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Anatomical and mesoscopic characterization of the dystrophic diaphragm: An *in vivo* nuclear magnetic resonance imaging study in the Golden retriever muscular dystrophy dog

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Abstract

Because respiratory failure remains a major issue in Duchenne Muscular Dystrophy patients, respiratory muscles are a key target of systemic therapies. In the Golden Retriever Muscular Dystrophy (GRMD) dogs, the disease shows strong clinical and histological similarities with the human pathology, making it a valuable model for preclinical therapeutic trials. We report here the first nuclear magnetic resonance (NMR) imaging anatomical study of the diaphragm in GRMD dogs and healthy controls. Both T1- and T2-weighted images of the diaphragm of seven healthy and thirteen GRMD dogs, from 3 to 36 months of age, were acquired on a 3 tesla NMR scanner. Abnormalities of texture and shape were revealed and consisted of increases in signal intensity on T2-weighted images and in signal heterogeneity on both T1- and T2-weighted images of the dystrophic diaphragm. These abnormalities were associated with a significant thickening of the muscle and we identified a clear 8-mm-threshold distinguishing clinically preserved GRMD dogs from those more severely affected. In this study, we demonstrated the feasibility of NMR imaging of the diaphragm and depicted several anatomical and mesoscopic anomalies in the dystrophic diaphragm. NMR imaging of the diaphragm shows a promise as an outcome measure in preclinical trials using GRMD dogs.

Keywords: NMR; Imaging; MRI; Diaphragm; Dog; GRMD; Dystrophin

1. Introduction

In Duchenne Muscular Dystrophy (DMD) patients, dystrophin deficiency leads to progressive dysfunction and destruction of skeletal muscles, which affect the respiratory muscles as well. Finally, this results in a restrictive lung disease, whose management is of paramount importance for DMD patient survival [1,2]. The decrease of functional residual capacity and maximal inspiratory effort was correlated with an increase of the diaphragm thickness due to connective tissue accumulation [3]. Respiratory failure was the main cause of death in the second decade of life until late in the twentieth century. The improvement of respiratory function management (mechanical ventilation etc.) has prolonged life by fifteen years shifting the cause of death toward cardiac origins [4]. Mean age of death was reported to be 17.7 years and 27.9 years for patients without and with mechanical ventilation, respectively [5].

The diaphragm remains nevertheless a key target for therapeutics. For example, the improvement of diaphragm and other respiratory muscle function prevented the development of cardiomyopathy in mdx mice [6].

Respiratory function is a key issue in management of DMD patients and this is most frequently an argument for systemic therapies. Nevertheless, locoregional therapeutic approaches in the Golden Retriever Muscular Dystrophy (GRMD) dog, which is the animal model closest to the human disease, have demonstrated an increased number of dystrophin positive fibers in the diaphragm muscle [7]. However, in most of these studies, *in vivo* qualitative and quantitative evaluations of respiratory muscles, and particularly the diaphragm, were lacking. Histological evaluations or *ex vivo* force measurements were the most frequent endpoint outcome measures. The paucity of precise *in vivo* evaluation of the diaphragm in therapeutic trials

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can probably be explained by the current lack of sensitive tools for monitoring respiratory and diaphragmatic structure and function.

The existence of macroscopic and histological alterations of the diaphragm is well-known, both in DMD patients and animal models. The canine dystrophic diaphragm muscle is reported to be as much as three times thicker compared to healthy muscle, as early as 6 months of age [8,9]. Histological assays point out an increase in endomysial and perimysial fibrosis and mild to moderate perimysial adipose tissue infiltration, which all contribute to pseudo-hypertrophy of the diaphragm. In addition, large hypertrophied fibers are occasionally encountered and signs of regeneration are present [8,10]. Furthermore, light and electron microscopy reveal a tortuous and discontinuous architecture of muscle fibers and the presence of type I collagen fibers in GRMD diaphragm [11]. *In vivo* radiographic studies point out abnormalities of the shape of dystrophic diaphragm, including diaphragmatic undulation, hiatal hernia and asymmetry, with an emphasized flattening or displacement of the left crus compared to the right one [12]. Finally, once present, the flattening appears to be a persistent finding [13]. More recently, an attempt to further characterize the functional respiratory impairment in GRMD dogs was proposed [14]. The range of motion of the diaphragm and the branching angle of the diaphragm to the vertebrae could be indices that help quantify respiratory disorders. These results seem to be rather well correlated to arterial blood gas analysis, tidal breathing spirometry, and respiratory inductance plethysmography which revealed higher tidal breathing peak expiratory flows and abnormal abdominal breathing in GRMD dogs [15]. The diaphragm dysfunction has been related to a remodeling of the muscle associated to a loss of sarcomeres in the muscular part of the diaphragm, a thickening of the muscle and an increase of its collagen content [9]. However, the evolution of the thickening and texture of the diaphragm with the progression of the disease was not evaluated whereas a strong link has been established in human patients.

In DMD patients as well as in animal models, NMR imaging, including T1-weighted and T2-weighted imaging, and associated indices such as the signal heterogeneity of T2-weighted images, have proven its capacity to discriminate healthy muscles from dystrophic ones [16–23]. Even more interesting is that in pre-clinical trials using GRMD dogs, NMR imaging and ^{31}P NMR spectroscopy were able to distinguish dystrophin-expressing limb muscles from untreated ones [24–26].

In humans, diaphragm structure and function can be assessed by several methods including static or dynamic NMR imaging, with its multi-planar capacity and excellent soft tissue resolution [27–29]. This was illustrated in a recent report showing diaphragmatic abnormalities in Pompe patients using T1- and T2-weighted NMR imaging [30].

The lack of indices that characterize the severity of the diaphragm involvement in muscle dystrophy and enable the follow-up of this muscle in gene therapy trials prompted us to identify NMR imaging indices that describe the anatomical structure and texture of the dystrophic diaphragmatic muscle. We investigated the canine diaphragm because it is more severely affected than the murine one, making the dog model a better clinical model of Duchenne patient respiratory failure [9,31].

2. Materials and methods

2.1. Animals

Seven healthy dogs, aged 7–24 months, and thirteen dystrophin-deficient dogs, aged 3–36 months, from a GRMD colony, bred at the Ecole Nationale Vétérinaire d'Alfort, were scanned at a single time point at the NMR Laboratory of the Institute of Myology, Pitié-Salpêtrière University Hospital. GRMD dogs were diagnosed with high serum creatine kinase activity and DNA analysis as previously described [32]. All of them remained ambulant after the age of 6 months, meaning that according to the neuromuscular grading schema no so-called "severe form" phenotype was included in this study [33]. All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Animal Use and Care Committee of the Ecole Nationale Vétérinaire d'Alfort, with respect to European legislation regarding the use of laboratory animals (Agreement No. 20/12/12-18).

Clinical examination, chest X-ray and blood biochemical analysis including venous [HCO_3^-] measurement were performed the day prior to NMR imaging. A clinical score encompassing 17 items (respiratory, digestive and locomotor) graded from 0 (no abnormality) to 2 (worst situation) was performed as previously described in order to clinically stratify the dogs [17,33]. Dogs with aspiration bronchopneumonia or life threatening respiratory distress were not imaged.

Anesthesia was induced by intravenous injection of propofol (PropoFlo™, Abbott Animal Health) at a dose of 6.5 mg/kg and maintained throughout NMR experiments with a mixture of 2% isoflurane (Forene, Abbott) and oxygen delivered through an endotracheal tube. Heart rate and oxygen saturation were constantly monitored (Luxtron One, Luxtron; 8600FO Pulse oximeter, Nonin Medical, Inc.) and body temperature was maintained at 38.5 ± 0.5 °C by a heating/cooling waterbed. During anesthesia, dogs were infused with isotonic sodium chloride solution (B. Braun Medical SAS).

2.2. NMR imaging experiments

NMR imaging experiments were performed on a 3 tesla whole-body Tim TRIO scanner (Siemens Healthcare, Erlangen, Germany). Dogs were positioned in left lateral recumbency. The volume body coil was used for transmission and a set of "body matrix" and "spine" surface receiver coils covered the thoracic and abdominal regions of the dog.

A sagittal 3D tidal breathing SPACE (Sampling Perfection with Application optimized Contrasts) T1-weighted sequence was acquired on the thorax and abdomen of all dogs at a 1 or 1.1 mm isotropic resolution (TE = 14 ms; TR = from 350 to 417 ms; FOV: 38 × 38 × 14.4 cm^3; matrix size: 384 × 384 × 144; slice thickness = 1 mm; NA = 1, TA = 6 min 57 s).