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Effect of pyridostigmine on *in vivo* and *in vitro* respiratory muscle of mdx mice

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The current work was conducted to verify the contribution of neuromuscular transmission defects at the neuromuscular junction to Duchenne Muscular Dystrophy disease progression and respiratory dysfunction. We tested pyridostigmine and pyridostigmine encapsulated in liposomes (liposomal PYR), an acetylcholinesterase inhibitor to improve muscular contraction on respiratory muscle function in mdx mice at different ages. We evaluated *in vivo* with the whole-body pletysmography, the ventilatory response to hypercapnia, and measured *in vitro* diaphragm strength in each group. Compared to C57BL10 mice, only 17 and 22 month-old mdx presented blunted ventilatory response, under normocapnia and hypercapnia. Free pyridostigmine (1 mg/kg) was toxic to mdx mice, unlike liposomal PYR, which did not show any side effect, confirming that the encapsulation in liposomes is effective in reducing the toxic effects of this drug. Treatment with liposomal PYR, either acute or chronic, did not show any beneficial effect on respiratory function of this DMD experimental model. The encapsulation in liposomes is effective to abolish toxic effects of drugs.

1. Introduction

Duchenne Muscular Dystrophy (DMD) is a recessive hereditary disorder linked to X chromosome (Hoffman et al., 1987), resulting of the lack of dystrophin (427 kDa) expression in all muscles and neurons (Van Den Bergh et al., 1995; Mehler, 2000). Dystrophin is cytoskeleton protein that limits sarcolemmal mechanical stresses developed during repeated cycles of muscle contraction-relaxation (Petrof et al., 1993). Dystrophin deficiency causes progressive muscle damage resulting in necrosis, fibrosis and muscle strength loss (Hoffman et al., 1988). Consequently, patients present ambulation loss, motor skills and gradually develop cardiac and respiratory failure (McDonald et al., 1995). Respiratory failure is the most common cause of death in DMD patients (Mah et al., 2014). Diaphragm degeneration leads to lung compliance reduction, nocturnal hypoventilation, hypoxemia and hypercapnia (Smith et al., 1988; Smith et al., 1989; Toussaint et al., 2007) progressing to hypoventilation during wakefulness (Hukins and Hillman, 2000). DMD patients also exhibit inefficient cough, which consequently makes them more susceptible to pulmonary infections and airway obstruction (Szeinberg et al., 1988).

Few pharmacological treatments for DMD exist. Glucocorticoids

such as prednisone and deflazacort (Beytía et al., 2012) are the only one, which have some beneficial effects on cardiac and respiratory functions of patients (Eagle et al., 2007; Markham et al., 2008; Moxley et al., 2010). They however have also important adverse effects such as body weight changes, vertebral fractures, hypertension, immunosuppression and gastrointestinal problems (Beytía et al., 2012) that limit considerably their prescription.

Among natural rodent and canine animal models of DMD, the mdx mouse model is the most commonly studied (McGreevy et al., 2015). The diaphragm of mdx mice is the only muscle, which exhibits degeneration pattern, fibrosis and severe dysfunction similar to human patients (Stedman et al., 1991). Signs of diaphragm dysfunction appear early in 6–10 weeks young animals as indexed by a force generation decrease evaluated *in vitro* (Stedman et al., 1991). However, this *in vitro* parameter only represents sarcomeric contractile function of the diaphragm fiber. Respiratory system dysfunction caused in part by respiratory muscle damage, can be also assessed by measuring *in vivo* using a pletysmograph, ventilatory parameters [respiratory rate (RR), tidal volume (VT) and minute ventilation (MV) as well as integrity of hypoxic and hypercapnic-ventilatory reflexes (HVR and HCVR, respectively) (Gosselin et al., 2003; Matecki et al., 2005; Gayraud et al.,

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2007; Mosqueira et al., 2008; Mosqueira et al., 2013; Burns et al., 2015; Burns and O'Halloran, 2016). These parameters integrate breathing pattern control, electric signal transmission, ventilatory mechanics and respiratory muscle contractile properties, which represent more clinically relevant deficits to be considered as main end-point for evaluating the efficacy of various therapies for muscular dystrophy.

However, this non-invasive *in vivo* approach has a lower discriminative power than *in vitro* evaluation of maximal diaphragm strength. Indeed, only old mdx mice present a blunted ventilation response to hypercapnia (Gayraud et al., 2007) or hypoxia (Mosqueira et al., 2013; Burns et al., 2015; Burns and O'Halloran, 2016) compared to control mice. The evolution of *in vivo* respiratory system dysfunction with age in mdx mice is not yet fully described. This is however important because numerous factors may contribute to respiratory system dysfunction in DMD and mdx mice, which are not considered by *in vitro* assessment of diaphragm strength.

Among them, alterations at neuromuscular junction (NMJ) may participate to DMD disease progression in addition to intrinsic muscle contractile property deficit (Pijl et al., 2016). Indeed, dystrophic muscles have altered nicotinic acetylcholine receptor (nAChRs) distribution pattern at postsynaptic membrane of NMJ (Nagel et al., 1990; Lyons and Slater, 1991). Evidences indicate that dystrophin absence could cause neuromuscular transmission defects at NMJ (Personius and Sawyer, 2006). Some studies showed decrease of miniature endplate potentials, that corresponds to depolarizations resulting from spontaneously single acetylcholine (ACh) quanta released in dystrophic muscle (Nagel et al., 1990; Lyons and Slater, 1991; Carlson and Roshek, 2001; Pijl et al., 2016), indicating a possible reduction of post-synaptic sensitivity to ACh. It is unknown whether this reduction participate to muscular weakness in DMD and whether drugs known to potentiate the neurotransmission at the NMJ such as cholinesterase inhibitors may improve *in vivo* respiratory muscle function in mdx mice.

Pyridostigmine (Pyr) is an acetylcholinesterase (AChE) inhibitor used to improve muscular contraction for diseases with a deficit in neurotransmission such as Myasthenia gravis (Maggi and Mantegazza, 2011). AChE is an enzyme found at NMJ and cholinergic synapses responsible for ACh hydrolysis (Quinn, 1987). Pyr promotes increase of endogenous ACh with a beneficial effect on muscular tone and strength (Adler et al., 1992) and may be efficient to limit muscular weakness in DMD, additionally Pyr has anti-inflammatory (Rocha et al., 2016; De Cuba et al., 2014) and antifibrotic effect (Liu et al., 2015), which could be contribute to prevent or reduced diaphragm damage and consequently, improve muscle force on mdx mice. However, Pyr has a short half-life and, it is poorly absorbed from the gastrointestinal tract, which results in a low oral bioavailability (Aquilonius et al., 1980). Additionally, Pyr induces side effects and toxicity, due to its parasympathetic effects. Muscarinic side effects include diarrhea, nausea, hypersalivation, urinary incontinence, increased bronchial secretion, miosis, and lacrimation. Nicotinic side effects are muscle cramps, fasciculations, and weakness (Pope et al., 2005). Such side effects and weak bioavailability may be reduced by a different formulation, encapsulated in liposomes and injected intravenously (Vidal et al., 2010) or subcutaneously (S.C.) (Souza et al., 2013). Liposomes are lipid bilayer vesicles composed of an aqueous compartment able to encapsulate hydrophilic and hydrophobic substances in their internal water compartment or into the membrane, respectively (Bangham et al., 1974). Liposomes confer sustained drug release (Blume and Cevc, 1990), increase drug effects and/or reduce its toxicity (Lasic, 1998). We already demonstrated that liposomal Pyr formulation increases the duration of the Pyr cardioprotective effects and prevent cardiovascular disturbances resulting from sympathetic hyperactivity in Wistar rats (Vidal et al., 2010).

We hypothesized that neuromuscular transmission defects at NMJ participate to DMD disease progression. To verify this hypothesis, we evaluated whether Pyr treatment would improve *in vivo* respiratory muscle function due to its ability to increase ACh availability at

endplate. Furthermore, we hypothesized that if the cholinergic nicotinic receptors at endplate under long-term overstimulation could be increase *in vitro* muscle force. We also verified for future clinical application if Pyr availability could be optimized by testing the efficacy of Pyr encapsulated in liposomes.

Thus, the first aim was to verify the discriminative power of *in vivo* respiratory muscle function assessment, by plethysmography, between control and mdx mice at different stage of muscular disease to select the best age of mdx mice, to be treated with Pyr. The second aim was to evaluate in mdx mice at the selected age the effect of free Pyr and liposomal Pyr on *in vivo* respiratory muscle function. Moreover, to individualize the specific effect of Pyr on the diaphragm, *in vitro* studies were performed on isolated diaphragm fiber.

2. Material and methods

2.1. Drugs and reagents

Pyridostigmine bromide was purchased from Sigma Aldrich France. Distearoyl phosphatidylcholine (DOPC) and cholesterol (CHOL) were purchased from Lipoid GmbH (Ludwigshafen, Germany). The solvents were analytical grade and all other chemicals were commercially available. Water was purified by reverse osmosis (Simplicity System 185, Millipore, USA).

2.2. Liposomes preparation and characterization

For the free Pyr formulation pyridostigmine bromide was dissolved the salt in phosphate buffered saline. Liposomal Pyr formulation was prepared according to our previously described protocol (Souza et al., 2013). In summary, multilamellar liposomes were composed of DOPC and CHOL at a molar ratio 5:4. Pyr encapsulation was carried out by freeze-thaw method proposed by Nayar et al. (1989). Empty liposomes were prepared using only PBS (0.15 M NaCl, 0.01 M phosphate, pH 7.2). Analysis by UV spectrophotometry (270 nm; Model B582, São Paulo, Brazil) was performed to evaluate encapsulation efficiency as described by Souza et al., 2013; final concentration of pyridostigmine in liposomal formulation was about 6.5 mg/ml. Particle size, polydispersity index (PDI) and zeta potential of liposomes were determined using Zetasizer 3000 HS (Malvern Instruments, UK).

2.3. Animals

Male wild-type (C57BL/10ScSnJ) and mdx mice (C57BL/10ScSnDmdmdx/J) were purchased at 6 month-old from Jackson Laboratories, Bar Harbour, ME). Animal were housed conventionally in controlled humidity and temperature, operating on 12 h of light and dark cycle with food and water available *ad libitum*. All investigations conformed to the European Parliament Directive 2010/63/EU and were approved by the local ethics committee (Comité d'éthique pour l'expérimentation animale Languedoc-Roussillon, n° CEEA-LR-12078).

2.4. Plethysmography

As already performed in our lab (Gayraud et al., 2007; Matecki et al., 2005), the respiratory function in conscious and unrestrained mice was measured using whole body plethysmography and signal was analyzed by iox2 software (EMKA Technologies, Paris, France). All mice were randomly placed into individual chamber. After one hour stabilisation, each animal was visually monitored and ventilatory parameters were recorded during inactive phase for 15 min under two experimental conditions. First, each animal was submitted to 100% room airflow (air breathing). Second, each animal was exposed to hypercapnia with 8% CO₂-enriched airflow (8% CO₂ breathing) to challenge respiratory function.

Air volume changes corresponding to spontaneously breathing were

obtained using a pressure transducer (EMKA Technologies, Paris, France). This pressure signal was used to calculate the ventilatory parameters: tidal volume (TV), respiratory rate (RR) and minute volume (MV).

2.5. Diaphragm isometric contractile measurement

In vitro diaphragm contractility was measured as previously performed in our lab in mdx mice (Pauly et al., 2012). Mice were euthanized and diaphragm together with the ribs was surgically removed. Briefly, the diaphragm was dipped into a petri dish containing Krebs solution (NaCl, KCl, MgSO₄, KH₂PO₄, CaCl₂, NaHCO₃) oxygenated and incubated at 28 °C. A transverse strip muscle (2 mm) was isolated and connected to an isometric force transducer (Aurora Scientific Instruments, USA). The force–frequency relationship was determined by sequentially stimulating the muscles for 600 ms at 10, 20, 30, 50, 60, 80, 100, and 120 Hz with 2 min between each stimulation train. Forces were normalized by muscle strip cross-sectional area determined by dividing muscle weight by its length and tissue density (1.056 g/cm³) and expressed in Newton per square centimeter (N/cm²).

2.6. Experimental protocols

Firstly, respiratory system was evaluated *in vivo* using whole body plethysmography under air and 8% CO₂ in C57BL10 and mdx mice at different ages, 6 month-old (C57BL10, n = 12; mdx, n = 10), 17 month-old (C57BL10, n = 10; mdx, n = 17), 22 month-old (C57BL10, n = 3; mdx, n = 3).

Secondly, in a preliminary experiment, we tested the dose-dependent toxicity of free PYR in 17 month-old animals. Some common symptoms used to characterized autonomic nervous system toxicity are lacrimation, salivation, defecation and urination, caused by excessive secretory stimulation at parasympathetic end organs (Pope et al., 2005). After single dose injection of PYR, the presence or absence of these adverse effects was qualitatively observed, as well as mortality rate. C57BL10 mice and mdx mice, both 17 month-old were then treated, subcutaneously (S.C.), with 0.01, 0.1 or 1 mg/kg of free PYR. The highest doses without any adverse effect concerning autonomic signs of toxicity and mortality were selected for evaluated the respiratory function.

Then, 17 month-old mdx mice (n = 6) were treated with free PYR (0.1 mg/kg) and compared to C57BL10 mice (n = 9) similarly treated. To evaluate the efficacy of liposomal PYR formulation compared to free PYR treatment, a third group of mdx mice (n = 5) were treated with liposomal PYR (1 mg/kg) and compared to the mdx group (n = 6) treated with free PYR (0.1 mg/kg). We also evaluated liposomal PYR (5 mg/kg) (n = 5).

Finally, a group of 10 month-old mdx mice were treated during 30 days with liposomal PYR (5 mg/kg, n = 6) or empty liposome (n = 5). *In vivo* respiratory function was evaluated before and after treatment at 2, 6, and 24 h, and days 15 and 30 by plethysmography. At the end of the protocol, animals were euthanized by cervical dislocation for *in vitro* measurement of isometric diaphragm contractile properties as previously described (Matecki et al., 2016).

2.7. Statistical analysis

All parameters are expressed as mean ± S.E.M. Data were statistically compared using two-way ANOVA (age and disease) or One-way ANOVA with Bonferroni post-hoc test. Value of P < 0.05 was considered statistical significant for all tests.

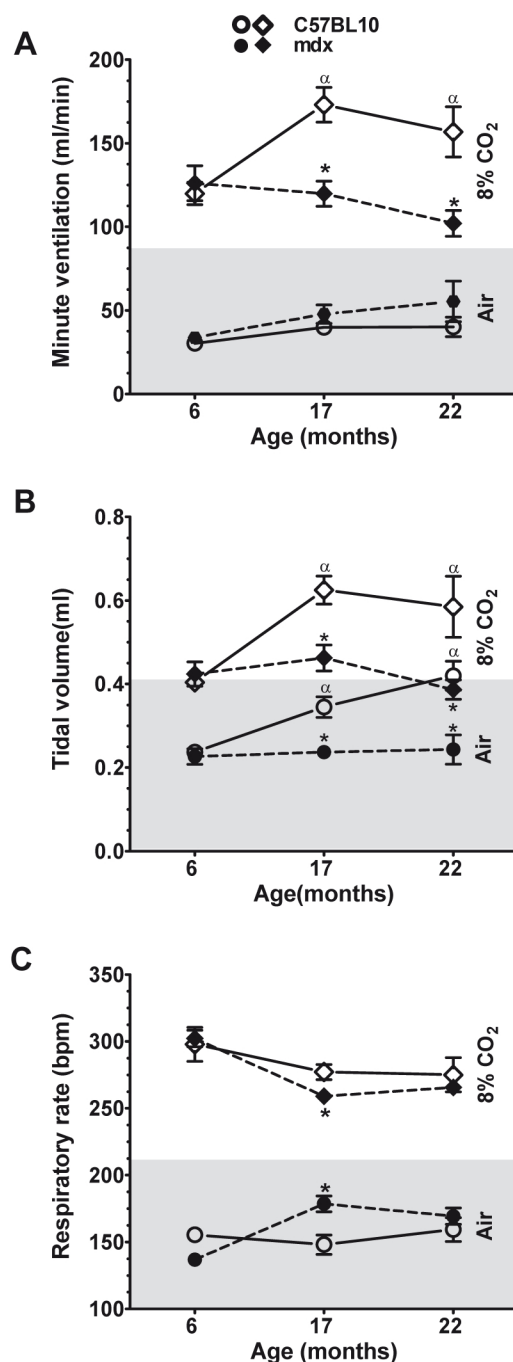


Fig. 1. *In vivo* characterization of respiratory function of C57BL10 and mdx mice by plethysmography at different ages. 6 month-old (C57BL10, n = 12; mdx, n = 10), 17 month-old (C57BL10, n = 10; mdx, n = 17), 22 month-old (C57BL10, n = 3; mdx, n = 3). C57BL10: air breathing, white circle; 8% CO₂, white diamond. Mdx: air breathing, black circle; 8% CO₂, black diamond. * P < 0.05: Significant difference between C57BL10 and mdx mice (ANOVA two-way). F values interaction (age x mutation) were 0.53, 6.00, 10.08, respectively for MV, TV and RR at air breathing and 6.53, 5.90, 1.21 for the same parameters at 8% CO₂. ^α P < 0.05: Significant difference compared to 6 month-old C57BL10 (ANOVA one-way). F values was 3.65, 14.08, 0.72, respectively for MV, TV and RR at air breathing and 9.88, 17.30, 1.24 for the same parameters at 8% CO₂.

3. Results

3.1. Respiratory function characterization of mdx mice

We tested the effect of age on respiratory function parameters in C57BL10 and mdx mice in normal conditions *i.e.* air breathing (Fig. 1). The respiratory function has been also evaluated under reduced O₂

environment (8% CO₂). Experiments have been performed in adult (6-month old) and old animals (17-month old). Very old animals of 22 month-old have been tested to test whether the respiratory declines dramatically at the end life of the mice. At 6 month-old mdx and C57BL10 mice had similar ventilatory response to both normocapnia (air breathing) and hypercapnia (8% CO₂) conditions (Fig. 1).

Under normocapnia condition, 17 and 22 month-old mdx mice present similar MV to age related C57BL10 mice (Fig. 1A) but with a different breathing pattern. Indeed, compared to age related control mice, the 17 and 22 month-old mdx mice present a reduced TV ($p < 0.001$; $p < 0.01$, respectively) (Fig. 1B) associated with a significant increase of RR ($p = 0.0003$) only for 17 month old mdx mice (Fig. 1C). The 22 month-old mdx mice presented similar RR to control mice.

Under hypercapnia, 17 and 22 month-old mdx mice had a reduced MV compared to age related C57BL10 mice ($p < 0.01$) which is underlined, compared to control mice, by a decrease of TV ($p < 0.01$) for both mdx mice groups, and RR ($p < 0.01$) only for 17 month-old mdx mice. The 22 month-old mdx mice presented similar RR than control mice.

Thus, these first results show the presence of an age dependent alteration of respiratory function, mostly characterized by a lower TV under normocapnia and hypercapnia, and MV only under hypercapnia in both 17 and 22 month-old mdx mice. Those 3 parameters were chosen as endpoint to evaluate the effect of PYR treatment on *in vivo* respiratory function.

3.2. Determination of nontoxic PYR dose

No toxic effect was observed in C57BL10 mice treated with 0.01 mg/kg ($n = 10$), 0.1 mg/kg ($n = 9$), and 1 mg/kg ($n = 7$) of free PYR. On the contrary, free PYR was toxic and lethal in mdx mice just after the injection. Two mdx 17 month-old animals died soon after 0.01 mg/kg free PYR ($n = 10$), one mdx with 0.1 mg/kg ($n = 9$) and all mdx mice treated with free PYR 1 mg/kg ($n = 6$). For this last dose, tremor, salivation, defecation, and sweating, preceding death were observed. We considered that acetylcholinesterase inhibition with free PYR was mortal for mdx mice that already presented cardiac failure and respiratory dysfunction at that age. Considering these results, we selected the dose 0.1 mg/kg as the safest dose to perform *in vivo* tests with free PYR.

To test the beneficial effect of liposomal formulation, we first tested liposomal PYR at 1 mg/kg that was lethal concentration for mdx mice when administrated free. Liposomal PYR did not induce any adverse effects or death at 1 mg/kg ($n = 6$). A higher dose of liposomal PYR was tested at 5 mg/kg. This 5 mg/kg dose administrated free PYR is known to cause muscarinic side effects in Sprague-Dawley rats (Richtsfeld et al., 2013). Liposomal PYR at 5 mg/kg did not induce any adverse effects or death ($n = 5$) to mdx mice or C57BL10 mice.

3.3. Effect of PYR single-dose on 17 month-old mice *in vivo* respiratory function

We observed, as showed in Fig. 2A and 2B, that free PYR (0.1 mg/kg) has no effect on the 3 main endpoints. Indeed, in 17 month-old mice, one and two hours after free PYR single-dose compared to baseline, under normo and hypercapnia, TV did not significantly increase, neither MV under hypercapnic condition.

We did not observe also any effect in RR under normo and hypercapnic conditions, neither MV under hypercapnic solution between baseline and one or two hours after free PYR injection. This was an expected result considering that these 3 parameters were not discriminant (Fig. 2C).

Considering that, free PYR is rapidly hydrolyzed and that the dose 1 mg/kg was toxic for mdx mice, we tested the effect of a single dose injection of its liposomal formulation at 1 mg/kg in 17 month-old mdx

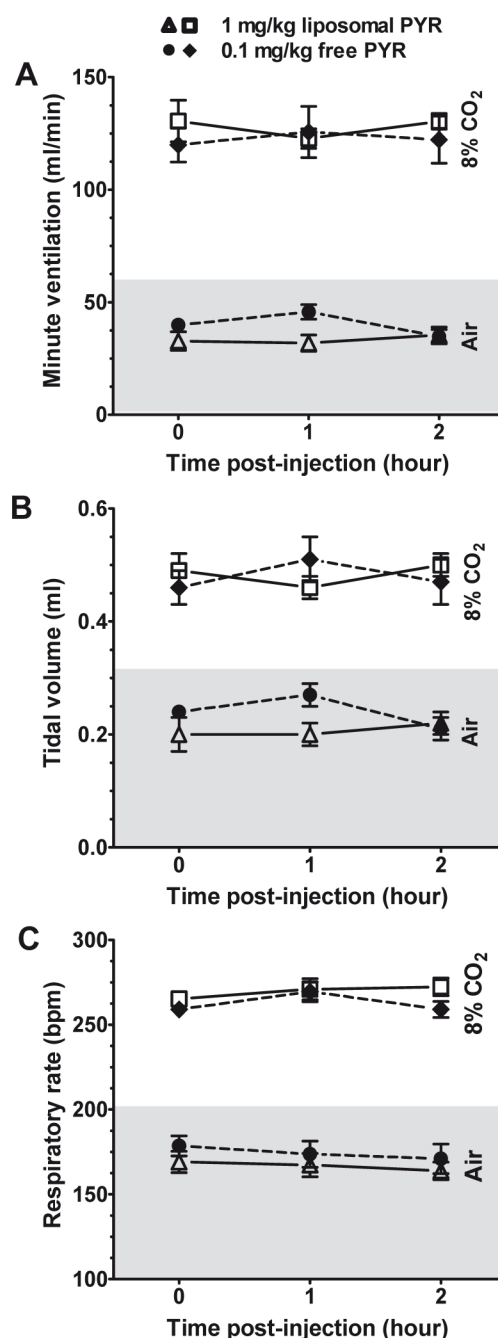


Fig. 2. Ventilatory parameters in response to air and 8% CO₂ breathing of 17 month old mdx mice treated with S.C. single dose of free PYR and liposomal PYR. 0.1 mg/kg free PYR ($n = 6$): air breathing, black circle; 8% CO₂, black diamond. 1 mg/kg liposomal PYR ($n = 5$): air breathing, white triangle; 8% CO₂, white square. One way-ANOVA. F values for dose comparison: 8.68, 2.0, 1.68 for MV, TV, RR, respectively at air breathing and 6.69, 3.55, 2.12 for the same parameters at 8% CO₂.

Table 1

Physicochemical parameters of liposomal PYR formulation (mean \pm SD).

	EE (%)	Particle size (nm)	PDI	Zeta potential (mV)
Liposomal PYR	17.5 \pm 4.8	2556 \pm 180	0.49 \pm 0.03	-5.0 \pm 3.7
Empty liposome	-	1194 \pm 72	0.7 \pm 0.42	-1.0 \pm 0.2

PDI: Polydispersity index; EE: Encapsulation efficiency.

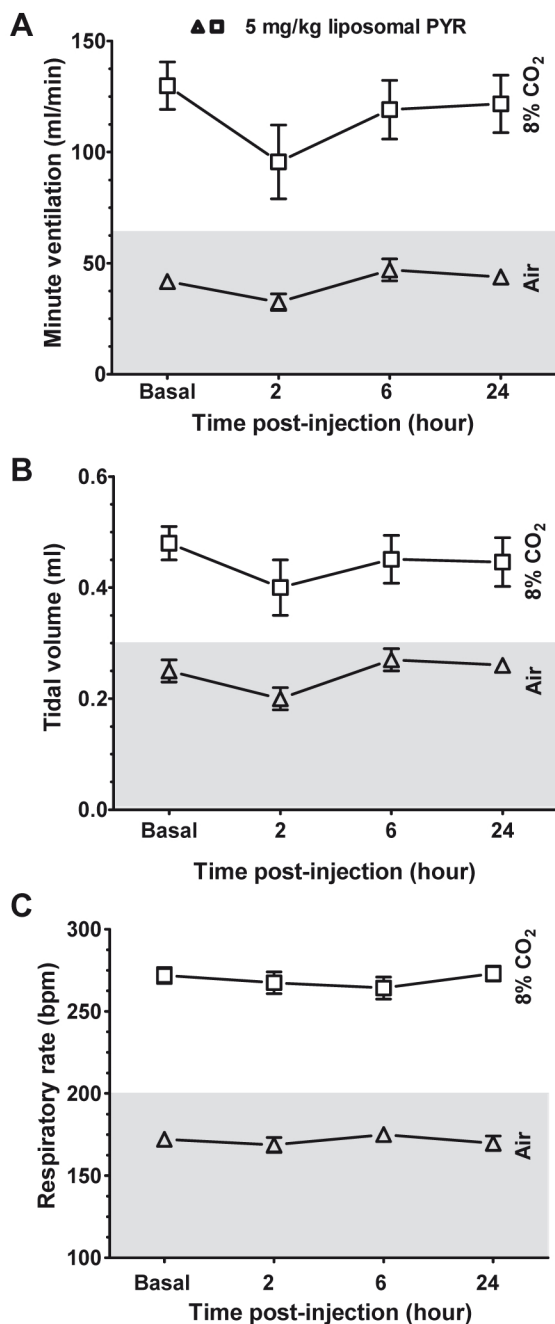


Fig. 3. Ventilatory parameters in response to air and 8% CO₂ breathing of 17 month-old mdx mice treated with S.C. single dose of liposomal PYR 5 mg/kg. Air breathing, (white triangle), 8% CO₂ (white square), n = 5. One way-ANOVA. F values for dose comparison: 10.34, 11.97, 3.79 respectively for MV, TV, RR at air breathing and 4.30, 8.24, 3.90 for the same parameters at 8% CO₂.

mice.

Physiochemical characteristics of liposomal PYR formulation were determined before *in vivo* use (Table 1). A liposomal formulation for subcutaneous administration was developed to permit a gradual release of the drug into systemic circulation (Souza et al., 2013). PDI determination (superior to 0.3) was important to demonstrate that it was obtained multilamellar liposomal PYR with large and polydisperse mean diameter. Formulation exhibited a negative zeta potential and PYR encapsulation efficiency in liposomes was similar to that described by Souza et al. (2013). Liposomal PYR formulation presents appropriate physiochemical characteristics for S.C. administration.

Similarly to free PYR treatment, none of the respiratory parameters

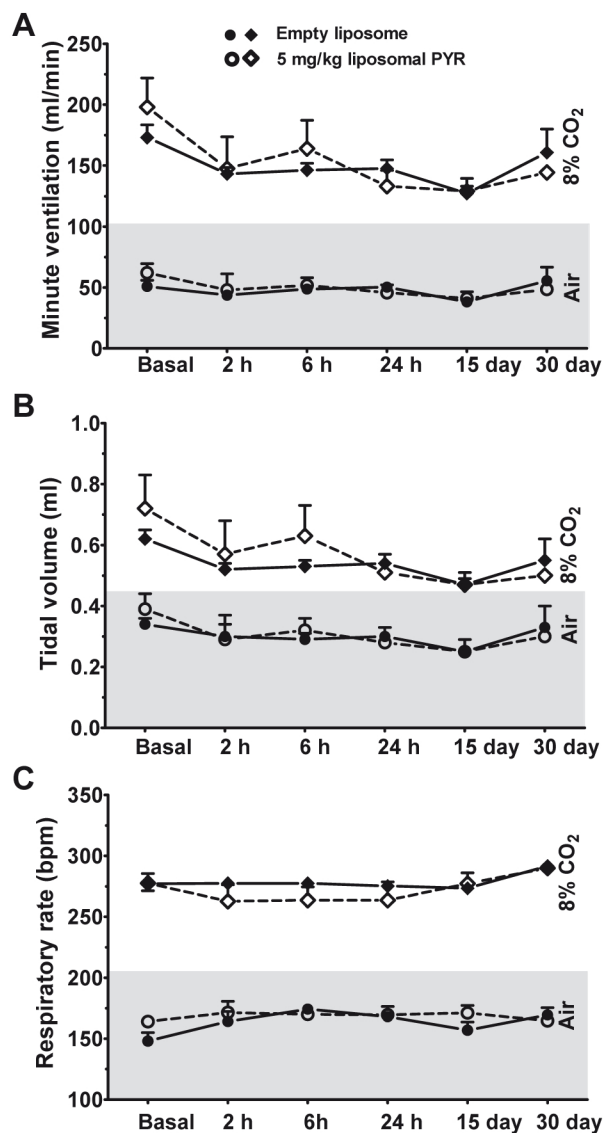


Fig. 4. Ventilatory parameters in response to air and 8% CO₂ breathing of 10 month-old mdx mice after 30 days of S.C. treatment with liposomal PYR. Empty liposome, n = 5 (air breathing, black circle; 8% CO₂, black diamond). 5 mg/kg liposomal PYR, n = 6 (air breathing, white circle; 8% CO₂, white diamond). One way-ANOVA. F values for dose comparison: 0.83, 0.63, 0.67 respectively for MV, TV, RR at air breathing and 1.47, 1.25, 2.65 for the same parameters at 8% CO₂.

were affected by a single dose injection of 1 mg/kg liposomal PYR (Fig. 2). Then, we tested a highest dose of liposomal PYR (5 mg/kg), but this dose had no effect either (Fig. 3).

3.4. Effect of liposomal PYR repeated doses on 10 month-old mdx mice in vivo and in vitro respiratory function

None of respiratory parameters were affected after first dose or 15 and 30 days of treatment of liposomal PYR (5 mg/kg) or liposome empty injection compared to basal values ($p > 0.05$) (Fig. 4). No difference was observed between the liposomal PYR or liposome empty groups.

The same treatment did not improve the diaphragm contractile force at any stimulation frequency used, since there was no difference between treated compared to non-treated groups ($p > 0.05$) (Fig. 5).

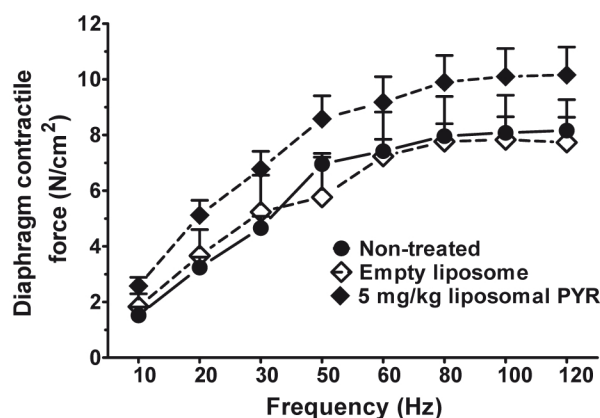


Fig. 5. Diaphragm contractile specific force-frequency relationships of 11 month-old mdx mice after 30 days of S.C. treatment with liposomal PYR. Non-treated mdx, $n = 5$ (black circle). Empty liposome, $n = 3$ (white diamond). 5 mg/kg liposomal PYR, $n = 6$ (black diamond). Two-way ANOVA, followed by Bonferroni post-test. F values for treatment 14.93 and frequency 27.22.

4. Discussion

In this study, using both *in vivo* and *in vitro* techniques, we demonstrated that PYR injection did not have any effect on the respiratory system and diaphragm contractile function in mdx mice.

Although we observed that mdx mice have *in vivo* normal respiratory function at 6 month-old, at this age, their diaphragm present already significant histological muscle fiber degeneration and loss of contractile force evaluated *in vitro* (Steldman et al., 1991). Those results are consistent with previous study showing that their respiratory intercostal muscles, which are less affected may compensate the diaphragm weakness to prevent respiratory system impairment evaluated *in vivo* (Gayraud et al., 2007). However, with the muscular disease progression, which affects all the muscle including intercostal muscles, we observed in older mdx animals (17 and 22 month-old) a blunted ventilatory pattern under normo and hypercapnia, compare to age related control mice.

In both conditions, due to the metabolic control of breathing, when carbon dioxide accumulate in the blood, MV increase to reestablish its baseline level, by increasing VT (Mosqueira et al., 2013). The decreased VT observed in both conditions with our 17 and 22 month-old mdx mice, is the result of an increase muscular weakness due to age related disease progression. To prevent a MV decrease and thus maintain blood carbon dioxide level homeostasis, 17 month-old mdx mice are able to increase at rest their RR to compensate their low VT due to respiratory muscle contractile defect. This particular breathing pattern, so called rapid shallow breathing have been clearly observed in DMD patient, due to an increase of respiratory system elastic load associated to presence of respiratory muscle weakness (Misuri et al., 2000). Indeed, the breathing pattern generator is predominantly influenced by ventilatory mechanics change to propose the best economical breathing pattern minimizing the work of breathing necessary to maintain a constant level of carbon dioxide (Younes et al., 1988).

Interestingly, with disease progression, under air, this compensatory mechanism is not any more efficient in 22 month-old mdx mice, which present the same level of RR compared to age related control mice. Indeed, considering their constant decline in VT with age, which decreased the ventilatory efficiency, 22 month old mdx mice, to correctly compensate their low VT should have increase their MV and their RR, with higher value compared to age related control mice.

Those defects of compensatory mechanism are accentuated under hypercapnic condition and thus can be detected earlier. Indeed, in our 17 month mdx mice, VT is lower compared to age related control mice, but on contrary to air ambient, we also observed a lower MV due to their inaptitude to sufficiently increase their RR which is lower than

control group.

The response to hypercapnia is a result of both muscular strength of respiratory muscles and sensitivity of the respiratory chemoreceptors to CO_2 and O_2 pressure variations. In mdx mice, the peripheral chemoreceptors located in carotid body have less sensitivity to O_2 pressure decrease (Mosqueira et al., 2013), which contributes, at least partially, to alterations observed on mdx mice. However, considering that carotid body are not mainly responsible for ventilatory response to carbon dioxide (Pan et al., 1995; Bernards et al., 1966; Coles et al., 2002; Gautier and Bonora, 1979; Dejours, 1963), and our 6 month-old mdx mice presented similar ventilatory response than age related control mice, we could consider that the difference in ventilatory response we observed was mainly due to muscular disease progression.

In this study, we wanted to ascertain if functional deficit at NMJs may aggravate muscle dysfunction and participate in this progressive degeneration of muscle fiber which is the main cause of muscle weakness in patients with DMD. ACh augmentation at muscle endplate of Myasthenia gravis muscle with PYR, which present some similarities with dystrophin deficient muscle, improve contractile function (Maggi and Mantegazza, 2011). Indeed, dystrophin is important to maturation and stabilisation of nAChR at muscle (Kong and Anderson, 1999; Grady et al., 2000; Jacobson et al., 2001). Dystrophic muscles have reduced density of nAChR at postsynaptic membrane of motor endplates (Nagel et al., 1990), increased degradation rates of nAChR (Xu and Salpeter, 1997) and upregulation of extrajunctional nAChR (Ghedini et al., 2008). Mdx mice present neuromuscular synaptic dysfunction, characterized by reduction of postsynaptic neurotransmitter sensitivity, demonstrated by reduced MEPP amplitudes (Nagel et al., 1990; Pijl et al., 2016), increased presynaptic ACh release, probably as a compensatory effect (Carlson et al., 2001).

Thus, we firstly hypothesized that treatment with free PYR could improve respiratory function and contractile force of diaphragm mdx mice, due to its ability to increase the availability of ACh at muscle endplate of 17 month old mdx mice.

We choose this age, because at this level of muscular disease progression, all parameters of ventilatory response to hypercapnia was able to discriminate from age related control mice.

However, the effect of (0.1 mg/kg) free PYR on 17 month-old mdx mice respiratory function measured by whole body plethysmography method was negative. Indeed, we did not observe any modification of MV, VT and RR, in both condition (ambient air and 8% CO_2) between baseline and 1 or 2 h after injection free PYR injection.

To ascertain that this negative effect was not simply due to an insufficient level of free PYR administration, we firstly tested higher dose (1 mg/kg), which induced mdx mice death, observed after S.C. administration. However, similar dose in control age related mice was never toxic. Thus, this observation strongly indicates that mdx mice are more sensitive to ACh overload than C57BL10 mice. The death probably was due to the overstimulation of muscarinic receptors. At respiratory tract, ACh has effects on bronchomotor tone and mucus secretion from airway submucosal glands regulation (Colebatch and Halmagyi, 1963; Baker et al., 1985). Thus, the possible causes of mdx mice death could be bronchoconstriction and/or increase of mucus secretion (Taylor, 2001), which may aggravate respiratory dysfunction already present in mdx mice. Though, any alterations of number, morphology or function of muscarinic receptors of mdx mice airways are known.

Multilamellar liposomes of PYR was developed for S.C. administration since it is more comfortable than intravenous route, generally used for unilamellar liposomes (Vidal et al., 2010). Liposomal PYR is safer than free PYR because PYR encapsulated in liposomes is gradually and sustainably release into the blood compartment, reducing the adverse effects and prolonging its effect, as demonstrated for the PYR cardioprotective effect when intravenously administered (Vidal et al., 2010).

Thus, to decrease side effect of higher dose of PYR and increase the

duration of delivery and action we tested the effect PYR liposomal formulation at 1 mg/kg single dose on 17 month-old mdx mice respiratory system. Indeed, considering that no side effect was observed in mdx treated with this dose, we hypothesized a potential benefit effect for this formulation on muscular function. However, single dose of liposomal PYR did not have any beneficial effect on respiratory function of 17 month-old mdx mice.

Finally, to evaluate a chronic increase of endogenous acetylcholine at NMJs which may have a better effect on muscular function compared to single dose, we finally, in a group of mdx mice of 10 month-old, injected a daily liposomal PYR 5 mg/kg dose or with empty liposomes during 30 days. As showed in Fig. 3, none of the ventilatory parameters were affected by 1, 15 and 30 days of treatment of PYR or empty liposomal injection compared to basal values. At the end of the protocol, contractile properties in isometric condition was determined, and no difference was observed between groups (Fig. 5).

Taking together, these results showed that increase of ACh concentrations is not sufficient to improve respiratory muscle function. These negative results could be due to advanced degree of muscle degeneration present in old mdx mice (Steldman et al., 1991. Gayraud et al., 2007). Future *in vitro* diaphragm contractile evaluation should be carried out with higher doses of liposomal PYR on younger mdx mice and the electrical activity of the membrane should be measures to ascertain that neuromuscular transmission defects at NMJ may not participate to DMD disease progression.

Moreover, although no benefit was observed on respiratory function, the total absence of adverse effects observed qualitatively and no death even after higher dose of PYR (5 mg/kg) indicate its potential for further therapeutic evaluation and even for mechanism studies on mdx mice.

Despite the result on the contractility of the diaphragm not being positive, the effect of PYR on muscle inflammation should be evaluated in future study. A recent study demonstrated that activation of nicotinic ACh receptors can reduce inflammation of muscle cells in mdx mice (Leite et al., 2014). Furthermore, the excitatory effect on skeletal muscle, ACh released from the vagal synaptic endings inhibits the secretion of TNF- α , IL-1 β , and a host of other inflammatory mediators by macrophages (Tracey, 2002). The PYR anti-inflammatory action has been demonstrated in other species, as in rats subjected to ischaemic heart disease, when increased cholinergic modulation by PYR induced recruitment of anti-inflammatory cells (Rocha et al., 2016).

In summary, we can conclude that free PYR (1 mg/kg) was toxic to mdx mice, unlike liposomal PYR, which did not show any side effect, confirming that the encapsulation in liposomes is effective in reducing the toxic effects of this drug. Treatment with liposomal PYR, either acute or chronic, did not show any beneficial effect on respiratory function of these DMD experimental model.

Conflict of interest

The authors declare no potential conflict of interest.

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