Muscle and Nerve Biopsy

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Biopsy of muscle and nerve is an essential consideration in the diagnosis of neuromuscular diseases in small animals. While neurological examination and electrodiagnostic procedures provide vital information relating to the distribution and severity of a disease process, it is only by means of biopsy that examination of specific components of the motor unit, and sensory or autonomic nerves is possible, and that definition and classification of pathological processes may be completed.

Muscle biopsy

Examination of most of the components of the motor unit, including intrafascicular nerve branches, neuromuscular junctions, myofibers, and supportive connective and vascular tissues, is possible by means of muscle biopsy. Conventional biopsy and formalin fixation techniques, as used with most organ systems, severely limit the quantity and quality of information that may be obtained. The development of specialized enzyme histochemical techniques using frozen skeletal muscle biopsy specimens, has greatly increased the understanding of both normal muscle and the underlying pathological processes of many neuromuscular diseases. Not only is it possible to recognize specific muscle fiber types within a biopsy specimen, but assessment of various oxidative and glycolytic metabolic enzyme systems may be done.

Selection of a muscle

1. The muscle selected for biopsy should be affected by the disease process. This choice may be based on:
    a. Electrophysiological data including abnormal electromyography (EMG).
    b. Clinical abnormalities suggesting muscle involvement (atrophy, hypertrophy, apparent pain, weakness). Ideally an affected but functional muscle should be selected for biopsy. In acute disease, a more severely affected muscle should be chosen. In chronic, end-stage disease, atrophy and replacement of myofibers with fat and connective tissue may make biopsy interpretation difficult. In this situation a less severely affected muscle may be selected.
2. The muscle selected for biopsy should be easily identified, the surgical approach should be minimal, and the muscle fibers should be oriented in a single direction.
3. The biopsy procedure should be associated with low morbidity relating to the muscle itself and the surrounding soft tissue structures.
4. If possible, biopsy specimens should be harvested from muscles for which there is previous interpretive experience, including data regarding myofiber size and distribution of myofiber types. Preferred biopsy sites have been published and normal morphometric data are available for several canine and feline muscles. Standard muscles used in the authors' laboratory are the lateral head of the triceps brachii (distal third), vastus lateralis (distal third), cranial tibial (proximal third) and temporalis muscle. Biopsy specimens from both a thoracic and pelvic limb muscle, or other distant locations, are necessary to permit a diagnosis of generalized neuromuscular disease. For example, diffuse disease affecting the spinal cord lumbar intumescence may result in neurogenic changes in several muscle groups in a pelvic limb. Clinical signs of neuromuscular dysfunction often are more pronounced in the pelvic limbs, at least early in the course of a generalized neuromuscular disease, and it is only by documenting pathological alterations in other distant muscles that the correct diagnosis may be made. Biopsy of the cranial tibial muscle is often rewarding when a disease process preferentially affects distal nerves or muscles, and has the additional benefit of allowing a combined muscle and nerve biopsy (see common peroneal nerve biopsy below).
5. Muscle biopsy specimens should be harvested from a site remote to tendinous insertions and aponeuroses where histological characteristics normal for these areas (central nuclei, fiber splitting, increased connective tissue) may be interpreted as pathological if the exact location of the biopsy was not known.
6. The site selected for biopsy should be free of artifacts induced by previous disease, intramuscular injections, and EMG needle insertion. Insertion of needles may induce a localized necrosis and phagocytosis (so called "needle myositis") and may make interpretation of a biopsy specimen problematical.
7. Some specialized procedures may require biopsy specimens from specific muscles or regions within muscles. For example, biopsy specimens harvested from the motor points of muscles will contain the highest concentrations of neuromuscular end plates should specific examination of this area of the motor unit be required. Diagnosis of congenital myasthenia gravis is based on the demonstration of decreased numbers of acetylcholine receptors in biopsies of external intercostal muscle.
8. Discussion of appropriate muscle biopsy site selection with the laboratory that will be processing and interpreting the specimens, prior to the procedure, will help to ensure that maximum diagnostic information is obtained.

Muscle biopsy procedures

Two basic procedures are available for muscle biopsy: (1) an open surgical biopsy procedure, and (2) a percutaneous needle (or punch) biopsy procedure.

Open Muscle Biopsy Procedure

Although open biopsies may be done under local anesthesia, they are most often done under general anesthesia following electrodiagnostic investigations. Normally EMG is done on one side of the body, and to avoid needle insertion artifacts, muscle biopsy specimens are harvested from indicated muscles on the contralateral side.

Routine surgical preparation of the skin overlying the biopsy site should be done prior to the biopsy procedure. Specimens for electron microscopy (EM) are harvested first as these samples must be collected prior to manipulation of the myofibers. These specimens are immersed in fixative, usually gluteraldehyde (either sodium phosphate buffered gluteraldehyde or Karnovsky's fixative). Special biopsy clamps are used to prevent contraction of myofibers following excision and fixation. The specimen, which should not be large, as this may prevent rapid fixation, is placed immediately into the fixative. Clamps may be removed after 24 hours, following fixation. If a muscle clamp is not available, the muscle biopsy specimen may be sutured to a 1-2 cm length of wood (e.g. the wooden stem of a cotton tipped applicator). The muscle specimen and wood are then removed together and placed in the fixative. Muscle specimens may also be sutured or stapled to a strip of balsa wood or a wooden tongue depressor prior to fixation.

Following collection of this specimen for EM, a larger specimen is harvested for routine histochemical staining. Collection of a specimen that is oblong in shape allows easy orientation of the biopsy specimen ensuring that well-oriented transverse sections are obtained. This muscle specimen should not be maintained in a stretched position. Electrocautery is normally not required and should be avoided if possible or at least until after the biopsy has been taken. If local anesthesia is used care should be taken to avoid infiltration of the region of the biopsy. Complications are uncommon and usually are the result of animals interfering with the sutures, or hematoma formation. As with all surgical procedures muscle biopsy is potentially contraindicated in patients with significant coagulopathies.

Percutaneous Muscle Biopsy Procedure

Percutaneous muscle biopsy has become more popular over the last decade in human medicine, and is advocated as standard practice by many clinicians. The procedure may be performed under local anesthesia, quickly and inexpensively and is minimally invasive with little scarring. Repeat biopsies may be obtained easily and the site of sampling may be guided ultrasonographically. The main disadvantages of needle biopsy are the small sample size, which makes orientation difficult, and the inability to keep the muscle fibers stretched when fixing samples for EM. Despite these problems, many clinicians harvesting biopsy specimens from human patients suggest that samples are adequate for full analysis in nearly all patients. Percutaneous muscle biopsy is used routinely in large animals, and its use has been reported in dogs with good diagnostic results. The necessity for general anesthesia is the major disadvantage of open muscle biopsy for both large animals and humans. However, almost all biopsy procedures in small animals will be coordinated with an electrodiagnostic examination, which, in itself, requires general anesthesia. The authors therefore advise that open biopsy should be done whenever possible to provide biopsies of optimum size, that are easily to orientated and that are as free of artifact as possible. Needle biopsies may be beneficial in research situations, where sequential biopsy samples may be required over a period of time.

Specimen processing and transport

Ideally, muscle biopsy specimens should be immediately frozen to preserve labile substrates such as glycogen, and prevent loss of soluble enzymes. Muscle specimens may be trimmed with a sharp blade to remove areas grasped by forceps, and approximately 0.5cm blocks are mounted on thin cork squares either directly, or using tissue embedding media such as gum tragacanth (Sigma-Aldrich Co.) or Tisue-Tek O.C.T. compound (Sakura Finetek). Muscle fibers are oriented vertical to the cork, thus facilitating cutting in the transverse plane. Specimens may be placed in the longitudinal plane, however little useful additional information is obtained from such sections in most circumstances. Proper freezing of the sample is critical for preservation of morphological detail and prevention of artifacts. Freezing too slowly will result in ice crystal formation within the myofibers, while freezing for too long a period may result in cracking or fissures within the tissue block. The recommended method of freezing involves immersing the sample for approximately twenty seconds in isopentane (2-methylbutane) which has been cooled to approximately $-150^{o}C$ in liquid nitrogen. Direct freezing in liquid nitrogen generally is not recomended due to the formation of an insulating layer of nitrogen bubbles around the sample that slows the rate of freezing. However, this problem may be reduced by coating samples with talcum powder prior to direct freezing in liquid nitrogen and artifact free sections may be obtained using this approach. Frozen blocks are transferred to a microtome/cryostat maintained at $-20^{o}C$ where consecutive 10μm sections are cut for histological and histochemical staining. Frozen blocks may be stored in airtight containers at $-80^{o}C$ or in liquid nitrogen storage vessels.

Most biopsy specimens will be transported to specialized laboratories for processing and interpretation. It cannot be overemphasized that the quality of the information that will be obtained from a biopsy specimen is dependant on the quality of the biopsy specimen when it arrives at the laboratory. Always obtain the laboratory's specific instructions for handling and transportation of biopsy specimens before completing the biopsy procedure. Cytochemical and histochemical properties of muscle biopsy specimens

are maintained for at least 30 hours in samples that are stored appropriately. [12] Samples should be wrapped in saline moistened swabs that have been thoroughly wrung dry, and placed in an airtight container. For optimal results the specimen should be maintained at 4°C using cold packs and shipped overnight to the laboratory within 30 hours. Biopsy procedures should be coordinated so that samples do not arrive at the laboratory during a weekend. If samples must be collected on a Friday they may be frozen as described above if facilities are available, or as a compromise, wrapped in aluminium foil and frozen in a slurry of dry ice and acetone or frozen at–80°C. Samples that have been frozen will need to be transported on dry ice to prevent freeze thaw cycles that result in severe ice artifact. Muscle specimens to be used for biochemical analysis, such as carnitine quantitation, may be transported in a separate container along with the main biopsy.

In the clinical setting, frozen biopsy specimens may provide the information necessary to allow categorization, or definitive diagnosis, of the underlying disease process in most cases. Samples for EM may be necessary in a small number of cases. It is advisable to take a suitable specimen for EM (see above) at the same time as the biopsy specimen for histochemistry is harvested, and to store this specimen for future processing, if required. Samples for EM should be placed into fixative as soon as possible after removal. While some laboratories may request additional material placed in formalin, the amount of additional information gained from muscle preserved in this way is of limited value, and a muscle biopsy procedure where only formalin fixed tissue is provided is of dubious value.

Nerve biopsy

In conjunction with muscle biopsy, examination of an affected nerve may provide valuable information for greater definition of underlying neuromuscular pathology. In most instances, nerve biopsy allows an accurate determination of the specific pathological processes involved in the disease, such as demyelination or axonal loss. In some cases, however a specific diagnosis may be made from examination of a nerve biopsy specimen. Any nerve potentially may be biopsied, whether peripheral or cranial, at the level of the nerve root, spinal nerve or plexus, or at a distal extremity. Ease of biopsy and associated morbidity varies widely, and the choice of a nerve to biopsy should be guided by clinical and electrophysiological data.

Choice of nerve

1. The nerve should be affected by the disease process as evidenced by:
    a. Abnormal electrophysiological investigation, and
    b. Neurological abnormalities in areas innervated by the nerve (atrophy, hypotonia, hyporeflexia, paresis, sensory deficits).
2. If the disease process is generalized, a nerve should be selected that:
    a. Is easily biopsied with low morbidity,
    b. Has established normal, electrophysiological and morphometric data available
    c. Innervates a muscle, that is routinely biopsied for which normal histochemical and morphometric data are available.

When a generalized neuromuscular disease is suspected based on clinical and electrophysiological data, the common peroneal nerve provides a satisfactory site for biopsy. The pelvic limbs often are affected either before, or at the same time, as the thoracic limbs, making a pelvic limb nerve a logical choice for biopsy. The common peroneal nerve is easily identified and relatively easy to biopsy due to its flat anatomy and easily identified fascicles. There are well established motor and sensory electrophysiological data for the peroneal nerve in both dogs and cats. Detailed age related morphometric data are available for the common peroneal nerve of dogs, although available information relating to the peroneal nerve of cats is less specific for the suggested area of biopsy.

The common peroneal nerve is a mixed nerve containing motor, sensory and autonomic nerve fibers. Other mixed nerves that may easily be biopsied include the tibial nerve and its branches in the pelvic limb, and the ulnar nerve in the thoracic limb. When a predominantly sensory neuropathy is suspected, biopsy of cutaneous sensory nerves such as the caudal cutaneous antebrachial nerve in the thoracic limb, and caudal cutaneous sural nerve in the pelvic limb, may be beneficial. Biopsy of nerve roots may be necessary when pathology is restricted to the most proximal portions of the peripheral nervous system. Nerve root biopsy is an invasive procedure requiring extensive surgical exposure and laminectomy. If both dorsal and ventral nerve roots are affected, biopsy of the dorsal nerve root is preferred and will normally result in minimal neurological deficits post operatively, even if the nerves of the cervical or lumbar intumescences are involved. Assessment of dorsal and ventral nerve roots may also be useful in determining whether motor, sensory or both types of nerve fibers are affected. Intrafasicular nerve branches, both motor and sensory, are often present in muscle biopsy specimens, and may provide useful information relating to distribution of neuropathic disease involving the most distal portions of a nerve. Biopsy of cranial nerves is an infrequently completed procedure, largely due to the inaccessibility of these nerves. Biopsy of the facial and trigeminal nerves has been described, and intrafascicular branches of the trigeminal nerve may be found in muscle biopsies of the masticatory muscles. The vagosympathetic trunk and recurrent laryngeal nerves are accessible distally in the cervical region, however biopsy is not without significant morbidity. The hypoglossal nerve may be visualized medial to the digastricus muscle and has been described as being accessible by means of a ventral approach.

Nerve biopsy technique (Common peroneal)

With fascicular versus full thickness biopsies, and attention to good surgical technique, biopsy of peripheral nerves may be done with minimal morbidity. Approaches and techniques for other less commonly biopsied nerves have been described previously.

Biopsies generally are done under general anesthesia following routine surgical preparation of the area. Severely diseased nerves may be markedly reduced in size and may appear almost translucent. Care should be taken to dissect as much fat and fascia from around the nerve as is safely possible. It is surprisingly easy to inadvertently take a fascicular biopsy of connective tissue that does not contain nerve. Combined biopsy of the ipsilateral cranial tibial muscle (usually via a separate incision) provides samples of both nerve and a dependant muscle (see above). External dressings normally are not required, however animals should be monitored to prevent their interference with the sutures. Some animals may exhibit proprioceptive deficits with knuckling of the distal pelvic limb on the side of the biopsy. This usually will resolve within 3-4 days, and long term deficits are extremely uncommon. It is always wise to warn the owner of the expected short term deficits prior to the procedure.

Nerve specimen processing

It is essential that specific details of fixation and transportation are obtained from the laboratory that will be processing the nerve, prior to the biopsy. Most laboratories will request nerve to be placed immediately into fixative, either 10% formalin, gluteraldehyde or usually both. In order to prevent significant artifact formation during fixation, the biopsy must be prevented from contracting during this time. Several methods may be employed for this purpose including:

a. Pinning the nerve at either end to a piece of balsa wood or a tongue depressor
b. Suturing around the nerve at either end onto the wooden stem of a cotton tipped applicator
c. Suspending the nerve in fixative using the silk suture placed during the biopsy, with a stainless steel weight hooked through the opposite end.

The sample may then be transported in sealed screw top bottles to the laboratory. In the laboratory samples are generally processed in one of three ways:

a. Routine histological staining of formalin fixed specimens e.g., hematoxylin and eosin, Gomori trichrome, luxol fast blue. This allows for basic assessment and screening for obvious loss of myelin, axonal degeneration or other gross abnormalities. Formalin fixed, paraffin embedded tissue is of particular value for assessment of cellular and nuclear detail such as perivascular infiltration/invasion.
b. Plastic embedding of gluteraldehyde fixed specimens. Semithin cross sections (1-2μm) of nerve stained with toluidine blue for light microscopy and ultrathin sections for electron microscopy, allow for more detailed investigation of peripheral nerve pathology. Morphometric data including numbers and diameter of axons (both myelinated and unmyelinated), and thickness of myelin sheathes may be obtained and results may be compared to normal values.
c. Single teased fiber preparations; Small strands of gluteraldehyde fixed nerve fibers are treated with glycerol and osmium tetroxide and teased apart under magnification until single fibers are present. This technique is used particularly to study myelinated fibers and allows assessment of consecutive myelin internodes in the same nerve fiber. It is therefore useful for determining the frequency of certain neuropathological abnormalities such as areas of demyelination/remyelination and the presence of ongoing nerve fiber degeneration.

If facilities are available, a section of the nerve biopsy specimen may be frozen unfixed as described for routine muscle processing. Non fixed frozen tissue allows the use of specific enzymatic stains (e.g. acid phosphatase to demonstrate macrophage activity) and may be necessary for specific immunohistochemical studies that are not possible with fixed tissu. Immunolabeling may allow the differentiation of inflammatory infiltrate from neoplastic cells such as lymphoma. A nerve specimen also may be frozen in liquid nitrogen if specialized biochemical analysis is required.

In most instances, adequate information may be obtained from cross sections of plastic embedded nerve and teased fiber preparations prepared from biopsies transported in fixative. Electron microscopy, although necessary to study unmyelinated fibers, is rarely required in the clinical setting. Although morphometry is essential for research purposes and may be used to monitor disease progression and treatment efficacy, repeated nerve biopsies may be difficult to justify clinically.