Synovial Membrane Microarthroscopy of the Equine Midcarpal Joint

ALBERTO SERENA, DMV, MS, MRCVS, R. REID HANSON, DVM, Diplomate ACVS & ACVECC, and STEVEN A. KINCAID, DVM, PhD

Objective—To evaluate the value of microarthroscopy in the equine midcarpal joint using the vital stains methylene blue, trypan blue, neutral red, and Janus green B to observe components of the synovial lamina propria, vascular architecture, and synoviocytes.

Study design—Experimental.

Animals—Ten horses.

Methods—Microarthroscopy of left and right midcarpal joints was performed with and without vital staining of the synovium. Four vital stains (methylene blue, trypan blue, neutral red, and Janus green B) were evaluated, with each stain used in 5 joints. Synovial biopsy specimens were collected from the dorsomedial and dorsolateral aspects of the joint.

Results—All dyes were biocompatible. At ×60 without vital staining, synovial surface topography, vascular network, and translucency were observed. Intra-articular vital dyes improved evaluation of synovial surface topography. At ×150 with vital staining, individual synoviocytes were clearly identified with all dyes, except neutral red. Although methylene blue provided the best in vivo microscopic differentiation of the structure of the intima, trypan blue had superior retention in conventionally processed synovial biopsies.

Conclusions—Methylene blue, trypan blue, neutral red, and Janus green B stains can be used safely for microarthroscopy. Good visualization of cells and vascular network can be obtained by microarthroscopy, and microarthroscopic evaluation of the synovium compares favorably with conventional histologic evaluation of biopsy specimens.

Clinical relevance—Microarthroscopy may be beneficial in both research and clinical diagnosis of equine articular diseases.

Key words: horse, joint pathology, synovial membrane, microarthroscopy, vital stains.

INTRODUCTION

EQUINE MUSCULOSKELETAL injuries, in particular those affecting synovial joints, are the most common reasons for poor performance and wastage.1 Even in non-racing horses, lameness associated with joint disease is the most common cause of early retirement.2 The synovial membrane has a central role in the pathogenesis of inflammatory joint diseases of the horse.3 The synovial membrane is composed of an intima made of 1–4 layers of synoviocytes and a subintima or lamina propria. In addition to blood vessels, lymphatics and nerves, the subintima is composed of connective tissue types (areolar, fibrous, adipose) that determine...
Synovial classification. Areolar type synovial membrane has a thick intima and abundant blood vessels, numerous folds and villi, and responds to inflammation primarily by an increase in the number of blood vessels. Fibrous type synovium usually serves as a gliding surface of the joint and contains minimal numbers of synoviocytes and vessels whereas adipose type synovium represents a transitional area and has various surface folds. Areolar type synovium is more metabolically active than the fibrous and adipose types.

Synoviocytes are a functionally heterogeneous population of cells. There are 3 types of synoviocytes within the synovial intima of the horse: type A synoviocytes are phagocytic cells, type B synoviocytes have fibroblastic and secretory properties, and type C synoviocytes represent a transitional form between types A and B synoviocytes.

Synovial membrane evaluation is typically performed by microscopy and arthroscopy. Optical and electron microscopy require small, chemically preserved samples of synovial membrane and yield detailed information on morphology and metabolic status of synoviocytes, and villus vasculature. These methods use isolated tissue samples and thus only provide limited regional information that may not necessarily be representative of an articular disease process.

Using conventional arthroscopy, gross inflammation of the equine synovium typified by synovial hyperemia and hypertrophy can be characterized. These relatively non-specific signs of inflammation are commonly observed with intra-articular fractures, septic arthritis, and degenerative joint disease and, to a minor extent, with osteochondritis dissecans (OCD). Depending on joint anatomy, conventional arthroscopy permits evaluation of most of the synovial membrane in vivo, but at magnifications ≤ × 10 so the synovial microarchitecture cannot be evaluated and only subjective evaluation of inflammatory status is possible.

To improve resolution of in vivo alterations an arthroscopy with magnification similar to that of a light microscope (up to × 150) has been used in humans. Arthroscopic observation of the joint at these magnifications permits excellent histologic examination of the synovium in vivo. Thus, microarthroscopy has potential value for both research on, and clinical diagnosis of, equine joint diseases, by bridging the gap that exists between conventional arthroscopy and histologic evaluation. In humans, microarthroscopy allows precise morphologic differentiation among types of joint disease, highlighting alterations of the synovial membrane typical of osteoarthritis (OA), post-traumatic synovitis, and rheumatoid arthritis.

Microarthroscopic diagnostic criteria used in human medicine are synovial membrane translucency, character of the vascular network, synoviocyte number and morphology. Normal human synovium is translucent and contains tortuous vessels and star-shaped cells (type A synoviocytes). In OA, the synovium is translucent/opaque, blood vessels are straight and hypertrophied, with evident neovascularization, and cells are numerous and round in shape (type B synoviocytes). Rheumatoid arthritis is characterized by villous hyperplasia, the synovial membrane is opaque because of congestion and edema, the vascular plexus is irregular with no clear outline, and synoviocytes are numerous and round (type B).

A sterile 1% solution of methylene blue is typically used for human microarthroscopy. Other vital stains have not been evaluated after intra-articular administration; however, differing affinities for cells and intracellular organelles could make real time identification of cells possible during microarthroscopy. Dyes that can be used are either intravitral dyes composed of particles that are engulfed by phagocytic cells or supravital dyes that diffuse into the cell cytoplasm to localize in vacuoles or organelles. Trypan blue (1% solution) is an intravitral dye used to localize phagocytic cells and supravital dyes include methylene blue, Janus green B, and neutral red. Methylene blue stains the nucleus, Janus green B localizes in mitochondria, and neutral red accumulates in lysosomes. Thus using dye properties evaluation of equine synovial membrane to determine synoviocyte distribution (type A and B) and functional/metabolic status of resident cells may be possible.

Precise morphologic characterization of normal equine synovial membrane in vivo using microarthroscopic techniques may contribute to the understanding of the synovium’s role in joint homeostasis. Microarthroscopic studies might allow characterization of morphologic changes that occur in vivo during pathologic processes, thus the morphologic response of the synovium to a particular intra-articular or systemic treatment could be documented by in vivo observation.

Use of microarthroscopic techniques for diagnosis of equine joint disease remains undefined. Our goals were to evaluate microarthroscopy in horses and to provide a precise microarthroscopic morphologic characterization of normal equine synovium. An additional goal was to evaluate the ability of intra-articularly administered vital stains to aid in identification of cell types.

**MATERIALS AND METHODS**

Ten healthy adult horses (> 360 kg) of mixed breed and sex, with no history or signs of carpal joint disease were studied. All horses had complete physical and lameness examinations performed to determine health status, both midcarpal joints were radiographed, and synovial fluid was collected for evaluation of cell count and total protein concentration. Criteria for inclusion in the study included an absence of
lameness at a trot on a hard surface, both in a straight line and in a circle, and at a trot after carpal flexion (60 seconds), no radiographic abnormalities, and normal synovial fluid analysis (total leukocytes < 500 cells/μL, total protein < 2.5 g/dL).

**Arthroscopy**

Horses were anesthetized, positioned in dorsal recumbency, and both carpi aseptically prepared for surgery. To avoid inflammation and morphologic changes of the synovium, synovial fluid samples were collected immediately before arthroscopy insertion. An 18-cm-long, 4-mm-diameter, 30° forward viewing, contact microarthroscope (Karl Storz “Andrea-Dias”, Karl Storz Veterinary Endoscopy, Goleta, CA; Fig 1) was used. A lateral arthroscopic portal was used, halfway between the extensor carpi radialis tendon and the common digital extensor tendon, and midway between the proximal and distal rows of carpal bones with the joint flexed at 90°.

The microarthroscope provided magnification up to 150x. A hand piece on the telescope was progressively turned in a clockwise direction to increase magnification and in a counterclockwise direction to return to a panoramic view of the joint cavity. The tip of the scope was maintained in close contact with the synovium at higher magnification and this was difficult, but was facilitated by stopping fluid ingress. Panoramic observation of the joint cavity was initially performed without vital staining. Increased magnification was used to observe microscopic details of the synovium (e.g., erythrocytes); however, identification of most cells (synoviocytes) required vital staining. Microarthroscopic images were digitally recorded and stored (Pro 2 DVD, Stryker Endoscopy, San Jose, CA).

After examination of the synovial surface and synovial biopsy, skin portals were closed with non-absorbable suture in an interrupted vertical mattress pattern. Horses were recovered from anesthesia and observed daily for 15 days for joint effusion and for lameness at the walk. In addition, from days 5–15, carpi were flexed daily for 1 minute, and horses were trotted on a hard surface both in a straight line and in circle. Both carpi were bandaged for 5 days. Horses were administered phenylbutazone (4.4 mg/kg orally once daily for 3 days) after surgery. Skin sutures were removed after 12 days.

**Vital Staining**

Filtered (0.22 μm filter, Milli-GV, Millipore, Carrigtwohill, Ireland) 1% sterile solutions of methylene blue (Eastman Kodak Co., Rochester, NY), trypan blue, neutral red, and Janus green B (Sigma-Aldrich Co., St. Louis, MO) were used. Vital stains were obtained as a pure powder and diluted to 1% in an isotonic solution. Each stain (5 mL/joint) was injected into 5 different joints through an 18-g needle inserted intra-articularly medial to the extensor carpi radialis tendon. After injection the joint was repeatedly flexed and extended slightly to allow stain diffusion and homogeneous contact with the entire synovium. Five minutes later the joint was thoroughly rinsed through the arthroscopic cannula with sterile Normosol-R solution (Abbott Laboratories, Abbott Park, IL) and inspection of the joint at increasing magnification was performed systematically from medial to lateral. To obtain a contact view, the tip of the scope was gently pushed against the area of the synovium to be evaluated, and then magnification increased.

**Synovial Biopsy**

Synovial membrane was collected from the dorsolateral and dorsomedial aspects of the synovium for histologic evaluation. Four specimens were collected from each joint using a 6 × 12 mm Ferris-Smith rongeur (Scanlan, Saint Paul, MN). Two specimens were collected from the lateral and 2 from the medial aspect of the joint. Histologic evaluation of synovium specimens was used to substantiate, compare, and contrast microscopic structure with microarthroscopic images to determine what morphologic features could be observed in vivo.

**Specimen Preparation**

Two different techniques were used to determine the best method for histologic evaluation of vitally stained synovial biopsies. Specimens were fixed in buffered 10% formalin and in 2-methylbutan silicate cooled by liquid nitrogen. Formalin-fixed specimens were dehydrated in graded alcohol and cleared in methyl salicylate. A single-edged razor blade was used to cut thin sections from the tissue specimens biopsies that were mounted in a synthetic resin on microscopic glass slides for viewing. Sections (15 μm) were cut from frozen specimens using a cryostat then mounted on microscopic glass slide. Additional specimens from 1 horse were prefixed in ammonium molybdate before primary fixation in buffered 10% formalin to stabilize methylene blue staining of the tissues.

**RESULTS**

Technically, microarthroscopy was comparable to conventional arthroscopy, the only difference being that the tip of the scope must be in contact with the synovium at higher magnification (contact view). Mild subcutaneous edema of the left carpus from fluid extravasation in 2 horses resolved within 3–4 days. No other adverse effects associated with microarthroscopy occurred.
Intraarticular Observations

Synovial villi were numerous on the dorsal aspect whereas the proximal and distal cul-de-sac and the most lateral and most medial aspects of the midcarpal synovium were smooth. At \( \times 60 \), the vascular pattern within the villi, and erythrocyte motion were easily appreciated (Fig 2). Some villi had a spiral arrangement of blood vessels whereas in others vessels had a straighter configuration.

Intraarticular Observations After Vital Staining

Synovial staining with vital dye was necessary to evaluate microscopic structure at high magnification. At \( \times 150 \), synoviocytes, numerous in some regions and sparse in others, were identified (Fig 3). Some synoviocytes were spherical whereas others were star-shaped cells. A consistent finding with all vital stains was that thorough rinsing of the articular cavity with sterile Normosol-R solution 5 minutes after dye injection was necessary to visualize components of the synovium. Without lavage, the synovium was over stained, which produced a very dark background that prevented good visualization of the synovium. Substantial differences in identification of synovial membrane components during microarthroscopy was documented for each vital stain tested.

Methylene Blue. A supravital dye that stains the nucleus, methylene blue provided the best localization of cells (Fig 3), highlighted the vascular network, and the translucency or opacity of the matrix. At \( \times 150 \), round- and stellate-shaped synoviocytes were differentiated.

Trypan Blue. An intravital dye that localized in phagocytic cells, trypan blue permitted identification of synoviocytes and appreciation of synoviocyte shape was good. Background matrix and blood vessels could be seen but were not well defined (Fig 4).

Neutral Red. Cells, cell shape, blood vessels, and background matrix were all poorly differentiated with this supravital dye that localizes in lysosomes. Synovial membrane constituents could not be satisfactorily recognized because of a diffuse, intense red staining of the synovium.

Janus Green B. Synoviocytes were identified and cell shape (round versus star-shaped cells) was appreciated using this supravital stain that localizes in mitochondria.

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Fig 2. Microarthroscopic view of equine synovium stained with methylene blue (\( \times 60 \)). The vascular loop at the tip of a single villus is visible.

Fig 3. Microarthroscopic view of equine synovium stained with methylene blue (\( \times 150 \)) depicting visible synoviocytes.

Fig 4. Microarthroscopic view of equine synovium stained with trypan blue (\( \times 100 \)).
Blood vessels were not well highlighted and background matrix could not be seen (Fig 5).

Histologic Observations of Vital Dye Stained Formalin-Fixed Specimens

Methylene Blue. Eluted during processing of both formalin-fixed specimens and ammonium molybdate/formalin fixed specimens. Synoviocytes and blood vessels could be identified but different synovial components lacked definition (Fig 6).

Trypan Blue. Cell identification was considered very satisfactory. Trypan blue had the best retention in formalin-fixed specimens and provided the best three-dimensional visualization of cells in the synovial membrane (Fig 7). Matrix background and blood vessels identification, however, was considered poor.

Neutral Red. Excellent identification of synoviocytes was possible, matrix background was clear, and blood vessels could be identified (Fig 8).

Janus Green B. Elution of dye occurred and cell differentiation was not possible.

Histologic Observations from Vital Dye Stained Frozen Sections

Sections of frozen specimens retained all stains except Janus green B. Histologic evaluation of frozen sections of synovial membrane was considered satisfactory, except for specimens stained with Janus green B, but not superior to evaluation of formalin-fixed specimens.

Good correlation existed between cell and vascular network characterization observed by microarthroscopy and histologic evaluation of both formalin fixed and frozen biopsy specimens.

DISCUSSION

Using a microarthroscopic technique in the equine midcarpal joint we were able to identify synovial vascular architecture, background matrix, and synoviocytes. These morphologic observations were substantially enhanced by the use of intra-articular administration of vital dyes, particularly methylene blue, which allowed distinct observation of synoviocytes morphology, to the point to be able to distinguish round-shaped versus star-shaped cells.
Microarthroscopy is used diagnostically especially in human rheumatology. Diagnostic criteria used are tissue translucency, structure and appearance of the vascular network, and number and morphology of synoviocytes.8 Our observations in the equine midcarpal joint support use of these criteria for evaluation of equine synovial membrane. Microarthroscopic identification of human synoviocytes has been based on shape extrapolated from ultrastructural studies.16 Star-shaped cells were identified as type A synoviocytes and round cells as type B synoviocytes. Interestingly, ultrastructural studies of equine carpal joints identified type A synoviocytes as spherical in shape, and type B synoviocytes as star-shaped because of cytoplasmic processes that extend radially.17 The discrepancy between humans and horses in morphological classification is speculative, but may be because of differences in joint capsule and synovium anatomy, and biomechanical demands. The currently accepted classification system accounts for some overlap in function between the 2 principal cell types.3 Synoviocyte morphology and proposed function might not be strictly correlated, as suggested by the description of an intermediate cell type (synoviocytes type C).4

Equine synovial membrane reportedly has nonspecific histopathologic changes when inflamed.3 However, the synovial membrane has a critical role during joint inflammation. Synoviocytes release IL-1 and other cytokines that stimulate the synthesis of collagenase, gelatinase, caseinase, and PGE2 from monolayers of articular cartilage.18 Microarthroscopy may provide additional real time information about the equine synovium, by observing in vivo synovial morphology of normal and diseased joints.

To evaluate cells within the synovium, microarthroscopy requires vital staining of the synovial membrane. Vital dyes are used regularly in human ophthalmic surgery but infrequently in joints with methylene blue being most commonly used. We had hoped that by use of different vital stains we might detect different synovial cell populations, or different metabolic states within specific cell types, because of differing stain chemical properties and affinities. Despite different chemical characteristics of the 4 stains used, selective staining of synoviocytes did not occur. Differentiation between the types of synoviocytes could only be based on their morphologic appearance (round or star-shaped) during microarthroscopy.

Vital staining of synovial tissues observed with the microarthroscope in our study was similar to that of in toto and en bloc techniques used for conventional histologic observation.14 These simplistic techniques allow identification of individual cells and groups of cells and permit observation of the 3-dimensional relationships and orientation of cells and tissues that are not possible using other imaging techniques.14 For these methods to be efficacious, dyes must readily penetrate tissues, have selective staining specificity, and for vital staining they must be biocompatible. Importantly, dyes must be retained in tissues and be able to withstand intra-articular lavage, or dehydration and clearing techniques used for conventional histologic processing. Methylene blue, neutral red, and Janus green B are cationic dyes that bond to anionic molecules. A characteristic of cationic dyes is their susceptibility to elution from cells and tissues by alcohol during dehydration19 but resistance to washing with water during tissue processing or saline during microarthroscopy. Thus, the intense intra-articular staining of synovial tissues by methylene blue, Janus green B, and neutral red and their elution during histologic processing can be explained by their ionic nature. Unfortunately, neither shortening times for fixation/dehydration/clearing nor pre-fixation in ammonium molybdate15 prevented loss of staining from our biopsy specimens. Because trypan blue is an anionic dye, it has good tissue retention in vivo and in biopsy specimens. Like Frizziero et al,7–10 we found that methylene blue was an effective vital dye for synovium and provided excellent intra-articular staining of cells and tissues.

Trypan blue staining provided the best visualization of cells and tissues in dehydrated and cleared biopsy specimens. Staining of nuclei was unexpected because trypan blue is an anionic dye. One possible explanation may have been the tissue pH. With neutral pH, the amine group of anionic dyes is capable of bonding to anionic molecules, such as nucleic acids of cells;19 thus it is possible that the physiologic environment of normal equine synovium facilitated such bonds resulting in nuclear staining. We had anticipated that trypan blue particles would be phagocyti-
zed by type A-synoviocytes, which would facilitate their intra-articular and in vitro identification. However, this did not occur which may reflect either the relatively short staining time required for microarthroscopy so that phagocytosis did not occur or that preparation of the dye did not result in particle formation.

Ultrastructural analysis of synoviocyte phagocytic properties has shown that 10 minutes after intra-articular injection, colloidal gold particles were located in the lamina propria of synovium but not within synovial injection. However, a similar study by Luckenbill and Cohen indicated that some carbon particles were identified in avian synoviocytes at 15 minutes after intra-articular injection. Thus in our equine joints, the short exposure of synoviocytes to trypan blue may have been insufficient time for phagocytosis to occur, yet trypan blue readily entered synoviocytes judged by the nuclear staining. Sheehan and Hrapchak suggested that intravital dyes should have the capacity to flocculate to a particulate size that could be phagocytized by cells. Dyes we used, including trypan blue, were filtered with a 0.22 μm filter (Millex-GV, Millipore, Carrigtwohill Co., Cork, Ireland) to minimize the potential for foreign body reaction or infection; however, filtration may have also minimized flocculation of dye molecules and prevented or delayed phagocytosis. In addition to filtration, dyes were sterilized (autoclave) before use. In retrospect, filtration could have been avoided, at least for trypan blue, to obtain larger particle sizes that could flocculate and be phagocytized by synoviocytes. This may have allowed selective type A synoviocytes (phagocytic cells) to be stained by trypan blue.

Supravital staining of synovial tissues presented an unusual histologic challenge compared with other organs because of the physiologic environment of joints. Janus green B and neutral red readily penetrated the synovium and obscured cellular detail. Staining of mitochondria by Janus green B may have occurred but was not visible. Another consideration is that Janus green B will only stain mitochondria if oxygen is present in cells and as oxygen content decreases so does Janus green B stain intensity. We did not evaluate oxygen tension in the synovium but joint lavage may have adversely affected oxygen tension. Elution of methylene blue from specimens processed for histology limited the value of biopsy specimens and was more pronounced for formalin fixed than frozen specimens. Neutral red was considered inappropriate for microarthroscopic synovium evaluation because cell identification was not possible. Interestingly, neutral red provided excellent synoviocyte and blood vessel identification for histologic evaluation of synovial biopsies.

Although histologic evaluation of frozen section offers a possible clinical advantage over intraoperative imaging, this method lacks the definition achieved with histologic evaluation of formalin fixed specimens. Overall, histologic evaluation of formalin fixed specimens was considered superior to frozen section for evaluation of vitally stained specimens.

Thus we found that methylene blue was the best stain of those evaluated for microarthroscopic evaluation of equine synovium. Trypan blue and Janus green B staining also permitted precise identification of synoviocytes during microarthroscopy, but the vascular network and matrix were not as well visualized as with methylene blue. Seemingly, microarthroscopy may be useful to document in vivo morphologic changes in equine synovium at magnifications up to ×150 and thus facilitate targeted biopsy for more specific synovial membrane evaluation. The excellent anatomic detail of normal equine synovium we observed suggests that microarthroscopy is very likely to be valuable for evaluating equine joint disease in both a clinical and an experimental setting. However, further studies are needed to determine the experimental and clinical value of the technique.

REFERENCES