SYNOVIAL MEMBRANE MICROARTHROSCOPY OF THE EQUINE

MIDCARPAL JOINT: TECHNIQUE APPLICATION AND

EVALUATION OF FOUR VITAL STAINS

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SYNOVIAL MEMBRANE MICROARTHROSCOPY OF THE EQUINE MIDCARPAL JOINT: TECHNIQUE APPLICATION AND EVALUATION OF FOUR VITAL STAINS

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THESIS ABSTRACT

SYNOVIAL MEMBRANE MICROARTHROSCOPY OF THE EQUINE MIDCARPAL JOINT: TECHNIQUE APPLICATION AND EVALUATION OF FOUR VITAL STAINS

Alberto Serena
Master of Sciences, August 8, 2005
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Directed by Drs. Reid Hanson, Steven Kincaid, Earl Gaughan, Joel Lugo, and James Wright

The objectives of this study were to evaluate the feasibility of microarthroscopy in the equine midcarpal joint using the vital stains methylene blue, trypan blue, neutral red, and Janus green B, to describe the components of the lamina propria, vascular architecture, and types of synoviocytes of the synovium.

Microarthroscopy of both left and right midcarpal joints of ten horses 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 randomly placed in five joints. Biopsy specimens of synovium

were harvested from the dorso-medial and the dorso-lateral aspects of the joint prior to completion of the procedure. Horses were recovered from anesthesia.

All dyes were biocompatible. At 60x without vital staining, the surface topography, vascular network, and translucency of the synovium were observed. Intra-articular vital dyes improved the evaluation of the surface topography of the synovium. At magnification of 150x with vital staining, individual synoviocytes were identified clearly with all dyes, except with neutral red. Methylene blue provided the best *in vivo* microscopic differentiation of the structure of the intima. Trypan blue had superior retention in formalin processed biopsies.

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. Microarthroscopic evaluation of the synovium compares favorably with conventional histological evaluation of biopsy specimens.

Microarthroscopy is a valuable tool for both research and clinical diagnoses of equine articular diseases.

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INTRODUCTION

Musculoskeletal injuries, in particular those affecting synovial joints (i.e. degenerative joint disease, articular fracture, synovitis, and septic arthritis), are the most common reasons for poor performance and wastage in the equine industry.[1,2,3] Even in non-racing horses, lameness associated with joint disease is the most common cause of early retirement. The synovial membrane has a central role in the pathogenesis of inflammatory joint diseases in horses.[4,5,6,7,8]

Evaluation of the synovial membrane typically is performed using conventional arthroscopy, as well as light and electron microscopy of biopsy samples.[9] While useful, these methods have limitations. Light and electron microscopes require small, chemically preserved samples of synovial membrane.[10,11,12] The conventional arthroscope permits the evaluation of almost the entire synovial membrane *in vivo*, but at magnifications not greater than 10x, which limits the ability to thoroughly evaluate the synovial membrane microarchitecture. To provide greater resolution of *in vivo* alterations of synovial membrane, an arthroscope with magnification similar to that of a light microscope (up to 150x) is used in humans.[13] This technique, called microarthroscopy, allows precise morphological differentiation among various types of joint disease, highlighting alterations of the synovial membrane typical of osteoarthritis, post-traumatic synovitis, and rheumatoid arthritis.[14,15,16]

A precise morphological characterization of the normal equine synovial membrane *in vivo* utilizing microarthroscopic techniques should contribute to the understanding of the role of the synovium in joint homeostasis. Further, microarthroscopic studies would allow investigators to characterize the morphological changes that occur during different pathological processes *in vivo*. The morphological response of the synovial membrane to a particular intra-articular or systemic treatment could be documented by direct *in vivo* observation.[17]

The main objective of this study was to evaluate the ability of microarthroscopy to provide a precise microarthroscopic morphologic characterization of the normal equine synovium. An additional goal was to evaluate the ability of intra-articularly administered vital stains to aid in identification of synovial cell types. Trypan blue, neutral red, and Janus green B were evaluated intra-articularly for the first time in this study.

REVIEW OF THE LITERATURE

Normal Anatomy of Synovial Membrane

Musculoskeletal injuries are the most common reasons for poor performance and wastage in the equine industry.[1,2,3] Even in non-racing horses, lameness associated with joint disease is the most common cause of early retirement. Among the aforementioned causes of lameness-related poor performance, degenerative joint disease, in its primary form or secondary to other joint pathology, has probably the highest prevalence and morbidity within equine populations.[4]

The synovial membrane has an important role in the pathogenesis of inflammatory joint diseases of the horse.[4,5,6,7,8] The synovial membrane is a modified mesenchyme composed of an intima, and a sub-intima or lamina propria. [9] The intima is an incomplete layer, one to four synoviocytes thick, with no basement membrane. Gaps between synoviocytes and the lack of a basement membrane allow for the efflux of the capillary exudates into the synovial cavity. Synovial fluid in fact is an ultrafiltrate of plasma.[10] The sub-intima is composed of areolar, fibrous, or adipose tissue with accompanying blood vessels, lymphatic vessels, and nerves. Based on the predominant type of connective tissue of the sub-intima, equine synovium can be classified as areolar, fibrous, or adipose types. The areolar type of synovial membrane has a

thick intima and abundant blood vessels, and numerous folds and villi are normally present. The fibrous type of synovium usually serves as a gliding surface of the joint and contains minimal numbers of synoviocytes and vessels. The adipose type of synovium represents a transitional area and has various surface folds.[10] The areolar type of synovium is more metabolically active than are the fibrous and adipose types.[18,19]

The synovial intimal cells, termed synoviocytes, are a functionally heterogeneous population of cells.[20, 21,22] Synoviocytes are responsible for the production of some components of synovial fluid, for adsorption of debris and foreign substances from the joint cavity, and for assisting with blood/synovial fluid exchanges.[23] There are three 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.[24,25] Type A synoviocytes are non-fixed cells that can phagocytose cell debris and waste products in the joint cavity, and possess antigen-presenting ability.[26] These cells are derived from blood-borne mononuclear cells, and can be considered resident macrophages (tissue macrophages).[23] Type B synoviocytes are characterized by abundant rough endoplasmatic reticulum and dendritic processes which form a regular network at the luminal surface of the synovial membrane.[27,28] Type B cells are involved in production of specialized matrix constituents including hyaluronan, collagens and fibronectin.[29-37] The proliferative potentials of type B cells are much higher than type A cells, although the transformation of subintimal

fibroblasts into type B cells cannot be excluded.[38, 39,40] Ultramicroscopic studies of the equine synovium have indicated that type A synoviocytes are concentrated around the tips of the synovial villi, at the superficial layer of the synovial intima, and have an antenna-like process that protrudes into the joint cavity with tips covered with long microvilli.[41] In the basal half of the villi, type B synoviocytes predominate, which are situated at various depth, frequently in the deeper layer of the intima, projecting thick processes horizontally and intertwined to form a regular network of processes on the synovial surface. The meshwork of horizontal processes and the antenna-like processes imply advantages in not only secretion but also sensation and regulation of the barrier function of the synovial membrane.[42]

Synovitis

The equine synovial membrane is reported to have nonspecific histopathologic changes with inflammation.[9] However, this tissue has a critical role in the inflammatory process within the joint.[43,44,45,46]

Inflammation of the synovial membrane and fibrous capsule (synovitis and capsulitis) often occur simultaneously.[47,48] This can be a common early consequence of day to day cyclic trauma in athletic horses. The synovitis process can result from direct trauma or alternatively from cartilage and bone debris stimulating the synovial membrane to produce prostaglandin E_2, cytokines (i.e. tumor necrosis factor-TNF, interleukin 1-IL1), and neutral metalloproteinases (i.e. collagenase, gelatinase).[49,50] Acute synovitis and capsulitis also can cause

substantial clinical compromise directly due to fluid accumulation and decreased range of motion. Pain results from direct stimulation of delta receptors in the capsular tissue as well as increased intraosseous pressure in the subchondral bone secondary to joint effusion.[51,52] The effusion and reduced range of motion can produce permanent changes in both the synovial membrane and fibrous joint capsule.[53]

Even more important is the contribution of synovitis to the degenerative process in joints by the release of enzymes, inflammatory mediators, and cytokines produced by inflamed synovial membrane.[54,55] Many of these enzymes are considered important: neutral metalloproteinases, serine proteinases, cysteine proteinases, and aspartic proteinases.[56,57,58] Other inflammatory mediators of importance include prostaglandins and free radicals.[59,60] In addition, cytokines, or intercellular messengers, are important. These are interleukin-1 (IL-1) and tumor necrosis factor (TNF).[61,62] Cytokines can be released by inflamed synovial membrane cells as well as cartilage cells (chondrocytes). These cytokines in turn can cause other cells, particularly chondrocytes and synoviocytes, to release additional enzymes.[63,64]

The most important enzymes in synovitis are neutral metalloproteinases (also called matrix metalloproteinases or MMPs). Those of particular importance are collagenase, stromelysin and gelatinase.[65] Collagenase is produced by a wide variety of cells and degrades collagen. Stromelysin can break down a wide variety of substances, but its ability to degrade proteoglycans of the cartilage is critical.[64] Gelatinase degrades denatured type II collagen (the collagen specific

for articular cartilage) as well as other substances.[64,66] These important enzymes are secreted in an inactive form and then activated; for example, collagenase is activated by stromelysin, which probably is most commonly activated by plasmin.[67] Some inhibitors of these enzymes have also been identified.[61,68]

Other important proteinases are serine proteinases. These are plasminogen activators and can also be produced by inflamed synovial membrane. They transform plasminogen to active plasmin and this cascade is important in activating the aforementioned metalloproteinase enzymes.[57,64] The role of cysteine proteinases in cartilage degradation is uncertain, but they are capable of breaking down proteoglycans in the cartilage.[64]

Prostaglandins (primarily E group) are also produced in inflamed joints and can cause a decrease in the proteoglycan content of the cartilage matrix by decreasing synthesis as well as enhancing degradation.[49,56] Prostaglandin E_2 can be released from synovial cells stimulated by interleukin-1.[62] The presence of prostaglandin E_2 in synovial fluid from inflamed joints has been demonstrated in the horse.[49] In addition to depleting proteoglycans, PGE_2 also enhances pain perception in joints as well as possibly promoting bone demineralization.[64]
In addition, oxygen-derived free radicals (super-oxide anion, hydroxyl radicals and hydrogen peroxide) also may be released from injured joint tissues.[59,60] Studies have demonstrated degradation of hyaluronic acid by free radicals.[60] Hyaluronic acid provides lubricating qualities to the synovial fluid. Free radicals also break down proteoglycans and there is evidence for a role in breaking down collagen as well.[59] The release of proteinases is controlled in part by cytokines.

Cytokines are small soluble proteins produced by one cell that affect the activity of other cells. Studies of cytokines in joint tissues suggest that IL-1 and TNF influence the synthesis of metalloproteinases by both chondrocytes and synovial cells.[64,65] IL-1 and TNF are produced by synovial cells and may therefore be very important in the deleterious effects of synovitis on articular cartilage. The normal turnover of the matrix of the articular cartilage is regulated by the chondrocytes under the control and influence of cytokines and mechanical stimuli. The degradation of cartilage associated with disease represents an exacerbation of these normal processes.[45,47,63] Cytokines induce proteoglycan depletion in articular cartilage by either increasing the rate of degradation or decreasing synthesis in association with the release of proteinases and prostaglandins from chondrocytes.[47]

The above pathways represent a number of options for the destruction of the extracellular matrix of articular cartilage. Therefore, the synovium plays an important role in perpetuating the inflammatory cycle within the joint cavity. This inflammatory cycle leads to articular cartilage degeneration, the critical factor in disease as osteoarthritis or degenerative joint disease.

Evaluation of Synovial Membrane

Clinical evaluation of the synovial membrane, and its potential inflammatory status, is typically performed using conventional arthroscopy, as well as light and electron microscopy from biopsy samples. While useful, these methods have limitations. The conventional arthroscope permits the evaluation of almost the entire synovial membrane *in vivo*, but at magnifications not greater than 10x,

which limits the ability to thoroughly evaluate the synovial membrane microarchitecture.[69,70] Also, the synovial membrane status can be indirectly evaluated to some extent by analyzing samples of synovial fluid. Typically, synovial fluid is analyzed for total white cell count, protein content and cytology.[9]

Conventional arthroscopy can be used to characterize the gross inflammatory reaction of the equine synovium, which is typified by hyperemia and hypertrophy of the synovial membrane. These relatively non-specific signs of inflammation are commonly observed with intra-articular fractures, traumatic synovitis, septic arthritis, degenerative joint disease and, to a minor extent, with osteochondritis dissecans (OCD).[70] Synovial hypertrophy without marked hyperemia, on the other hand, is typical in the dorsal aspect of the metacarpophalageal joint affected by chronic proliferative synovitis.[71,72] A conventional arthroscope can be used to observe most of the synovial membrane of most joints, but the image is limited to a magnification not greater than 10x.[70] Therefore, only subjective evaluation of an inflammatory status is possible.

Optical and electron microscopy of biopsy specimens of the synovium provide detailed information on morphology and metabolic status of synoviocytes, and highlight the vasculature of villi.[10] On the other hand, these methods, making use of isolated tissue samples, provide information concerning only a small portion of synovium, and results are not necessarily representative of an articular disease process.

Microarthroscopy

To evaluate the less evident alterations of the synovial membrane *in vivo*, an arthroscope with magnification similar to that of a light microscope (up to 150x) was developed and employed clinically in human medicine.[13] This innovative instrument, called a microarthroscope, is a derivation of the microhysteroscope developed in 1979 by Karl Storz together with a French gynecologist, Hamou.[73] The microarthroscope can uniquely provide a panoramic view of the articular cavity at magnifications up to 150x, thus permitting an excellent histological examination of the synovial lining *in vivo*.[13]

Endoscopic examination is performed by gradually increasing magnification. Magnification of 10x affords a conventional panoramic view of the articular cavity; a more restricted, but detailed observation is obtained at 20x magnification. The synovial and villous architecture, the terminal vessels, and red blood cell streams are all visible at 60x. Contact microscopic view of the cellular synovial layer is offered at 150x.[13]

Microarthroscopy allows precise morphological differentiation among various types of joint disease, highlighting alterations of the synovial membrane typical of osteoarthritis, post-traumatic synovitis, and rheumatoid arthritis.[14-16] Microarthroscopic diagnostic criteria employed in human orthopedics/rheumatology are synovial membrane translucency, character of the vascular network, and morphology and number of synoviocytes.[13-16] Normal human synovium is translucent and contains tortuous vessels and star-shaped cells (type A synoviocytes). In osteoarthritis, the synovium is translucent/opaque,

blood vessels are straight and hypertrophied with evident neovascularization, and cells are numerous and round in shape.[14] 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.[15]

Although the vascular network architecture within the synovium can be observed directly with the microarthroscope, synoviocyte identification requires intra-articular administration of particular stains using a procedure called vital staining.

Vital Staining

Vital staining of the synovial membrane is required to visualize and differentiate components of the synovium during the microarthroscopic procedure. A vital stain is defined as a substance that can be introduced into a living animal without causing any toxic effects and, at the same time, be taken up by and stain certain cells.[74]

Most biological stains are textile dyes. Routine biomedical application of these dyes began only in the early twenties. However, the synthesis of many of these stains is dated much earlier, such as 1876 for methylene blue.[75] Trypan blue was widely used for direct dying of cotton. The food industry is another area where these types of dyes are employed; for example, trypan blue is used to give chewing-gum the flavor of peppermint. Fundamental characteristics of vital dyes are their biocompatibility and absence of toxicity. Dyes employed for *in vivo* use need to be certified for medical use.[76]

The nomenclature of dyes is confusing. A dye might have different names, because manufacturers want their product to have their own trade name. For instance, trypan blue can be referred as niagara blue, congo blue, benzo blue, or azidine blue.[77,78] Also, acid and basic dyes are not acids and bases, despite such a classification in the literature. Rather, these ionic dyes bind to tissue components that are anionic and cationic respectively.[78-80] Trypan blue is an anionic dye, while methylene blue, neutral red, and Janus green B are cationic dyes.[81]

The physical/chemical features of a dye are the bases for its “affinity” for particular cells or organelles: the electric charge carried by a dye, its size, and its hydrophobic-hydrophilic nature, all influence which type of cells or organelles will be stained.[82,83]

Two subcategories of vital dyes are used to visualize and study live cells:

1) intravital dyes, which are composed of particles that are endocytosed by phagocytic cells
2) supravital dyes that diffuse into the cytoplasm of cells to localize in vacuoles or organelles.[84]

Trypan blue (1% solution) is an example of an anionic intravital dye that is used to localize phagocytic cells. Intravital dyes are composed of particles sufficiently small for uptake by single cells and have a strong tendency to flocculate into particles of colloidal dimension. A dye that does not possess this flocculating ability, that is, a dye that exists simply as a small ion, can diffuse in and out of a cell very easily and cannot be used for intravital staining for this

reason. Examples of supravital dyes include methylene blue, Janus green B, and neutral red.[76,84] These three stains are all cationic. Methylene blue is a dye of the thiazine group (tetramethylthionine) that vitally stains the nucleus of cells.[85,86] Janus green B localizes in mitochondria, depending upon enzymatic activity of the cell.[84] The staining will occur under partial anaerobic conditions. Neutral red is freely soluble in water and alcohol, and accumulates in lysosomes of cells.[84,87]

Anionic and cationic dyes are considered to react with oppositely charged tissue components, predominantly by means of electrostatic bonds.[75,82] However, there are a number of factors that influence the staining reaction, such as temperature, dye concentration, solvents, pH, dye hydrophobic-hydrophilic nature, and presence of impurities.[82,84] Also, any eventual processing of tissue after *in vivo* use, such as fixation and embedding, might influence the stability and retention of the dye as well.[81] Cationic dyes show more resistance to water in staining procedures than anionic dyes and the reverse is true for stability of the two types of dyes in dehydrating alcohols.[74]

Based on the chemical properties of these dyes,[84] the synovial membrane may allow uptake of a given stain dependent on the synoviocyte predominance (type A versus type B) and functional/metabolic status.

Vital dyes are utilized quite regularly in different fields of human medicine.[88-93] In ophthalmic surgery, trypan blue is used to stain the anterior capsule of the eye.[90] In gastroenterology, methylene blue can be used to stain the gastric and esophageal mucosa before endoscopy to aid in identification of

neoplastic tissue.[91] Methylene blue is also used in general surgery, for example, to visualize fistulous tracts or nerves during surgery.[88]

Vital staining of joints remains an unexplored area of study. The only dye that has been employed intra-articularly is methylene blue.[13] No information is available in the literature regarding the use of other vital dyes such trypan blue, Janus green B, or neutral red in synovial tissues. Further, specimens stained using methylene blue *in vivo* have not been evaluated following processing to verify the localization of the dye and microarchitecture of the synovium viewed with the microarthroscope. Consequently, the stability of the dyes in the joint tissues during post-biopsy processing for histologic observation is not known. To date, vital dyes other than methylene blue that accentuate morphological and functional aspects of cells have not been evaluated in synovial joints using the microarthroscope in humans and horses.

OBJECTIVES

The goal of the study was to assess the use of the microarthroscope with different vital staining to visualize *in vivo* histomorphological features of the normal synovial membrane of the equine midcarpal joint. To accomplish this goal, the objectives were:

1) to evaluate the ability of microarthroscopy to provide a precise microarthroscopic morphologic characterization of the normal equine synovium without vital staining,
2) to determine if methylene blue, trypan blue, Janus green B, and neutral red are biocompatible in equine synovial joints,
3) to determine what histological features of normal synovial membrane of the equine midcarpal joint stained with methylene blue, trypan blue, Janus green B, and neutral red can be visualized with a microarthroscope,
4) to determine which vital dye provides the best visualization of histomorphological features of equine synovial membrane using the microarthroscope
5) to confirm observations using the microarthroscope by analysis of biopsies of vitally stained synovial membrane following *in vitro* tissue processing.

MATERIALS AND METHODS

Animals

Ten healthy adult (body weight ranging from 360 Kg to 620 Kg) horses of mixed breed and sex, with no history or signs of carpal joint disease were included in the study. To be included in the study, all horses had normal physical and lameness exams, no major radiographic signs of joint disease in both midcarpal joints, and a normal complete synovial fluid analysis.

A microarthroscopic procedure and biopsy collection of synovial membrane was performed in both midcarpal joints of each horse. Horses were recovered from anesthesia and observed daily for 15 days for signs lameness and for joint effusion. Post-operatively, carpi were bandaged for five days. Horses received 4.4 mg/Kg of phenylbutazone per os, once a day for three days after surgery. Skin sutures were removed 12 days after surgery.

The Auburn University Animal Care and Use Committee approved the experimental design and use of animals.

Microarthroscopy

Each horse was placed under general anesthesia in dorsal recumbency, and both carpi were aseptically prepared for surgery. In order to avoid the possible inflammatory reaction and morphological change of the synovium caused by the

procedure, synovial fluid samples were collected immediately before insertion of the arthroscope into the joints.

Joints were examined using a 30 degree forward Karl Storz “Andrea-Dias” contact microarthroscope (Karl Storz Veterinary Endoscopy, Goleta, CA) (Fig.1), 18 cm long, 4 mm in diameter.

To approach the midcarpal joint, a lateral arthroscopic portal was used, halfway between the extensor carpi radialis tendon and the common digital extensor tendon and midway between the two rows of carpal bones with the joint flexed.[70] The arthroscopic procedures for the midcarpal joint are described in detail elsewhere.[70,94]

The microarthroscope provided magnification up to 150x. A hand piece on the scope was turned in a clockwise direction to increase the magnification during the procedure, 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. A panoramic observation of the joint cavity was first performed without staining (Fig.2). Greater magnification was used to visualize microscopic details of the synovium (Fig. 3).

Vital staining of the synovial membrane was performed to distinguish cell populations. A filtered (0.22 μ filter, Millex-GV, Millipore, Carrigtwohill Co. Cork, Ireland; Fig. 4) 1% sterile solution of methylene blue, trypan blue, neutral red, and Janus green B were used. Each stain (5ml per joint) was injected into five different joints through an 18-gauge needle placed intra-articularly medially to the

extensor carpi radialis tendon (Fig. 5 and 6). After injection of 5 ml of 1% stain solution, the joint was repeatedly flexed and extended slightly to allow diffusion of the stain and a homogeneous contact with the entire synovium. Five minutes later the articular cavity was thoroughly rinsed through the arthroscopic sleeve with sterile Normosol-R solution (Abbott Laboratories, IL) and inspection of the joint at increasing magnification was performed. Inspection of the synovial membrane 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 the magnification increased.

Tissue Collection and Processing

Synovial membrane biopsies were harvested from the dorso-lateral and dorso-medial aspect of the synovium for histological evaluation. A total of four biopsies were harvested for each joint using a 6x12 mm Ferris-Smith rongeur (Scanlan) (Fig. 7). Two biopsies were harvested from the lateral aspect of the joint, and two from the medial aspect of the joint. The goal of histological evaluation of synovium specimens was to substantiate, compare, and contrast the morphology of biopsied tissue from images obtained during microarthroscopy and determine what morphological components of the synovial membrane can be observed *in vivo*. Microarthroscopic images were digitally recorded and stored.

Biopsy samples were processed by two different methods: immersion fixation in aqueous buffered 10% formalin, and fixation by quenching (quick freezing) in non-aqueous 2-methylbutane (isopentane) cooled by liquid nitrogen.

Dyes used in this study are soluble in water. This characteristic of the dyes means that they might elude from cells of the biopsies when they are exposed to aqueous fixatives and subsequent dehydration, clearing, and mounting of the tissues for observation with a microscope. Thus, to determine the best method for histological evaluation of vitally stained synovial biopsies, in situ preservation of tissue biopsies and localization of dye molecules in the tissue was accomplished by two different methods: immersion fixation in aqueous buffered 10% formalin, and fixation by quenching (quick freezing) in non-aqueous 2-methylbutane (isopentane) cooled by liquid nitrogen.

Formalin-fixed biopsies were dehydrated for 10 minutes in graded ethyl alcohol and cleared in methyl salicylate.[95] A single-edged razor blade was used to cut thin sections from the biopsies that were mounted in a synthetic resin on microscopic glass slides for viewing. Fifteen micrometer thick sections were cut from frozen biopsies using a cryostat and mounted on microscopic glass slides. Additional biopsies from one horse were prefixed in ammonium molybdate before primary fixation in buffered 10% formalin to stabilize methylene blue staining of the tissues.[96]

RESULTS

The microarthroscopic procedure was technically comparable to conventional arthroscopy. Two horses had mild subcutaneous edema of the left carpus after extravasation of fluid during the procedure. This resolved within 3-4 days. No other systemic or local adverse effects of microarthroscopy were observed during and after the procedure.

Synovial villi were numerous on the dorsal aspect of the synovium. The proximal and distal cul-de-sac, together with the most lateral and most medial aspects of the synovium, was smooth (Fig. 8). At 60x, the vascular pattern within the villi, and even the flow of erythrocytes were easily appreciated (Fig. 9). Some villi had a spiral arrangement of blood vessels, while in others blood vessels had a straighter configuration (Fig.10).

Staining of the synovium by vital dyes was necessary to evaluate microscopic structure at high magnifications. At 150x, synoviocytes composing the synovial lining were identified (Fig. 11). In order to visualize synoviocytes, the tip of the scope was maintained in close contact with the synovium at higher magnification. This procedure was difficult but was facilitated by stopping the ingress of fluid. Synoviocytes were numerous in some areas and sparse in others (Fig. 12 and 13), but without a consistent pattern in different joints. Some synoviocytes were spherical in shape and others were star-shaped. A consistent

finding using all stains was that thorough rinsing of the articular cavity with sterile Normosol-R solution (Abbott Laboratories, Illinois) after 5 minutes from injection was necessary to visualize components of the synovium. Without this procedure the synovium was over-stained, which produced a very dark (or red when neutral red was used) background that prevented good visualization of the synovium.

A substantial difference in identifying synovial membrane components during microarthroscopy was documented for each vital stain evaluated.

Methylene blue, a supravital dye that stains the nucleus, provided the best localization of cells. Methylene blue also highlighted very well the vascular network, and the translucency or opacity of the matrix. At higher magnification (150 x), round and stellate shaped synoviocytes were differentiated (Fig.11).

Using Trypan blue, an intravital dye that localizes in phagocytic cells, identification of synoviocytes and appreciation of their shape was good. Background matrix and blood vessels could be seen, but without good definition (Fig.14 and 15).

Neutral red is a supravital dye that localizes in lysosomes. Cells and their shape, identification of blood vessels, and background matrix were poorly differentiated using this stain. Components of the synovial membrane could not be satisfactorily recognized, because of a diffuse and intense red appearance of the synovium (Fig. 16 and 17).

Using Janus green B, a supravital stain that localizes in mitochondria, synoviocytes were identified and the shape of the cells (round versus star-

shaped cells) was appreciated. However, blood vessels were not highlighted well, and the background matrix could not be seen (Fig.18-23).

Biopsy samples consisted of intima and sub-intima, and for each sample entire villi could be observed. For histological evaluation of biopsy samples, methylene blue eluted during the processing of formalin fixed specimens. Using this stain synoviocytes and blood vessels could still be identified but lacked definition of different components of the synovium (Fig. 24). This vital stain also eluted when synovium samples were fixed in a solution of formalin/ammonium molybdate instead of formalin alone.

Histological identification of cells in samples stained with trypan blue and fixed in formalin 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. 25). Matrix background and blood vessels identification, however, was judged poor.

Synovium samples stained with neutral red and fixed in formalin allowed excellent identification of synoviocytes when observed using light microscopy (Fig. 26). The matrix background was clear, and blood vessels could be identified.

Histological evaluation of synovial membrane stained with Janus green B and fixed in formalin was disappointing. Janus green B appeared to elute drastically from tissues and differentiation of cells was not possible.

Sections of frozen specimens retained all stains except for Janus green B. Histological evaluation of frozen section of synovial membrane was considered satisfactory, except for samples stained with Janus green B.

Despite different chemical characteristics of the four stains evaluated in this study, synoviocyte type differentiation was not achieved.

A good correlation exists between cells and vascular network characterization obtained by microarthroscopy and histological evaluation of biopsy specimens.

DISCUSSION

Microarthroscopy is one of the diagnostic tools currently utilized in human orthopedics and rheumatology. The diagnostic criteria employed in human beings include tissue translucency, structure and appearance of the vascular network, and morphology and number of synoviocytes.[13,14] The results of this study in horses support the use of these criteria for the evaluation of the equine synovial membrane. Identification of human synoviocytes based on their shape have been extrapolated from ultramicroscopic studies.[11] Star-shaped cells were identified as type A synoviocytes, and round cells as type B synoviocytes. Interestingly, ultramicroscopic studies of the equine carpal joints identified type A synoviocytes to be spherical in shape, and type B synoviocytes to be star-shaped due to cytoplasmatic processes extending radially.[42]

The equine synovial membrane is reported to have nonspecific histopathological changes when it is inflamed; however, the synovial membrane has a critical role during joint inflammation. [9] Synoviocytes release IL-1 and other cytokines that stimulate the synthesis of collagenase, gelatinase, caseinase, and PGE2 from monolayers of articular cartilage.[57-62] Microarthroscopy may provide additional real time information about the equine synovium, by observing *in vivo* synovial morphology of normal and diseased joints.

In general, but with variation depending on the stain used, at lower magnifications, the general architecture of the synovium of the midcarpal joint could be evaluated, and it was comparable to the morphologic description previously published.[69,70,94]

To evaluate cells within the synovium, microarthroscopy requires vital staining of the synovial membrane. Vital dyes are utilized regularly in different fields of human medicine, yet vital staining of joints remains an unexplored area of study. The only vital dye previously evaluated in joints is methylene blue.[13-15] Additional vital stains, not used intra-articularly before, were evaluated in our study, to determine if different cell populations, or the same cell population in different metabolical states, would retain a specific stain according to its chemical properties and affinities. Differentiation of synoviocyte type could not be achieved despite different chemical characteristics of the four stains evaluated in this study.

Vital staining of synovial tissues observed with the microarthroscope was similar to that of *in toto* and *en bloc* techniques used for the conventional observation of histological specimens.[95] These simplistic procedures allow identification of individual cells and groups of cells and have the advantage of observation of the 3-dimensional relationships and orientation of cells and tissues that are not possible using other imaging techniques.[95] For these techniques to be efficacious, dyes must readily penetrate tissues, have selective staining specificity, and for vital staining they must be biocompatible.[77,79] Importantly, dyes must be retained in tissues and be able to withstand intra-articular lavage or

dehydration and clearing techniques used for conventional histological processing. The chemical nature of dye molecules is the basis to meet these criteria and is the foundation for dye classification. Dyes used in this study are soluble in water. This characteristic of the dyes means that they might elude from cells of the biopsies when they are exposed to aqueous fixatives and subsequent dehydration, clearing, and mounting of the tissues for observation with a microscope. Thus, to determine the best method for histological evaluation of vitally stained synovial biopsies, *in situ* preservation of tissue biopsies and localization of dye molecules in the tissue was accomplished by two different methods: immersion fixation in aqueous buffered 10% formalin, and fixation by quenching (quick freezing) in non-aqueous 2-methylbutane (isopentane) cooled by liquid nitrogen.

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 dehydration procedures,[81] but resistance to washes 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 histological processing can be explained by their ionic nature. Unfortunately, neither shortening times for fixation/dehydration/clearing nor pre-fixation in ammonium molybdate[96] prevented the loss of staining from biopsies obtained in this study. Because trypan blue is an anionic dye, it has good retention in biopsies as well as good tissue retention *in vivo*. As reported by Frizziero,[13-15]

methylene blue was an effective vital dye for the synovium and the present study confirms that it provides excellent intra-articular staining of cells and tissues.

Trypan blue provided the best visualization of cells and tissues in dehydrated and cleared biopsies in this study. The staining of nuclei by trypan blue was unexpected since it is an anionic dye, which usually should not stain nuclei, and not a cationic dye, which should stain the anionic radicals of nuclear nucleic acids. The reason for this result probably resides in the pH of the tissue environment in which vital staining occurred. In the vicinity of neutral pH, the amine group of anionic dyes is capable of bonding to anionic molecules, such as nucleic acids of cells.[81] Thus, in the present study the physiologic environment of normal equine synovium facilitated bonding between amine groups of trypan blue to nucleic acids resulting in nuclear staining. It was anticipated that particles of trypan blue would be phagocytized by type A-synoviocytes to facilitate their intra-articular and *in vitro* identification. This did not occur and may have been related to either the short staining time required by microarthroscopy or the preparation of the dye for injection. An electron microscopic analysis of the phagocytic properties of synoviocytes indicated that ten minutes following intra-articular injection, colloidal gold particles were located in the lamina propria of synovium but not within synoviocytes.[97] However, a similar study by Luckenbill and Cohen[98] indicated that some carbon particles were identified in avian synoviocytes at fifteen minutes after intra-articular injection. Thus, the short exposure of synoviocytes to trypan blue may have been insufficient time for phagocytosis to occur. Trypan blue readily entered synoviocytes in this study as

evidenced by the presence of nuclear staining. Sheehan and Hrapchak[84] suggested that intravital dyes should have the capacity to flocculate to a particulate size that could be phagocytized by cells. Dyes used in this study, including trypan blue, were filtered with a 0.22 μ filter to minimize the potential of an intra-articular foreign body reaction or infection. However, filtration may have also minimized flocculation of dye molecules and prevented or delayed phagocytosis of trypan blue.

Supravital staining of synovial tissues presented an unusual histological challenge compared to other organs of the body because of the physiologic environment of synovial joints. Janus green B and neutral red readily penetrated the synovium to such an extent that they obscured resident cells. Consequently, staining of mitochondria by Janus green B may have occurred but was not visible with the microarthroscope. A secondary factor for mitochondrial staining was the presence of oxygen in the cells. Janus green B will only stain mitochondria if oxygen is present in the cells.[83] As oxygen content decreases, so does staining by Janus green B to a point that no staining occurs in the absence of oxygen. In the present study, the oxygen tension in the synovium was not evaluated but may have been influenced by the lavage solution used during the microarthroscopy procedure.

None of the stains employed in this study caused clinically detectable synovitis and/or lameness. A transitory light pain is sometimes reported in human patients after microarthroscopy, where methylene blue is used.[13] Even after vital

staining with methylene blue, or any of the stains employed, no discomfort was observed in any horse in this study.

Elution of methylene blue from specimens processed for conventional histological evaluation limited the value of the biopsy procedure. The loss of staining was more substantial when biopsies were fixed in formalin than when biopsies were frozen.

Neutral red was considered inappropriate for microarthroscopic synovium evaluation, because cell identification was not possible. Interestingly, this stain provided excellent synoviocyte and blood vessel identification when synovial biopsies were evaluated histologically. We hypothesized that the synovium was over-stained during microarthroscopy, but processing and clearing of biopsy specimens removed the stain present at the extra-cellular level, making cell identification possible on histologic examination.

Results of our study show that methylene blue is the best stain of those evaluated for microarthroscopic evaluation of the synovium. Trypan blue and Janus green B also permit a precise identification of synoviocytes during microarthroscopy, but the vascular network and the matrix are not as well visualized as with methylene blue.

In conclusion, microarthroscopy is a new technique that can document *in vivo* morphological modification of natural or experimentally induced joint diseases. The structural changes of the synovium during septic arthritis or after intra-articular or systemic medications, such as hyaluronan and triamcinolone, may also be amenable to real time evaluation by microarthroscopy. In addition,

the possibility of performing *in vivo* observation of the synovium up to 150x can allow targeted biopsy procedure for more specific synovial membrane evaluation. This study indicates that microarthroscopy is a useful procedure for the study of the equine synovial membrane *in vivo*. Studies evaluating the usefulness of microarthroscopy of diseased equine synovium are necessary to determine the clinical value of the technique.

Microarthroscopy has potential value for both research and diagnosis of equine joint diseases, by bridging the gap that exists between conventional arthroscopy and microscopic histological evaluation.

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FIGURES

Fig. 1: Karl – Storz “Andrea-Dias” microarthroscope. The hand piece is turned in a clockwise direction to increase the magnification.

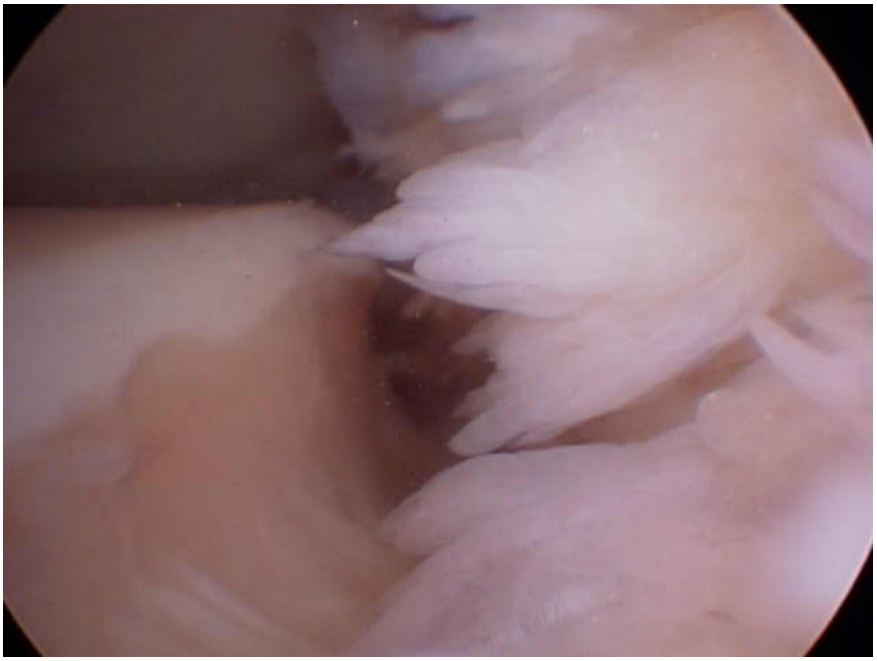

Fig 2: Panoramic arthroscopic appearance of the dorsal reflection of the synovium of the midcarpal joint without vital staining. The dorsal, non-articular aspect of the intermediate carpal bone has a more opaque color than the articular cartilage.

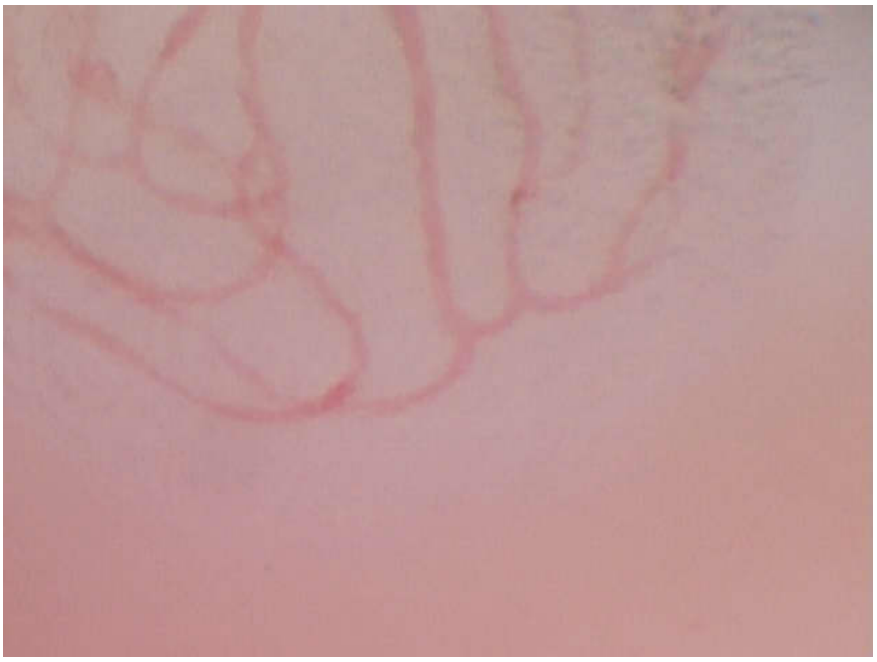

Fig. 3: Microarthroscopic view of the non-stained synovium (60x). Detail of the vasculature arcade at the tip of a single villus. Due to the lack of stain, synoviocytes are not visible.

Fig. 4: A 0.22 µ filter, Millex-GV, Millipore, Carrigtwohill Co. Cork, Ireland. All stains were filtered before autoclave sterilization.

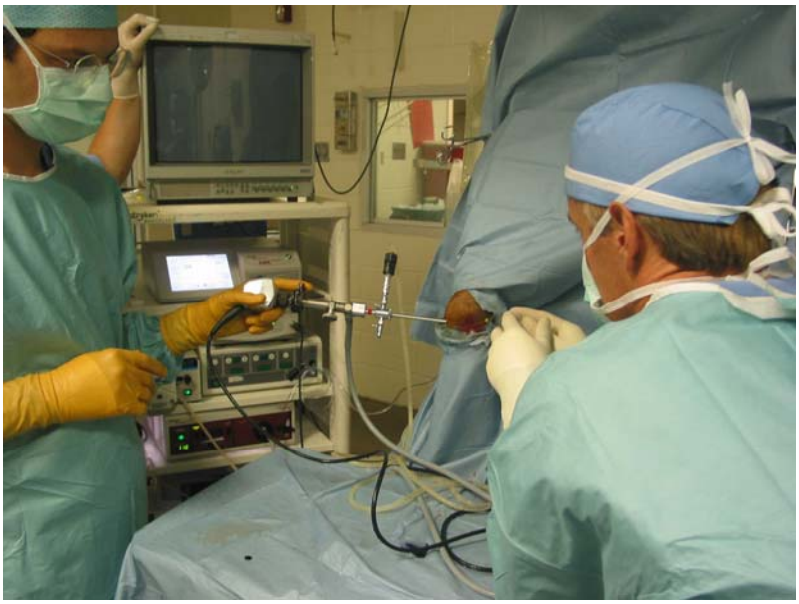

Fig. 5: Overview of the microarthroscopic procedure. One operator injects vital stain into the midcarpal joint; the second maintains the arthroscope position.

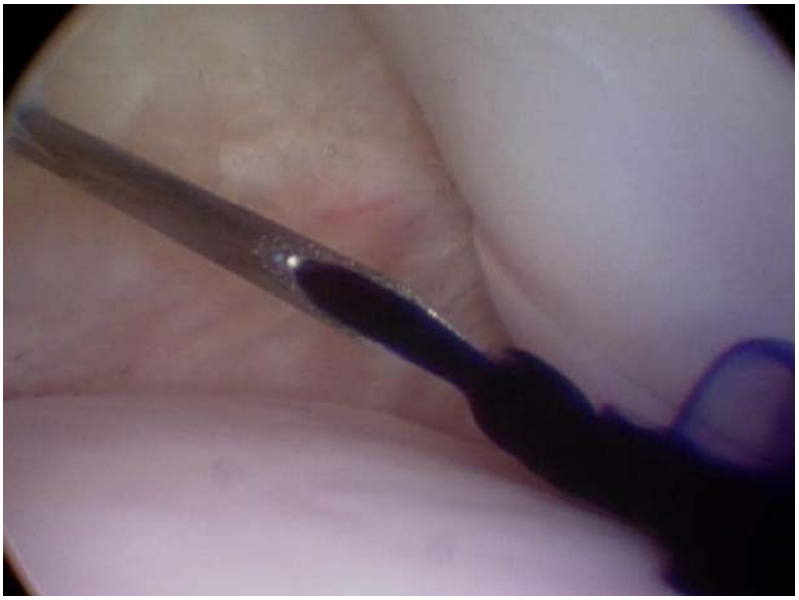

Fig.6: Methylene blue is injected into the midcarpal joint through an 18G needle. (20x)

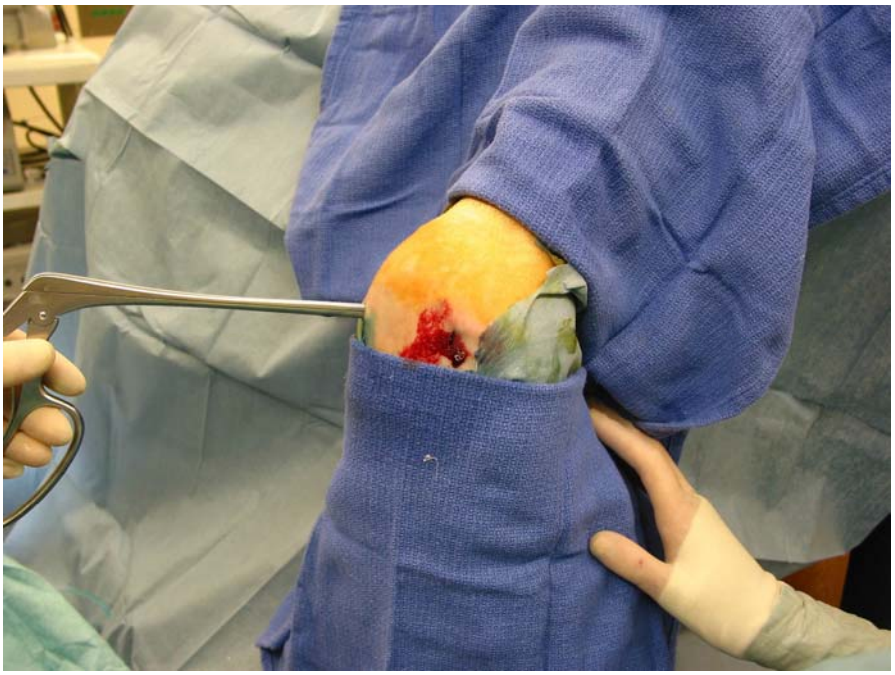

Fig. 7: Biopsies are harvested from the dorso-lateral and dorso-medial aspect of the synovium using a Ferris-Smith rongeur.

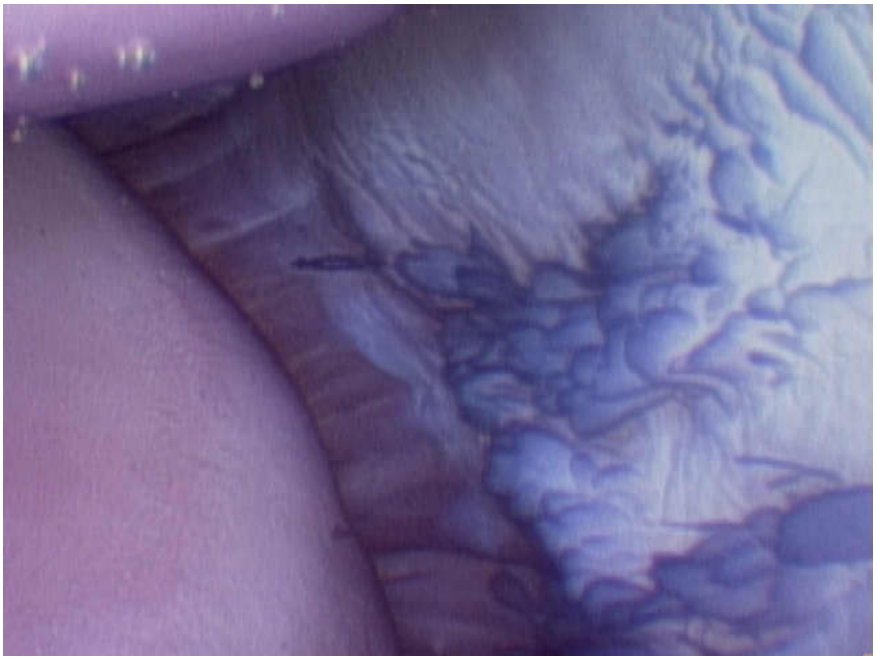

Fig.8: Microarthroscopic view of the lateral portion (cul-de-sac area) of the synovium vitally stained with Janus green B (60x). Note the transition between the villous synovium and the smooth synovium.

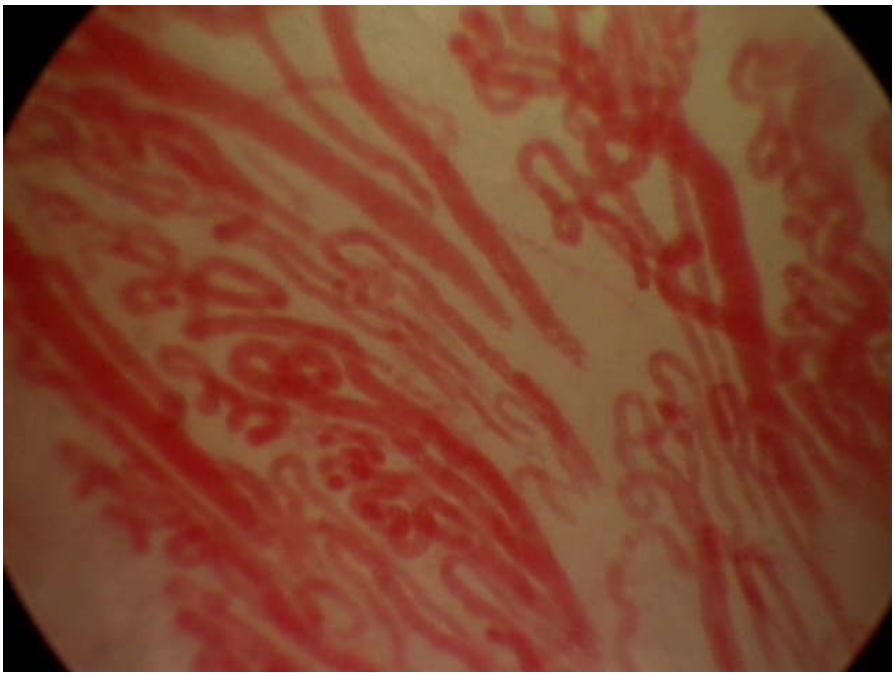

Fig. 9: Microarthroscopic appearance of the vascular network of the synovium without vital staining (60x).

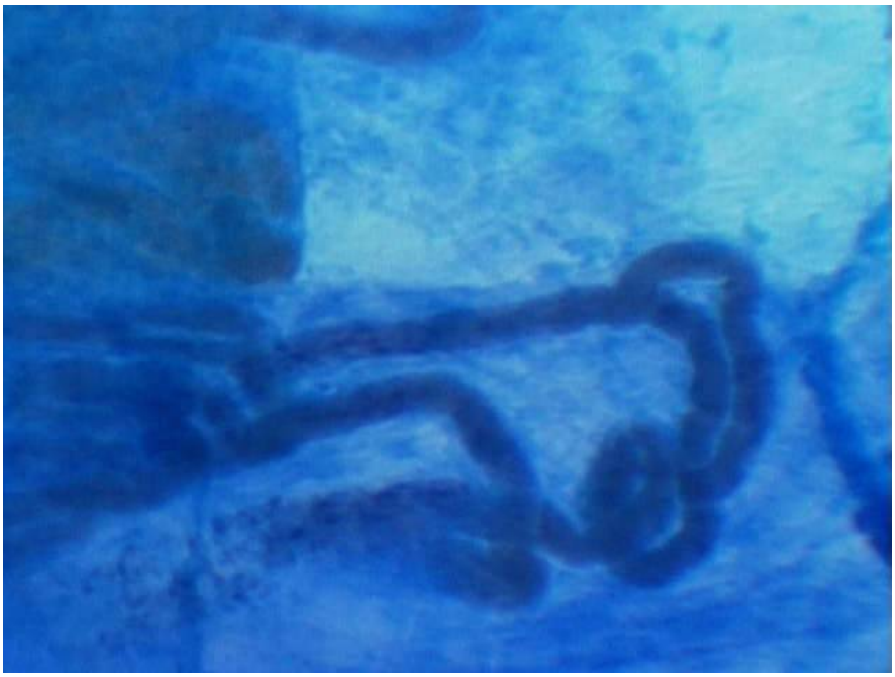

Fig.10: Microarthroscopic view of the synovium stained with methylene blue (60x). The vascular loop at the tip of a single villus is visible.

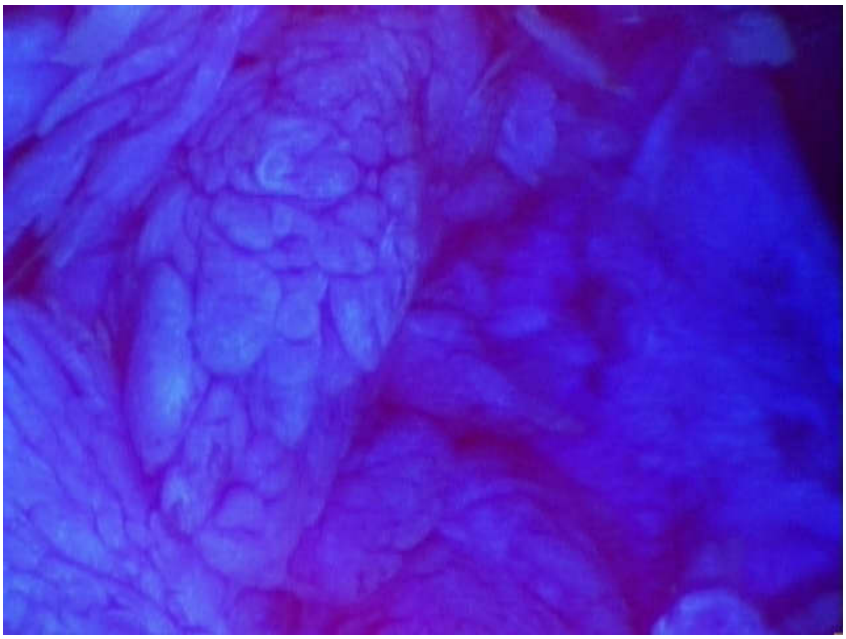

Fig.10 B: Microarthroscopic view of the dorso-medial aspect of the synovium vitally stained with methylene blue (30x). Close-up view of a cauliflower-like villus.

Fig. 11: Microarthroscopic view of the synovium stained with methylene blue (150X). Synoviocytes are readily visible.

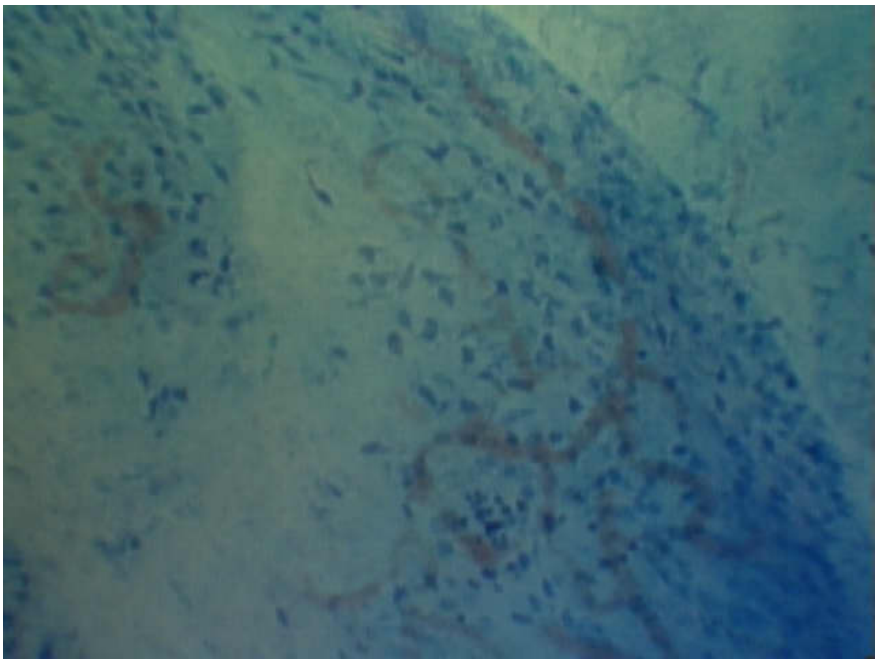

Fig.12: Microarthroscopic view of the synovium vitally stained with methylene blue (80x). Note the increased cellularity towards the periphery of the villus.

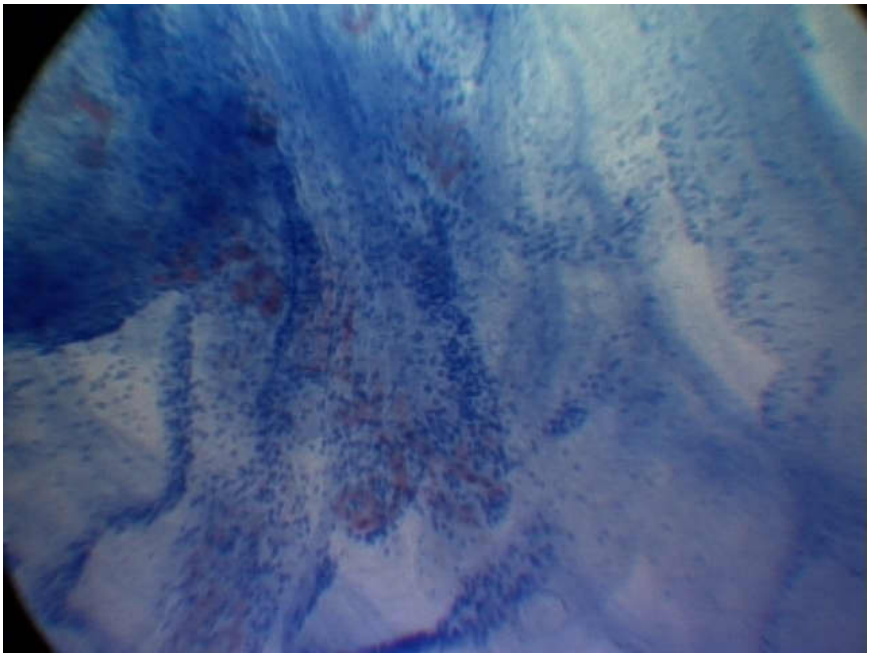

Fig.13: Microarthroscopic view of the synovium vitally stained with trypan blue (40x). Areas of the synovium with high cellularity are visible.

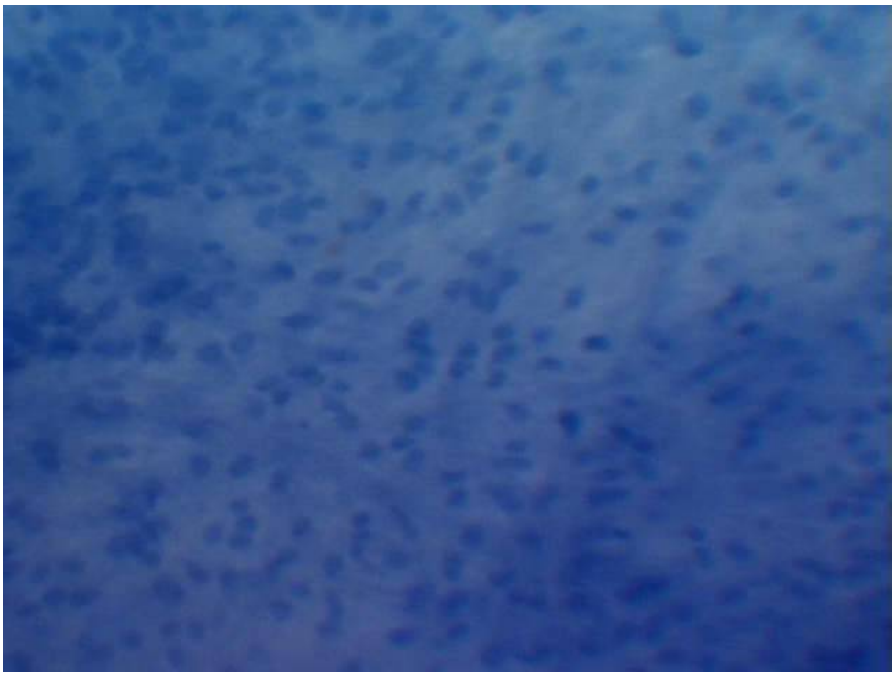

Fig. 14: Microarthroscopic view of the synovium stained with trypan blue (100x). Numerous synoviocytes are identifiable.

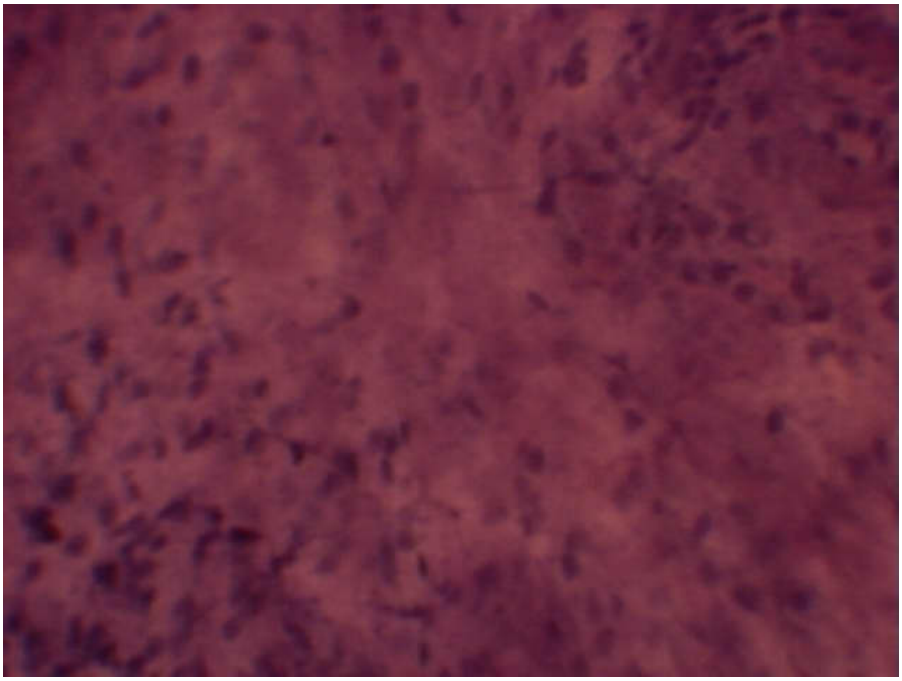

Fig.15: Microarthroscopic view of the synovium vitally stained with trypan blue (100x).

Fig. 16: Microarthroscopic view of the synovium stained with neutral red (60x). Synoviocytes and blood vessels are not identified at all, due to the diffuse, homogeneous staining of the synovium.

Fig.17: Microarthroscopic view of the synovium vitally stained with neutral red (100x). Due to the homogeneous over-staining, synoviocytes are barely discernable from the background matrix.

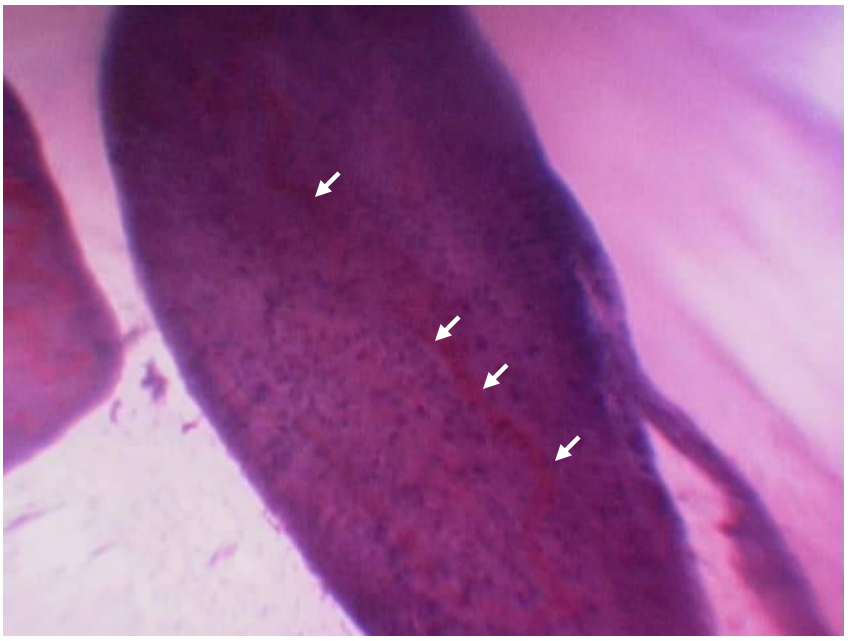

Fig. 18: Microarthroscopic view of the synovium stained with Janus green B (80x). Single villus. Synoviocytes and the vascular architecture (arrows) are visible.

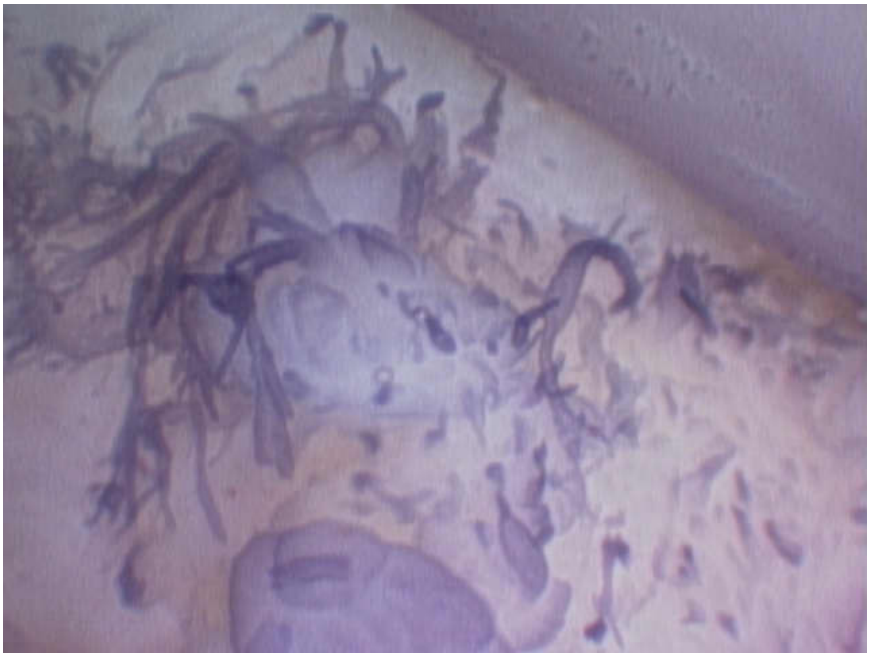

Fig.19: Microarthroscopic view of the synovium vitally stained with Janus green B (60x). Note the secondary villi emerging from the main villus. This morphology, referred as fringe-like, is one of the different villi's morphology in the midcarpal equine joint.

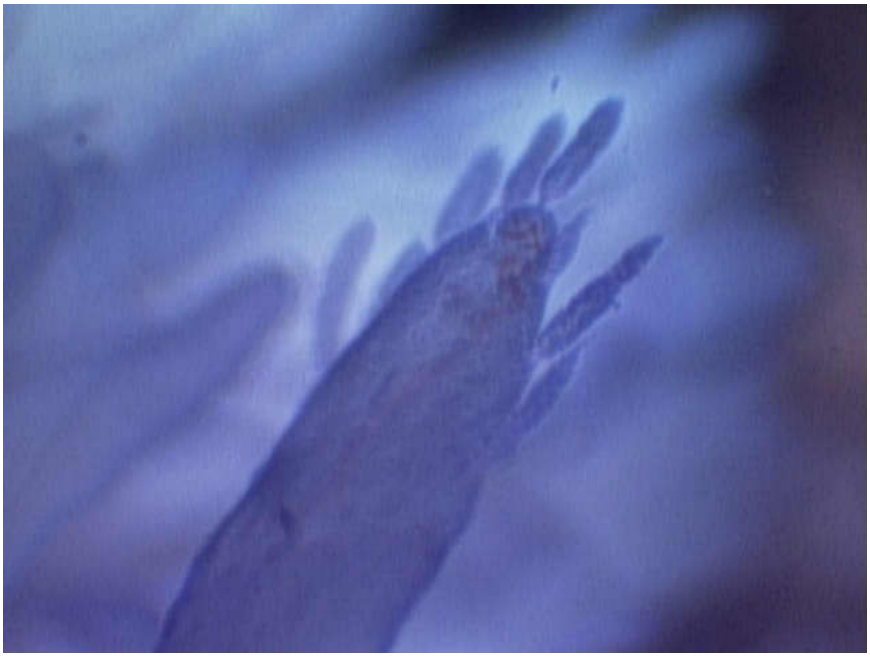

Fig.20: Microarthroscopic view of the synovium vitally stained with Janus green B (60x). Close-up view of the filamentous morphology of the villi.

Fig.21: Microarthroscopic view of the synovium vitally stained with Janus green B (60x). Secondary twig-like villi emerging from the main villus are visible.

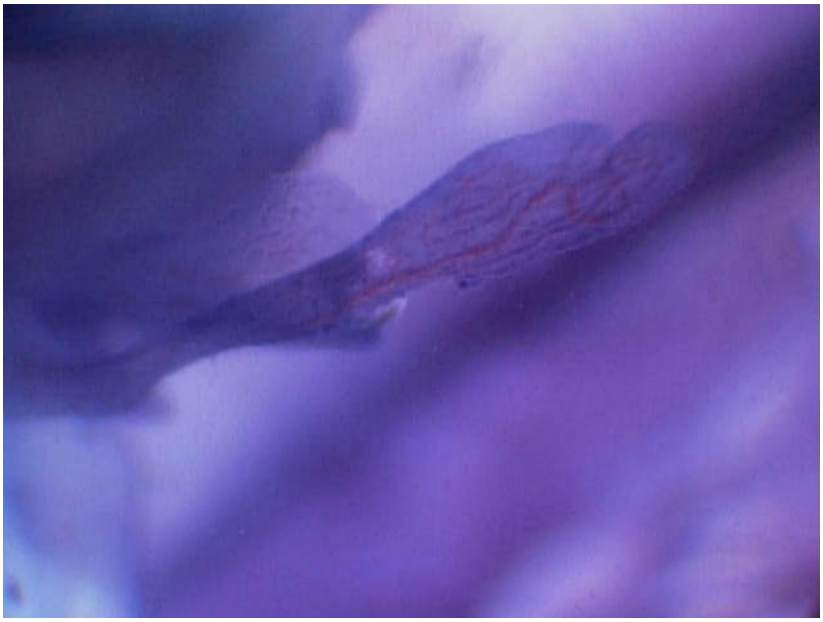

Fig.22: Microarthroscopic view of the synovium vitally stained with Janus green B (60x). Close-up view of the vasculature within a single polyp-like villus.

Fig.23: Microarthroscopic view of the dorso-medial aspect of the synovium vitally stained with Janus green B (30x). In this area of the synovium polyp-like villi predominate. Villi have different morphology in different areas of the synovium.

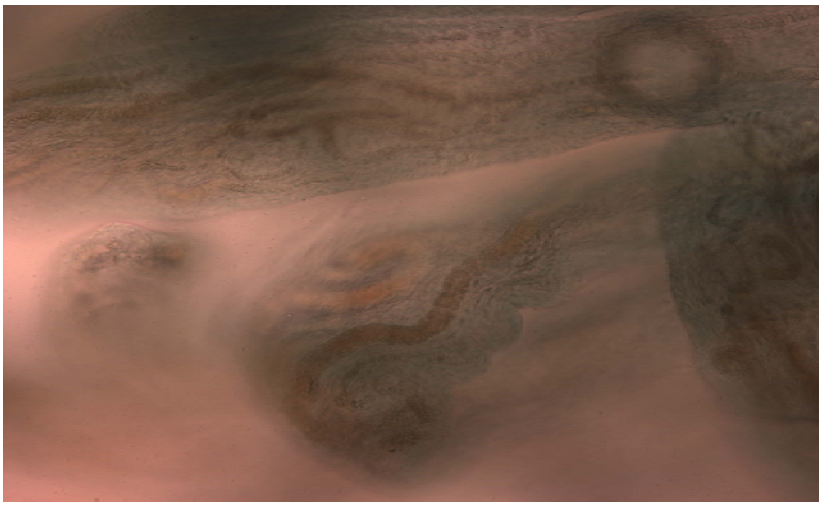

Fig. 24: Histological picture of a sample of synovium vitally stained with methylene blue and fixed in formalin (40x). Due to elution of the stain during fixation, there is no clear outline among different structures composing the synovium.

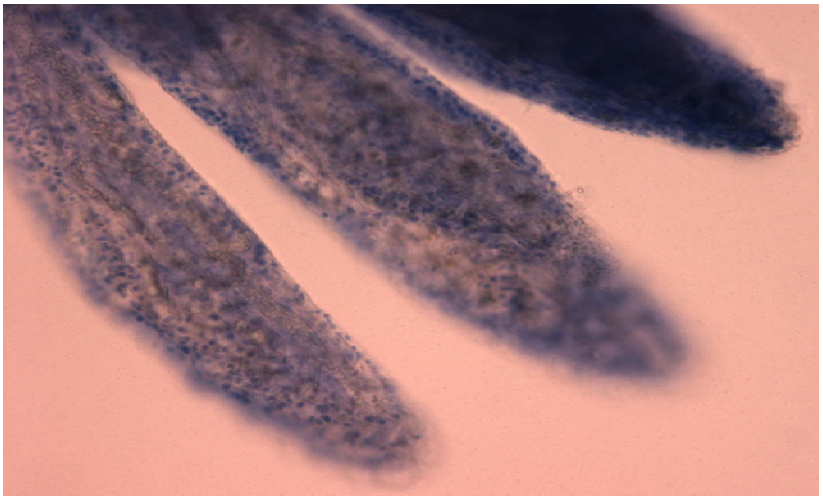

Fig. 25: Histological preparation of synovium vitally stained with trypan blue and fixed in formalin (40x). Synoviocytes composing the intima are well identified. Trypan blue was retained by tissues despite processing and fixation in formalin.

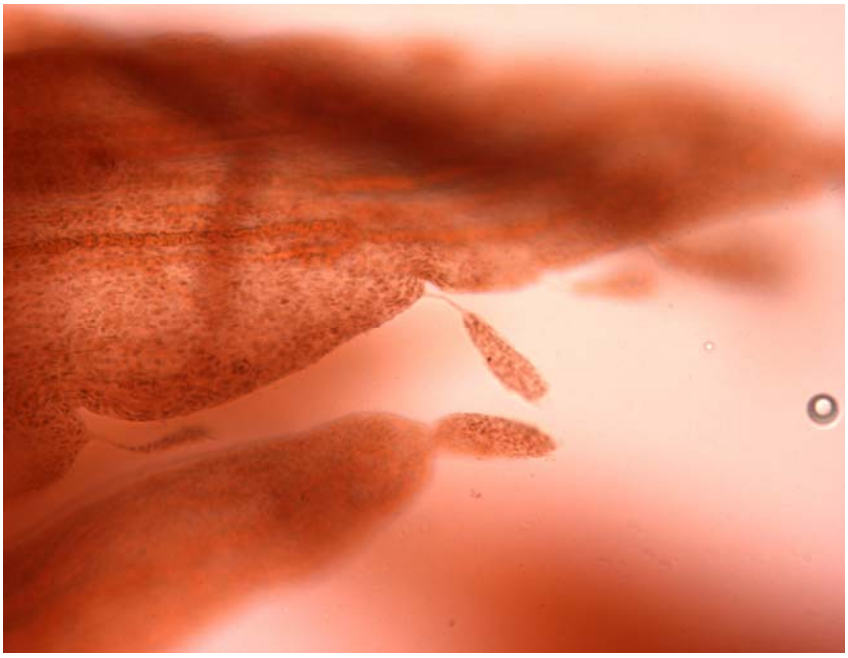

Fig. 26: Histological picture of a sample of synovium vitally stained with neutral red and fixed in formalin (40x). Synoviocytes, as well as blood vessels, are identified precisely.