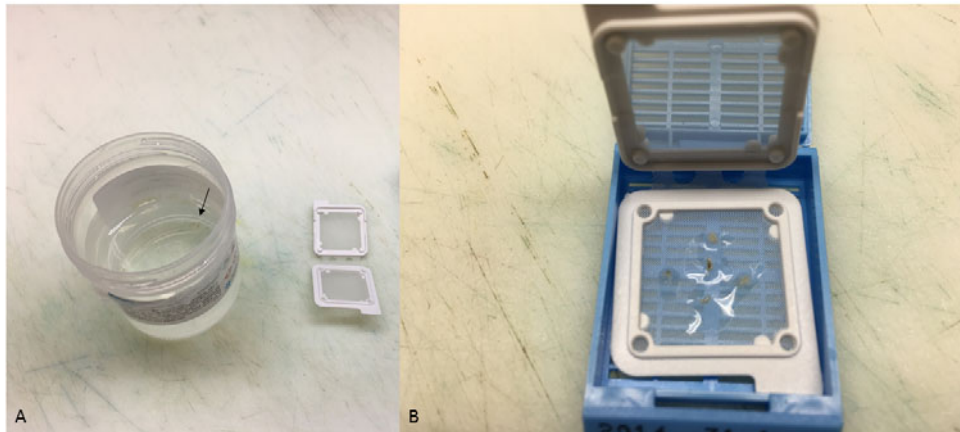


Figure 3. A. Transcervical endometrial biopsies in 10% NBF. A mesh tissue cassette is adjacent to the container. B. Transcervical endometrial biopsies in the mesh cassette and a standard histology tissue cassette. Note the size of the endometrial biopsies compared to the size of the grid of the tissue cassette. The mesh cassette prevents tissue loss during processing. The mesh cassette (or a folded paper towel) can be used to insure that the specimens stay together during transport of the fixative container.

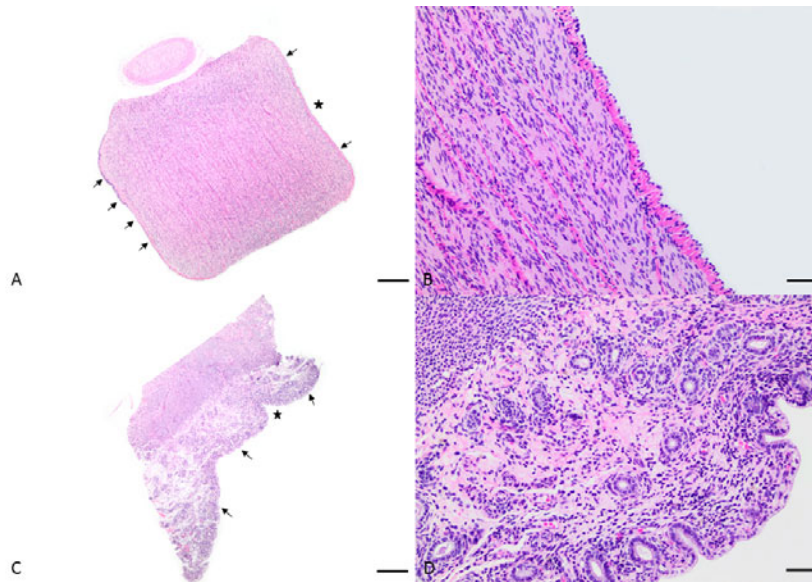


Figure 4. Orientation of a uterine punch biopsy can affect what is visible on the slide to the pathologist. A. In this section, the endometrium is oriented away from the microtome blade, resulting in a section that contains only myometrium and perimetrium. Note that the perimetrium is visible on both sides of the specimen (arrows). The star indicates the approximate location of the image shown in Figure 2B. Bar = 500 μ m B. 200X magnification photomicrograph depicting the perimetrium and myometrium. Bar = 50 μ m C. The same specimen following melting of the paraffin block and reorientation of the specimen by 90 degrees. Endometrium is now visible on the section. The star indicates the approximate location of the image shown in Figure 2B. Bar = 500 μ m. D. 200X magnification photomicrograph depicting the endometrium. Bar = 50 μ m.

(Editor's Note: The photographs in this manuscript are available in color in the online edition of Clinical Theriogenology.)