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Arginine butyrate *per os* protects *mdx* mice against cardiomyopathy, kyphosis and changes in axonal excitability

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ABSTRACT

Duchenne muscular dystrophy (DMD) is a progressive neuromuscular disease caused by lack of dystrophin, a sub-sarcolemmal protein, which leads to dramatic muscle deterioration. We studied in *mdx* mice, the effects of oral administration of arginine butyrate (AB), a compound currently used for the treatment of sickle cell anemia in children, on cardiomyopathy, vertebral column deformation and electromyographic abnormalities.
Monthly follow-up by echocardiography from the 8th month to the 14th month showed that AB treatment protected the *mdx* mice against drastic reduction (20–23%) of ejection fraction and fractional shortening, and also against the ≈20% ventricular dilatation and 25% cardiac hypertrophy observed in saline-treated *mdx* mice. The phenotypic improvement was corroborated by the decrease in serum CK level and by better fatigue resistance. Moreover, AB treatment protected against the progressive spinal deformity observed in *mdx* mice, another similarity with DMD patients. The value of the kyphosis index in AB-treated mice reached 94% of the value in C57BL/10 mice. Finally, axonal excitability parameters such as the membrane resting potential, the threshold and amplitude of the action potential, the absolute and relative refractory periods and the supernormal and subnormal periods, recorded from caudal and plantar muscles in response to excitability tests, that were modified in saline-treated *mdx* mice were not significantly changed, compared with wild-type animals, in AB-treated *mdx* mice. All of these results suggest that AB could be a potential treatment for DMD patients.
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Introduction

DMD is a progressive neuromuscular disease affecting 1/3500 male at birth, caused by lack of dystrophin, the central component of a complex that links cytoskeletal actin to extracellular matrix, thus maintaining muscle fiber membrane integrity. Lack of dystrophin results in weakness of skeletal, smooth and cardiac muscle fibers, leading to dramatic muscle deterioration (Blake et al., 2002).

Although various curative therapeutic approaches, such as cell, gene and pharmacological therapies, are currently being investigated, they have limitations (Finkel, 2010; Goyenvalle and Davies, 2011; Meregalli et al., 2010; Pichavant et al., 2011). The problem of efficiently targeting all muscles (40% of the total body mass), including the diaphragm and heart, remains a challenge. Currently the only approved treatment is corticosteroids, which have limited benefits and a number of side effects, such as weight gain, mood changes, and a loss of bone mineral density.

Recently we successfully tested arginine butyrate (AB) (Vianello et al., 2013) and covalent derivatives (Vianello et al., 2014), which combines two pharmacological activities described as alleviating dystrophic phenotype in *mdx* mice and human DMD myotubes (Archer et al., 2006; Barton et al., 2005; Benabdellah et al., 2009; Brunelli et al., 2007; Chazalette et al., 2005; Colussi et al., 2010; Consalvi et al., 2013; Gurpur et al., 2009; Hnia et al., 2008; Marques et al., 2010; Sciorati et al., 2011; Ségalat et al., 2005; Thomas et al., 2012; Uaesoontrachoon

Abbreviations: AB, arginine butyrate; CK, creatine kinase; CMAP, compound muscle action potential; DMD, Duchenne muscular dystrophy; EF, ejection fraction; FS, fractional shortening; HDAC, histone deacetylase; IVS,s, interventricular septum, systole; KI, kyphotic index; LV, left ventricle; LVPW,s, left ventricular posterior wall, systole; LVID, left ventricular inner diameter; LVOD, left ventricular outer diameter; MRI, magnetic resonance imaging; NO, nitric oxide; TEd, depolarizing threshold electrotonus; TEh, hyperpolarizing threshold electrotonus.

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et al., 2014; Voisin et al., 2005): nitric oxide (NO)-pathway activation by arginine and histone deacetylase (HDAC) inhibition by butyrate. AB is currently used for the treatment of sickle cell anemia in children (Perrine, 2008). *In vivo* and *in vitro* experiments demonstrate that the drug combination acts synergistically. Intraperitoneal administration of AB to *mdx* mice resulted in a 2-fold increase in the level of utrophin correctly localized at the membrane. The improvement of the dystrophic phenotype in skeletal muscles and diaphragm was observed in adult and newborn mice (Vianello et al., 2013).

In the present work we focused our attention on three deficits described in DMD patients: cardiomyopathy, kyphosis and electromyographic abnormalities. Preclinical abnormalities are detectable by electrocardiography in 59% of patients between 6 and 10 years of age and progressively develop into clinically apparent cardiomyopathy in 100% of patients over 18 years of age. Cardiomyopathy begins by affecting particularly the posterobasal segment of the left ventricle (LV) (Goodwin and Muntoni, 2005) leading to a rise in wall stress and to excessive afterload (Colan, 2005). Progressively, systolic LV function decreases and myocardial oxygen consumption increases, leading finally to LV dilation dysfunction (Goodwin and Muntoni, 2005). *Post-mortem* examination of cardiac tissue shows that extreme fibrosis is the most characteristic histological feature of the disease (Moriuchi et al., 1993). Hearts of *mdx* mice share similar features, although the disease is less severe: no signs of cardiomyopathy are observed in young animals (6 weeks old), only slight differences are present in *mdx* mice at the age of 29 weeks, but at the age of 42/43 weeks cardiomyopathy is patent and electrocardiographic abnormalities, autonomic dysfunction, impaired conduction, arrhythmias and dilated cardiomyopathy are observed. Also, connective tissue accumulates in *mdx* hearts, suggesting that fibrosis is responsible for some features of the cardiomyopathy. (Au et al., 2011; Quinlan et al., 2004; Spurney et al., 2008).

In addition, DMD patients suffer from progressive spinal deformity (Oda et al., 1993). Kyphosis exacerbates respiratory dysfunction in affected boys and negatively impacts their quality of life, leading to surgery or chronic pain. The kyphotic index (KI) of *mdx* mice decreases progressively from month 9 to month 11 and then stabilizes (Laws and Hoey, 2004).

Finally, electromyography and nerve conduction studies have been performed in DMD patients. Although nerve conduction is normal in early DMD, the compound muscle action potential (CMAP), which idealizes the summation of a group of action potentials from several muscle fibers in the same area, decreases in amplitude as the disease progresses (Yiu and Kornberg, 2008). In *mdx* mice the absence of dystrophin in the postsynaptic membrane has little effect on the function of the neuromuscular junction, but the degeneration and regeneration of fibers lead to remodeling of the pre- and postsynaptic components (Banks et al., 2009; Hollingworth et al., 1990; Lyons and Slater, 1991; Nagel et al., 1990; Torres and Duchen, 1987).

We studied in *mdx* mice the effects of AB treatment on these changes by using an intermittent protocol (one week on/one week off) and force-feeding administration. The dose of AB used (800 mg/kg/d) was validated (by intraperitoneal injection) in our previous work (Vianello et al., 2013). A range of functional and structural approaches demonstrated that AB has a protective effect on progressive cardiomyopathy and on vertebral column deformation, as seen using two techniques (magnetic resonance imaging (MRI) and radioscopy). To assess whether the excitability properties of the neuromuscular system are affected when dystrophin is lacking, a multimodal evaluation of excitability was performed in *mdx* mice using a minimally invasive method that has been developed to supplement conventional electrophysiological methods and to investigate ion conductance and membrane properties in humans (Bostock et al., 1998), and then adapted to mouse models (Boerio et al., 2009): QTrack© protocol (software written by Prof. H. Bostock, Institute of Neurology, London, UK). The protocol includes different tests designed to measure more than 30 parameters related mainly to nerve excitability. All abnormalities detected in *mdx* mice were corrected by the treatment with AB.

Materials and methods

AB preparation

L-arginine and n-butyric acid (Sigma-Aldrich, Lyon, France) were prepared in a 26% stock solution (1 M arginine and 1 M butyrate, pH 7), aliquoted and stored at −20 °C. Weekly a tube was diluted in 0.9% NaCl, stored at +4 °C and used for ≈2 weeks.

Mouse experimental procedures

Experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals (EEC86/609 Council Directive, Decree 2001-131). The protocols were approved by the "Museum National d'Histoire Naturelle" Ethics Committee (68-024) and by the French Departmental Direction of Animal Protection (A91-453).

Adult male *mdx* and littermate wild-type (C57Bl/10) mice were 6 weeks of age at the start of experiments. The animals were weighed before each treatment administration. For the force-feeding procedure (1 mL/100 g body weight), flexible cannulas (PHYMEP, Paris, France) were used. The animals were assigned to the saline (0.9% NaCl) or treated group (AB at dose 800 mg/kg/day) in an intermittent protocol consisting of a series of 4 consecutive daily administrations every 2 weeks for 7 weeks or 14 months, depending on the parameters studied. All experiments were undertaken by an experimenter blind to treatment. Echocardiography was performed monthly from months 8 to 14, when KI was measured by X-ray and MRI. Neuromuscular excitability properties were recorded at week 13, after 7 weeks of treatment. At the end of the treatment, force (grip strength test) and fatigue (inverted grid test) were measured. The mice were then anesthetized with 55 mg/kg pentobarbital (CEVA Sante Animale), blood samples were taken from the heart for measurement of serum creatine kinase (CK) levels, the animals were euthanized by cervical dislocation and heart tissue was frozen for structural analysis.

Echocardiography

Recordings were performed using a high resolution Vevo 770 system, including a high-frequency (40 MHz) transducer (Visualsonics, Toronto, Canada). Mice were anesthetized by isoflurane (2–3% in oxygen). Heart rate and body temperature were monitored. Echographic studies were performed in the parasternal long- and short-axis views. Measurements were made off-line using the analytic software of the system (Visualsonics, Toronto, Canada). 2D and M-mode measurements were performed at the level of papillary muscles to obtain the left ventricular inner and outer diameters (LVID, LVOD) during systole and diastole. In addition, interventricular septum thickness (IVS,s) and left ventricular posterior wall thickness at systole (LVPW,s) were measured. Left ventricular systolic function was assessed by ejection fraction (EF) and fractional shortening (FS), which were calculated using M-mode, according to the formulas: EF (%) = 100 * (LV Vol,d − LV, Vol,s) / LV Vol,d and FS (%) = 100 * (LVID,d − LVID,s) / LVID,d. The average of at least three representative cardiac cycles was considered.

Magnetic resonance imaging

Mice were imaged under isoflurane anesthesia. Respiratory parameters were monitored and body temperature was maintained using a heated mattress. Measurements were performed using a 7-T horizontal bore magnet (Oxford, UK) driven by Paravision (Bruker, Wissembourg, France), equipped with a 300 mT/m actively shielded gradient device (internal diameter [ID] = 90 mm, Bruker). Animals were in a "birdcage" 1-H coil (ID = 63 mm). After scouting, gradient-echo imaging in the 3 orthogonal directions and the shimming process, the spinal cord and intervertebral spaces were explored using a 2D multi-slice Turbo Spin Echo Bruker MRI sequence (2D-TurboRARE).

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