Canine inherited myopathy as a translational model

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Abstract

The inherited myopathies include channelopathies, myotonias, metabolic and mitochondrial conditions, congenital myopathies, and the muscular dystrophies. This review focuses on a single centronuclear (congenital) myopathy, termed X-linked myotubular myopathy (XLMTM). X-linked myotubular myopathy of Labrador retrievers have recently become a valuable model for preclinical studies. In developing preclinical trials in dogs, the clinical course of the XLMTM syndrome in Labrador retrievers evolves rapidly, so affected dogs must be treated and assessed as early as ten weeks of age. Although interventional therapeutic studies in XLMTM dogs are in the early preclinical stages, promising results have already been seen. Having a dependable breeding program is absolutely essential to the success of these preclinical experiments. Future studies using this canine model should shed further light on the human disease.

Keywords: Inherited myopathies, centronuclear myopathies, X-linked myotubular myopathy (XLMTM), myotubularin, preclinical trials

Introduction

Inherited and acquired myopathies in dogs are becoming more widely recognized for how well they recapitulate their respective human conditions. From clinical presentation to the underlying ultrastructural and biochemical etiology of disease initiation and progression, canine muscle resembles human muscle to a remarkable degree. Smaller laboratory animal models, like mice, have provided a powerful tool to study the molecular pathogenesis of disease for decades, partly because transgenic or gene knock-down/out strains can be created quickly and relatively inexpensively. However, it is becoming clear that drug trials tested exclusively in mice often fail to be predictive of efficacy and/or toxicity in human clinical trials.[1] This dependence on model species that do not extrapolate to humans with high fidelity, because of differences in size, drug biodistribution, anatomy or physiology,[2, 3] has resulted in an expensive and time-consuming "fail late" phenomenon in the drug discovery pipeline.[4] These shortcomings are mostly avoided by utilizing canine models because of their similarity to humans in metabolic, physiological and anatomical characteristics.[5] In addition, our understanding of the genetic similarities between dogs and humans is growing, augmented by the sequencing of the canine genome more than ten years ago.[6] Therefore, the translational value of naturally occurring canine models of disease will likely increase as more studies corroborate their ability to predict therapeutic efficacy and safety in humans.[7]

Several sporadic and inherited myopathies have been described in dogs and have provided the opportunity to study the underlying disease mechanisms that are analogous in human conditions and to develop preclinical models. Acquired myopathies include both infectious[8] and immune-mediated inflammatory conditions,[9] as well as metabolic and endocrine diseases.[10] The inherited myopathies include channelopathies, myotonias, metabolic and mitochondrial conditions, congenital myopathies, and the muscular dystrophies.[11] Inherited muscular dystrophies and congenital myopathies, arising from mutations in genes coding for several components of the dystrophin-glycoprotein complex and proteins involved in membrane remodeling, respectively, have been described in dogs (Table 1).[12-15]

This review focuses on the condition homologous to a centronuclear myopathy (CNM) of human beings--specifically,XLMTM. At this stage, a clear distinction should be made between the centronuclear myopathies and the more familiar muscular dystrophies. Dystrophies typically present with cycles of degeneration and attempted regeneration, leading to myofiber necrosis, fibrosis and fatty deposition.

Historically, muscular dystrophies were classified based primarily on their pattern of inheritance and the particular muscle groups involved.[16, 17] Over the last decade, distinct subtypes of muscular dystrophies have arisen as specific mutations are identified, primarily in the proteins that make up the dystrophin-glycoprotein complex.[17] In contrast, the centronuclear myopathies are caused by mutations in genes whose proteins are involved in membrane remodeling within the myofibers.[18,19] In general terms, myopathies are distinguished from dystrophies because of their relatively non-progressive histologic nature and the presence of specific structures, like cores or nemalin rods within muscle fibers.[20]

Canine model of X-linked myotubular myopathy

The diagnosis of centronuclear myopathy refers to a subset of congenital myopathies characterized by skeletal muscle weakness and a majority of muscle fibers with centrally located nuclei and/or cores on biopsy. Among the congenital myopathies, murine and canine models exist for most of the genetically distinct CNMs, including dynamin2, protein tyrosine phosphatase-like member A (PTPLA),[21,22] amphiphysin *(BIN1)*[23,24] titin and the ryanodine receptor,[25] (Table 2). The most common CNM, XLMTM, is caused by mutations in the myotubularin 1 gene (*MTM1*) and affects about 1 in 50,000 male births.[26] Newborn males with XLMTM show a diverse clinical presentation that includes decreased fetal movement, low Apgar scores, large head circumference, areflexia, cryptorchidism, a need for immediate ventilator support and, of course, profound hypotonia.[26-28] Boys with severe mutations who survive their first two years will never walk and will be dependent on a ventilator so development of speech is precluded. Two murine models have been valuable in studying initiation and progression of the pathology and have served as a proof-of-concept platform for gene therapy efficacy and safety, but have inherent limitations for preclinical testing.[29-32] The Labrador retriever model of XLMTM, with a p.N155K mutation in the *MTM1* gene,[33,34] has proven enormously valuable in that it recapitulates the pathology observed in human patients and is a similar size to affected infants and young boys. This model will be the focus of this review, but it should be noted that a recent paper identified a second mutation, p.Q384P in a family of Australian Rottweilers that showed similar generalized, progressive and fatal myopathy in the second to third month of life.[35]

To appreciate the pre-clinical utility of the canine model of XLMTM, it is important to understand the disease onset and progression from birth to humane euthanasia in the laboratory setting, and how it compares to patients with this devastating disease. A comprehensive natural history study from our laboratory[36] documented the decline in limb strength, walking gait and respiratory function in young dogs as a consequence of the appearance of typical CNM histological biomarkers. These results corroborated and expanded on earlier studies of this particular p.N155K mutation.[33,34,37-39] Labrador retriever puppies with XLMTM have lower birth weight, grow more slowly and display mild muscle atrophy and pelvic limb weakness up to about seven weeks of age.[33,34] Patellar hyporeflexia, dysphagia, dropped jaw and a hoarse bark gradually appear over time. We have been struck by how little phenotypic variation occurs in our laboratory colony, even after outcrossing Labrador female carriers with beagle males in an attempt to interject genetic diversity and decrease the size of the dogs.[36] By ten weeks of age clear signs of the myopathy are present, and muscle weakness and atrophy progress rapidly leading to loss of ambulation, necessitating euthanasia by four to six months of age. An age equivalency paradigm used for a canine muscular dystrophy model was proposed.[40] Assuming a parallel between the first three months of a Labrador retriever's life and first five years of a human's, the four to six month course of XLMTM in Labradors is somewhat delayed compared to the progression of XLMTM in humans.[28]

Our principal outcome measure has always been limb strength, because assessing muscle function during the downward progression of the disease and to test potential therapeutic interventions should have the greatest impact on quality of life of the patients. Using physiology instrumentation to measure joint forces, torque generated across tibiotarsal or metatarsal joints in the hindlimbs or forelimbs, respectively by XLMTM dogs at ten weeks is ~40% lower than normal. By 18 weeks-of-age, torque generated by affected dogs is only ~15% of that of wild-type dogs.[36,39] Subjecting the dogs to a series of eccentric contractions and testing their force output gives us a glimpse into the underlying pathophysiology of XLMTM. In contrast to the progressive force drop observed in a canine muscular dystrophy model,

XLMTM dogs display a progressive *increase* (summation) in the isometric phase. Despite a progressive increase during repeated contractions, maximum isometric torque never exceeds values observed in normal controls or carriers.[39] Mutant dogs generate a gradual increase in isometric torque during a series of eccentric contractions until activations cease. Following a brief rest, the next eccentric contraction begins at a lower initial torque. The pronounced torque *increase* following repeated stretch activations in XLMTM dogs is in keeping with findings in ryanodine receptor (RyR1) mutant mice.[39,41] Both *MTM1* mutant dogs and *Mtm1* mutant mice display similar increases in force summation with repetitive nerve stimulation. While the precise molecular mechanism underlying these observations is unknown, a defect in excitation-contraction (E-C) coupling is likely responsible. In addition, affected dogs exhibit right-shifted torque-frequency curves compared to those of the normal and carrier dogs indicating that an E-C coupling defect is a central feature of this mutant canine model.

The single most burdensome aspect of XLMTM for young male patients, their families and full-time nurses is the absolute dependency on ventilator support. Therefore, careful assessment of respiratory indices has been a primary component of the phenotypic characterization of the canine model.[32,38] Affected dogs demonstrate paradoxical breathing at ~10 weeks and, when compared to wild-type dogs on serial measurements, have reduced inspiratory and expiratory flow rates before and after dosing with doxapram, suggesting involvement of the diaphragm. Ultrasonography images of the diaphragm were acquired from normal and affected XLMTM dogs[42,43] and showed that the normal diaphragm appeared thicker and less echogenic than in the XLMTM dog.

Lastly, walking gait of the XLMTM dogs was assessed using video-based motion capture analysis and an instrumented carpet.[36,37] Instrumented carpets with imbedded sensors coupled to customized software allows investigators to measure spatiotemporal gait characteristics, such as overall speed, step length, step width and stride length. However, spatio-temporal data alone do not capture postural changes or range-of-motion (ROM) excursion; kinematic (motion-capture) analysis is required to assess these parameters. X-linked myotubular myopathy dogs appear to maintain relatively normal joint range-of-motion, but walk slower than normal with shorter stilted strides. These differences become greater over time, suggesting that slowing of walking gait generally correlates with progressive limb weakness.

Production of forelimb and hindlimb torque, diaphragmatic strength as assessed by peak inspiratory flow and walking gait speed and stride length constitute the core outcome measures used to track disease progression. The fact that these assessments are sensitive, repeatable and non-invasive make them well suited to test for correction of the disease phenotype after systemic treatment with AAV8-MTM1 gene therapy. To summarize briefly, p.N155K mutant dogs were assessed at nine weeks to establish baseline readouts, then given approximately $1X10^{14}$ of an adeno-associated viral vector carrying the normal copy of the MTM1 transgene at ten weeks of age.[44] Two dogs treated with this regimen showed normalized tibiotarsal joint torque and peak inspiratory flow rates six to seven weeks after infusion. Moreover, all treated dogs remained ambulatory for more than three years, well beyond the age of 18 weeks when untreated XLMTM dogs can no longer walk. These two treated males remain in the XLMTM colony and have successfully bred to carrier females producing several litters of dogs, indicating the sustainability of gene replacement therapy.

XLMTM canine colony breeding program

These experiments, plus a much larger dose-finding study involving 22 dogs could not have been completed without a successful breeding program. To our knowledge, this is the first instance in which two dogs, affected with a fatal monogenic disease of muscle, have been treated and successfully bred to carrier females to produce multiple offspring. This is important given the X-linked mode of inheritance of myotubular myopathy. Before this breakthrough, carrier females were bred with normal males, producing one-quarter carrier females and one-quarter affected males. However, now by breeding affected-treated males with carrier females we can produce one-quarter affected males, but also carrier and homozygous affected females, thus enabling us to keep breeding and colony management costs down. In addition, XLMTM human females have been reported, although they are rare but having affected

female dogs in our colony allows us to test the gene therapy on the opposite sex, thus fulfilling another Food and Drug Administration requirement for approval in clinical trials. Because of the central importance of the XLMTM dog colony breeding program, the remainder of this review will focus on the reproductive methods employed to increase the likelihood of a successful natural coupling, and guarantee the long-term survival and inexpensive dissemination of this valuable model through semen preservation. The following summary is adapted from the following book chapters and journal articles.[45-50]

Female reproduction

The estrous cycle in the female dog is unique and different from other domestic animals. It starts with proestrus. Most breeders refer proestrus as start of the 'season'. Bitch owners notice spotting or bleeding from the vagina. During proestrus, the bitch attracts a male but will not allow him to breed. Estrogen produced by ovarian follicles causes change in the epithelial cells of the vaginal mucosa, which we will discuss in great detail. Estrus or the time of receptivity follows the proestrus, when the bitch accepts the male for breeding. The duration of proestrus and estrus cited in textbooks is nine days each. The authors agree with these times as an average but unfortunately they have not found many bitches following the textbook and have a nine-day schedule. The authors have seen bitches with a range of 2-21 days of each phase. During proestrus, the vulvar lips of the bitch become turgid or firm which become soft and 'wrinkly' towards receptivity phase of the bitch. The bloody discharge of the proestrus also changes to straw colored discharge towards estrus in most bitches but some bitches bleed all the way through estrus.

The production of progesterone (P4) is also unique during estrous cycle of the bitch. The early rise of the P4 during estrus is from the luteinized ovarian follicles and is useful in breeding management which will be discussed later. The bitch ovulates with the help of luteinizing hormone (LH). Ovulation occurs about three days after the LH peak. After ovulation, the follicles are replaced by corpora lutea (CLs) which produce P4. The production of P4 continues throughout diestrus, the next phase of estrous cycle. The duration of diestrus is the same as the pregnancy, about 62-63 days from the LH peak. Even the bitch is not pregnant; P4 will be produced for the duration of diestrus. It appears that there is no production of prostaglandin F2alpha (PGF2alpha; as in other domestic animals) from the endometrium to cause luteolysis (CL regression). During diestrus, many bitches go through 'pseudopregnancy'. The bitch may gain weight with an enlarged abdomen and start showing overt signs of pseudopregnancy during late diestrus, including mammary gland enlargement, nesting, adopting toys and shoes, and giving impression that she is about to whelp. Pseudopregnancy (or diestrus) is considered a normal occurrence, and may not need any treatment.

Ovulation

The ova released from the ovary during ovulation in the bitch are at the primary oocyte stage, i.e. the first polar body has not come out of the ovum and sperm are not able to penetrate the ovum. It takes about three days for the ovum to become a secondary oocyte and ready to be penetrated by sperm. Dog sperm can survive up to ten days in the bitch's reproductive tract. It can become a diagnostic challenge when a bitch's owner asks for a cesarean section 62 days after breeding. Theoretically that pregnancy (62 d after breeding) could be as early as 52 days. A few tools are avilable to predict whelping. Hypothermia (2-3°F) or a decrease in rectal temperature, caused by P4 decrease 12 to 36 hours before whelping is fairly reliable to predict delivery. The indication of diestrus day 1 (D1) by vaginal cytology is another reliable way to predict whelping 57-58 days from D1. Following is a discussion of the use of vaginal cytology in breeding management.

Reproductive anatomy. For successful vaginal swabbing technique, the operator needs to appreciate the unique reproductive anatomy of the bitch. The vagina of the bitch is very long, about 20 cm (~9 inches) in a medium size bitch. The cervix in the bitch is located in the abdominal cavity and is different than other domestic animals (e.g. cow, mare) where cervix is located at the pelvic inlet. Therefore, cervix in the bitch cannot be visualized by a speculum examination but can be visualized with a flexible endoscope and light source. This becomes important when performing artificial insemination

(AI) and wish to deposit semen transcervically into the uterus. Artificial insemination and semen deposition will be discussed in more detail below. The size of the uterus and ovaries vary considerably with the breed.

Vaginal cytology. A clean cotton tipped swab is commonly used for swabbing the vagina for the cytology sample. One can use vaginal speculum to guide the swab. A right-handed operator holds the bitch's vulva with the gloved left hand, opening the vulvar lips with the thumb and the middle finger, while placing the index finger behind the vulva to support it. The swab is inserted with the right hand almost vertically into the vagina avoiding the clitoral area, dorsally and over the brim of the pelvis. The swab is moistened with warm tap water before use which is helpful to advance the swab especially in small size bitches and bitches without a serosangious discharge. If the swab is 'stuck' in the vaginal folds, it is slightly withdrawn, redirected and advanced. Once the swab is in the vagina for at least 6-10 cm (2-4 inches), the swab is rolled a few times in one direction (if rotated back and forth, the cotton may unroll and drop in the vagina). The authors recommend advancing the swab as far as possible as there is less debris on swabs taken from the cranial vagina compared to the caudal vagina. Getting into the habit of reaching the cranial vagina is also be helpful for taking vaginal cultures and for AI. The swab is rolled on a clean microscope slide, air-dried and stained with Romanowski stain (Diff-Quick, Harleco, Gibbstown, NJ). Other stains used by practitioners include methlyene blue, eosin-nigrosin, gram stain, etc but the authors have found Romanowski stain satisfactory for staining epithelial cells as well as white blood cells (WBCs). The slides with smears are dipped five to seven times in each solution, rinsed with tap water, air-dried and examined under the microscope. The authors start under 200X magnification to get an overall impression and distribution of the cells before moving on to 400X magnification to closely examine the desired field.

Various theriogenologists, clinical pathologists and other practitioners have interpreted vaginal cytology of the bitch differently but for this presentation discussion will be limited to that previously published.[51] The vaginal mucosa is responsive primarily to estrogens (E2), and vaginal cytology is useful only during estrogenic phase of the cycle. Parabasal cells are small and round with large and distinct nuclei. The total area of the cytoplasm of the cell is smaller than the nucleus. These cells (along with red blood cells; RBCs) are present during proestrus. Superficial intermediate cells are larger than parabasal cells with small nuclei and irregular/folded borders. Large numbers of these cells are observed during late proestrus to early estrus. Superficial cells, also called cornfield or anucleated, are the largest of the epithelial cells present during estrus. Under a simple microscope, the nuclei of these cells appear faded or absent. The cells also appear 'light in weight', multi-layered, and have folded borders. The appearance of 80-90% of these cells in the smear is used as an indication to start breeding. A series of vaginal cytology samples is needed to observe the progressive change in epithelial cells. A single sample is unreliable. Some bitches display same type of epithelial cells for many days, whereas others change within 24 hours. Many bitches reach to 80-90% cornification during estrus but many bitches never go above 70% cornification. Obviously, if one waits for 80-90% cornification to breed, these bitches will be missed. The other tool useful in managing breeding of these bitches is to measure blood P4, which will be discussed below. If multiple cytology samples are obtained, neutrophils appear and this is the first day of the diestrus or D1. This finding is important because the bitch is going to whelp 57-58 days after D1. The authors recommend taking cytology every day, because in some bitches the change from cornfield to superficial intermediate and the appearance of neutrophils happens within 24 hours. Most breeders and bitch owners are able to obtain vaginal cytology samples at home after brief training. The swabs are brought to the clinic, stained and evaluated.

Progesterone assay. As discussed above, in addition to the CL, P4 also is secreted by luteinized follicles in the ovaries. The early P4 rise can be used to determine ovulation in the bitch. Ovulation takes place at about three days after the LH peak which is very short. Luteinizing hormone assays are available and it is recommended to measure LH daily when ovulation is expected. Progesterone assays are commonly used for canine breeding management. Enzyme linked immunosorbent assay (ELISA) kits (Synbiotics Corp., San Diego, CA) are available to determine P4. These are qualitative tests based upon a color change which is interpreted as a range of a P4 concentration. Many clinicians prefer to send the

blood samples to an endocrinology laboratory for P4 determination by radioimmunoassay (RIA). Progesterone is the same hormone in all species, including humans; therefore blood samples can be analyzed for P4 in veterinary or in human hospital laboratories.

Progesterone assays are an excellent tool to determine ovulation time for breeding at the appropriate time. This becomes critical when using chilled transported or frozen-thawed semen for AI. Like vaginal cytology, P4 concentration is also monitored starting few days after the start of proestrus; P4 concentrations in range of 2-10 ng/ml, with 2-2.9 ng/ml indicative of ovulation in about two days, 3-3.9 in one day, and 4-10 ng/ml indicative of the ovulation day.

Basic understanding of canine semen preservation and artificial insemination

For semen preservation and AI, semen is collected and evaluated in the same manner as for male breeding soundness examination (BSE). Depending upon the use of semen - fresh, chilled or frozen - semen is processed accordingly.

AI with fresh semen

Artificial insemiation is performed when the male and the female are at the same location but are unable to breed. The semen is usually deposited immediately after collection without adding any extender. Two to five ml of semen is inseminated. Some of the reasons that bitch and the stud dog would not breed and may require AI include:.

The bitch is not ready. The most common reason for fresh semen AI is when the dog owner 'believes' the bitch is ready but she does not stand for the male. It is important that the veterinarian examine the bitch including vaginal cytology to determine if she is ready. In few instances, the authors have performed AI when cytology showed only 50-60% cornification (superficial anucleated vaginal epithelial cells). The reason is that some bitches don't reach more than 70% cornification and they ovulate. Progesterone assay would be helpful to confirm this, but for the owner of the bitch and the stud dog, it is a less expensive option to have an 'extra' AI performed.

For AI, the authors use half of a cow AI pipette attached to a rubber connector, and other end of the connector is attached to the syringe with semen. Canine AI pipettes are available commercially. The pipette is inserted into the vagina in manner similar to that used for taking vaginal smear. The authors prefer to pass the pipette up and over the brim of the pelvis, depending on the size of the bitch, at least four inches, and deposit the semen in the cranial part of the vagina. The bitch's rear legs are raised for about 20 minutes to prevent semen back-flow and encourage the sperm transport through the cervix.

AI with chilled transported semen

This is a common procedure used when male and female are separated by long distances. Frequent airline transportation has made it possible to ship semen across the country, and in many locations between North America and western Europe within 24 hours. With strict airline regulations related to animal transport, it has become very difficult or impossible to ship a dog by air.

Appropriate coordination and communication between the owners of the female and male is important for success of AI with transported semen. The bitch owner works with a veterinarian to manage the breeding by monitoring the bitch's vaginal cytology and blood P4. The authors recommend that examinations of the bitch begin two to three days after onset of proestrus (spotting or bleeding) and that monitoring continues at one to two day intervals.

Steps in shipping semen

Collect semen

Dilute the semen by adding semen extender. Dilution rate will depend upon the total number motile and morphologically normal sperm in the ejaculate (refer to discussion of dog BSE below). It is a common practice to dilute semen 1:2 (1 part semen: 2 parts extender). The basic concept of a semen extender is to provide appropriate environment and energy for the sperm to survive. Skim milk, tris-egg

yolk and many other extenders have been utilized for dog semen. If using a commercial semen extender, follow the manufacturer's directions very closely.

Semen packaging and shipping. Semen cooling rate and temperature control are two important factors when selecting a shipping container. Numerous closed-cell extruded polystyrene foam boxes and other containers are available commercially. The authors' experience when receiving semen in homemade containers has been mixed with sperm motility ranging from 5 to 70%. In many cases, semen is packed touching the ice packs, resulting in cold shock to sperm. The semen is shipped for next day delivery.

Insemination timing. As discussed above, proper insemination timing is crucial for successful outcome of AI with chilled or with frozen-thawed semen. In addition to vaginal cytology, blood P4 assay (preferably by RIA) is very helpful to monitor the time of ovulation. Like vaginal cytology, P4 concentration is monitored starting few days after start of proestrus, using P4 concentrations in range of 2-10 ng/ml, with 2-2.9 ng/ml indicative of ovulation in about two days, 3-3.9 ng/ml in one day, and 4-10 ng/ml indicative of the day of ovulation.

As epithelial cells change from parabasal to intermediate superficial cells, P4 is measured to obtain a baseline of 2ng/ml and repeated every 24 hours, until concentrations reach 5 ng/ml. Insemination may be performed two to three days later.

AI with frozen-thawed semen

Semen freezing technology was developed in the 1960's to preserve desirable genetic material (semen) from bulls for future use. Since then this technology has been perfected to the point that more than 60% of the dairy cows in the US and Canada are bred with frozen-thawed semen. It is also interesting to note that proven (progeny tested) bulls in the US are kept in a few locations, semen is collected, frozen, stored and sold almost all over the world.

Even though reports of freezing dog semen were published in late 1960's, it was not until recently that frozen-thawed dog semen has been used on commercial basis. Obvious advantages of the frozen semen are that semen can be collected from a valuable dog (preferably at a young age), frozen and kept for very long time (almost forever) and used even after the dog's death. Semen is collected (as discussed below) and extended. Semen extender used for freezing has an additional component of cryoprotectant (e.g., glycerol) to protect the sperm membrane from injury by ice crystals formed during freezing and thawing process. Semen is commonly packed in mini straws (0.25-0.5 ml), frozen in liquid nitrogen (LN) vapor and stored in LN.

The procedure for thawing semen varies depending upon the freezing rate of the semen. Therefore, it is important to follow the thawing instruction received from the semen supplier. One common thawing technique used is to thaw frozen semen in straws at 37^0C in a water bath for 60 seconds. It is important to note that semen and water do not mix. Make sure water does not come into contact with semen during the thawing procedure.

Artificial insemination with frozen-thawed semen requires deposition of semen into the uterus of the bitch at the appropriate time. Even though the dog deposits semen in the vagina during copulation, and chilled semen is deposited in the vagina, frozen-thawed sperm lives for a much shorter time therefore it is desirable to deposit semen directly intp the uterus. Some practitioners have been successful getting bitches pregnant by vaginal deposition two to three times with frozen-thawed semen and monitoring vaginal cytology and blood P4. The following techniques are commonly used for AI with frozen-thawed semen.

Surgical AI. A 4-6cm midline incision over the pubis is made in an anesthetized bitch after routine surgical preparation for a laparotomy. The uterus is exteriorized and the semen is deposited into the lumen of the uterine body at a 45^0 angle with the bevel of the needle facing up. The operator should be able to see the distension of the uterine horns as the semen in injected, otherwise needle should be repositioned. Saline moistened gauze is held over the injection site for a minute or so after the needle is withdrawn. The uterus is replaced in the abdomen and the incision is closed.

Norwegian catheter. A stainless steel catheter protected by a plastic sheath is used for transcervical intrauterine AI. Three different length catheters are available for various size dogs. The cervix is fixed between operator's fingers through the abdominal wall of the standing bitch. The catheter is passed through the vagina and the tip of the catheter is passed through the cervix and the frozen thawed semen is deposited. The authors' experience based upon few bitches is that practice is required to locate the cervix and pass the catheter tip through the cervix. The technique is commonly used in Scandinavian countries with successful outcomes.

Transcervical insemination. Transcervical insemination (TCI) is primarily used to inseminate female dogs with frozen-thawed semen. However, it can be used for fresh chilled semen, or semen of poor quality. A special endoscope is used in a bitch during "standing estrous" which avoids the need to anesthetize the bitch for intrauterine insemination by laparotomy.

The long vagina in the female dog requires an elongated endoscope. In addition, the cervix appears to exit the vagina at a right angle to the lumen and there is a blind cul-de-sac just cranial to the external vaginal os. Therefore, for TCI the cervix is directly visualized. A specialized 29 cm long cystoscope with a biopsy channel for the insemination catheter is used. The commonly used equipment is a rigid cysto-urethroscope from Storz. It includes a telescope, a sheath, bridge, xenon light source, and a video camera. Insemination timing (breeding management), semen handling and appropriate insemination technique are key factors for a success.

Pregnancy diagnosis

The three most commonly used pregnancy detection techniques include abdominal palpation, ultrasonography and radiography. Other less commonly used techniques are relaxin and other assays. How early after breeding these techniques can be used can be highly variable depending upon ovulation time, sperm survival (dog sperm can live up to ten days in the bitch's reproductive tract), etc.

Abdominal palpation

This method is relatively easy to learn, inexpensive and requires some experience to be reliable but can be difficult if the bitch is obese or her abdomen is tense. Reliable results are obtained during 20-30 days after breeding. Embryonic vesicles are felt as 'little marbles'. Before 20 days and after 30 days after breeding, it is difficult to be certain because uterus feels like a tubular structure full of uterine fluid, which could be confusing and may mimic pyometra. Other limitations of this technique include inability to assess the fetal health.

Ultrasonography (US)

This is an excellent technique to confirm pregnancy, determine embryo viability and diagnose abnormalities of the pregnancy. Familiarity with US equipment and the image interpretation is critical for correct diagnosis. Depending upon the time of fertilization, which is difficult to determine, this techniques reliably can be used 25 days after breeding. At this time, fetal heartbeat can be detected in most bitches. In the authors' study involving ultrasonography of six German Shepherd bitches during pregnancy the stage of pregnancy was determined retrospectively, counting back from whelping (63 days). Pregnancy duration is more reliable if based upon the LH surge which causes ovulation. Embryonic tissue and heartbeats were first detected between 23 and 27 days after the LH surge in 10 bitches. In the pregnant queen, we were able visualize fetal heartbeats on days 21-23 after breeding. Pregnancy duration variability in the queen is less than in the bitch because cats are induced ovulators and breeding takes place within few hours compared to many days in the bitch.

Radiography

This is the best technique for determining the number (litter size) of developing fetuses. The technique is useful after fetal mineralization, which takes place after 42 days after breeding. The limitations are the inability to assess fetal health and having to wait until last trimester of pregnancy.

Whelping

Pregnancy in the female dog lasts about 65 days, but predicting the timing of whelping can be difficult. A drop in rectal temperature (hypothermia) usually precedes delivery by about eight to 24 hours, which coincides with a decrease in blood P4. Labor and delivery in dogs is divided into three stages. Stage I lasts 12 to 24 hours. During this stage uterine contractions begin, but are not visible externally. The cervix also begins to dilate. During stage II, abdominal contractions can be seen and the puppies are delivered. The puppies are usually delivered at intervals of one to two hours, but that can vary considerably. Stage II can last up to 24 hours. Stage III is defined as the delivery of the placenta. Dogs typically alternate between stages II and III until delivery is complete.

Male breeding soundness examination

The purposes of semen collection and evaluation are to assess an animal for breeding soundness; to perform artificial insemination; to preserve semen; to attempt to localize the site of disorder in some infertile dogs; and to determine treatment response in infertile dogs. Collection of the prostatic fluid and submission for cytology and bacterial culture may help differentiate prostatic disorders from other problems. Semen evaluation may be performed before a dog is purchased, before he is first used for breeding, or if he shows signs of infertility. Semen collection and evaluation are relatively easy and can be done at any small animal practitice. The results should be recorded on the canine Breeding Soundness Examination (BSE) form, which was devised by the Society for Theriogenology.

Semen collection

Equipment. Semen is easily collected from the dog using an artificial vagina (AV), a device composed of a latex rubber cone (Reproduction Resources, Hebson, IL) connected to a 15 mL calibrated plastic centrifuge tube. The AV should be washed with plain tap water (no soap, detergent, or other cleansing agent is used), rinsed thoroughly with distilled water, and air dried. Gas sterilization, if available, may be used on the AV. Some dogs may ejaculate into other containers under the pressure of hand massage alone. However, semen is collected successfully from most dogs with an AV because the AV simulates the pressure normally felt by the male during copulation. The calibrated collection tubes used in the procedure should also be washed with tap water, rinsed with distilled water, and sterilized with gas or autoclaved before use to prevent the formation of formaldehyde residues which are harmful to sperm. For optimum results, collection should be done in a quiet room with the dog on a nonslip surface and with the owner present. Semen may be collected before other examinations are performed, including the physical examination. For better responsiveness, the semen can be collected in the presence of a teaser bitch in proestrus or estrus. If a bitch in estrus is not available, a commercially available pheromone (Eau d' Estrus, International Canine Genetics, Malvern, PA; or methylparaben; Sigma Chemical, Saint Louis, MO) or vaginal swabs from a disease-free estral bitch can be used. The top of the AV should be folded down, and a scant amount of lubricant should be applied to facilitate later removal of the device from the dog's erect penis.

Procedure. The male and female are brought on leashes into a collecting room and allowed to play for a few minutes. The semen collector, if right handed, kneels at the dog's left side and, holding the AV in the left hand, uses the right hand to move the prepuce up and down the penile shaft to achieve protrusion of the penis into the AV. (Left-handed collectors may reverse the sides described above.) As the male begins to thrust the penis into the AV, the collector moves the sheath above the engorging bulbus glandis, keeping firm, steady pressure around the circumference of the penis to maintain the erection. Most male dogs ejaculate the presperm (clear) and sperm-rich fractions (cloudy) of the semen during pelvic thrusting; they then dismount and lift one hind leg as though trying to step over the bitch and achieve the tie. At this point the penis should be rotated 180° so that it is directed caudally and held still with tight pressure maintained around the circumference until the third prostatic fraction of semen has been ejaculated. The prostatic fluid is ejaculated after a short pause in association with anal contractions and rhythmic pulsations of the penile urethra, which the collector can detect. After a few milliliters of prostatic fluid have been collected, the AV may be removed from the penis; the dog may

continue to ejaculate prostatic fluid for some time after removal of the AV. The dog should not be kenneled or sent home until the erection has subsided completely and the entire penis is back in the preputial sheath. Normally, this takes about five to ten0 minutes after semen collection.

Evaluation

Five parameters should be evaluated and the results recorded for each semen collection: color and volume of the sample, motility, concentration and total number of sperm, and morphology of the sperm.

Color. The color of the sample is observed grossly in the collection tube. The first fraction should be clear, whereas the second (sperm-rich) fraction should be milky white. The third fraction (prostatic fluid) should be clear. Yellow discoloration may indicate urine contamination or the presence of purulent exudate. A red or brown color may indicate prostatic disease, trauma, urethritis, or ulceration.

Volume. The volume of semen can be read directly from the calibrated collecting tube. Volume varies among breeds and among individual dogs. Semen volume is not an important parameter with regard to the BSE, but it is necessary for determining the number of sperm per ejaculate. The volume of the prostatic fluid need not be recorded because it varies according to the length of time prostatic fluid is collected.

*Sperm motility***.** Motility testing should be performed immediately after collection. The examiner places a drop of undiluted semen on a slide with a cover slip, examines the specimen at 100x to 200x magnification (because canine spermatozoa are resistant to cold shock, the slide need not be warmed), and estimates the percentage of sperm cells moving in a progressive and forward manner across the field. Although sperm motility estimation is not precise, it is a quick functional test.

Concentration/total number of sperm. The total number of sperm in the ejaculate is more important than the concentration of sperm per milliliter because the concentration changes dramatically depending on the amount of prostatic fluid collected. The sperm concentration can be measured with a commercial WBC Unopett system (Becton Dickinson, Rutherford, NJ). The semen sample is stirred gently, drawn up into the capillary pipette provided with the kit, and dispensed into the diluent-containing reservoir chamber. Diluted semen then is dispensed into both chambers of a Neubauer hemocytometer (Becton Dickinson) and the contents of the 1 mm central square (composed of 25 small squares) are counted on each side of the hemocytometer and averaged (the central square fills the field of a light microscope when the 10x objective is used). The number of spermatozoa in the central square is the concentration in millions of spermatozoa per milliliter of semen. The total number of sperm in the ejaculate equals the concentration multiplied by the volume. The normal total number of sperm ranges from 300 to 2000 x 10^6; large breed dogs ejaculate more sperm than small breed dogs (direct cell counts done with the hematocytometer are time-consuming; quicker methods used to determine the sperm concentration are an electronic blood cell counter or spectrophotometer.) The total number of sperm may be decreased in young, old, and inbred dogs.

Morphology. Sperm morphology is usually assessed by staining the semen sample and observing the sperm under oil immersion (1,000x) objective. Various stain techniques may cause some artifactual changes in sperm morphology. Defects vary with the stain used, but the percentage of morphologically normal sperm should be fairly consistent regardless of method. Eosin/nigrosin stain and Romanowski stain (Diff Quik) are the stains most commonly used. For staining with eosin-nigrosin, a drop of semen and a similar-sized drop of stain are placed on one end of a clean glass slide and mixed gently with a wooden applicator. A second slide then is used as a spreader slide. A thin film, similar to a blood smear, is made and air-dried. For staining with rapid Romanowski stain, the semen smear is made as for a blood smear, air-dried, and immersed in each of the three solutions for five minutes each. The slide then is rinsed and allowed to dry. One hundred sperm cells should be examined and the findings recorded, including normal sperm, primary abnormalities (all head defects, proximally coiled tails, proximal cytoplasmic droplets) and secondary abnormalities (detached heads, bent tails, and distal cytoplasmic droplets). Primary abnormalities are abnormalities that occur in the testis during spermatogenesis. Secondary abnormalities occur during epididymal transport or as a result of sample-handling techniques. Most dogs have greater than 80% normal sperm per ejaculation.

Conclusions

Inherited myopathies cause debilitating limb weakness and respiratory dysfunction in affected humans. Animal models provide platforms to study underlying disease mechanisms and test potential therapies. The clinical courses of spontaneous canine conditions, such as XLMTM, largely parallel those of the human disease. This makes the canine myopathies particularly attractive models for studying their human counterparts making tried-and-true breeding methodologies essential to experimental success. In the context of preclinical trials, particular attention must be paid to onset and progression of the disease. Studies in XLMTM dogs have provided insight on the efficacy of treatments intended for human patients and hold great promise for future investigation.

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Table 1. Inherited Canine Dystrophinopathies and Centronuclear Myopathies with Confirmed Mutations				
Disease	Breed	Gene/Mutation	Clinical Signs	Reference(s)
X-linked Dystrophinopathy	Golden retriever	*DMD* – Base change (A-G) in the 3' splice site of intron 6, with deletion of exon 7 in the mRNA transcript	Weakness and respiratory difficulty possible at birth; stunted growth; progressive weakness and contractures, especially over the ages of 3 to 6 months; respiratory and cardiac involvement.	Kornegay JN, et al. 2012; Sharp NJH, et al. 1992.
	German shorthaired pointer	*DMD* – Large deletion encompassing entire gene		Schatzberg SJ, et al. 1999.
	Pembroke Welsh corgi	*DMD* – repetitive element-1 (LINE-1) insertion in intron 13		Smith BF, et al. 2011.
	Cavalier King Charles spaniel	DMD – Base change (G-T) in the 5' splice site of intron 50, with deletion of exon 50 in the mRNA transcript		Walmsley GL, et al. 2010.
	Rottweiler	DMD – Base change (G-T) in exon 58 resulting in stop codon		Winand N, et al. 1994.
	Cocker spaniel	*DMD* – Deletion of four nucleotides in exon 65, resulting in stop codon		Kornegay et al. 2012.
	Tibetan terrier	*DMD* – Deletion of exons 8-29		Kornegay et al. 2012.
	Labrador retriever	*DMD* – 184 nucleotide [pseudoexon] insertion between exons 19 and 20, resulting in stop codon		Kornegay et al. 2012.
X Linked Myotubularin Myopathy	Labrador retriever	*MTM1* – Base change (C-A) in exon 7; p.N155K	Stunted growth; muscle atrophy, and pelvic limb weakness at 7 weeks. Patellar hyporeflexia, dysphagia, and hoarse bark; paradoxical respiration at 10 weeks. Progressive weakness and muscle atrophy, with loss of ambulation, by 4 to 6 months.	Beggs et al. 2010; Cosford et al. 2008
Hereditary (Centronuclear) Myopathy of Labradors	Labrador retriever	*PTPLA* – Short interspersed repeat element (SINE) insertion in exon 2	Abnormal head and neck posture; stiff, hopping gait; muscle atrophy; signs stabilize somewhat at 1 year.	Blot et al. 2002; Kramer et al. 1976; Maurer et al. 2012
Inherited (Centronuclear) Myopathy of Great Danes	Great Dane	*BIN1* – Base change (A-G) in the 3' splice site of intron 10, with deletion of exon 11 in the mRNA transcript	Weakness, muscle atrophy, exercise intolerance, trembling, and characteristic posture with the pelvic limbs held under the body. Onset of signs at 6 to 19 months (median of 7), with variable progression.	Böhm et al. 2013; Luján-Feliu-Pascual et al. 2006

Table 2. Centronuclear Myopathies					
Structural Features	Disease	Gene	Protein	Mammalian models	Reference(s)
Congenital Myopathies with central nuclei	Myotubular myopathy	*MTM1*	Myotubularin	*Mtm1* KO mouse R69C mouse XLMTM dog p.N155K XLMTM dog p.Q384P	Buj-Bello et al, 2002. Pierson et al, 2012. Beggs et al, 2010. Shelton et al, 2015
	Centronuclear myopathy	*DNM2*	Dynamin 2	R465W mouse	Durieux et al, 2010.
		PTPLA	Protein tyrosine phosphatase-like member A	PTPLA dog	Gentilini et al, 2011.
		BIN1	Amphiphysin	shRNA-Bin1 knockdown mouse IMGD dog	Tjondrokoesoemo et al, 2011. Bohm et al, 2013.
		RYR1	Ryanodine receptor	*Ryr1I4895T/wt* (IT/+) mouse	Zvaritch et al, 2009.
		TTN	Titin	Ttn(mdm) mouse	Garvey et al, 2002.