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Redox balance following magnetic stimulation training in the quadriceps of patients with severe COPD

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Abstract
In severe COPD patients, oxidative stress, which is involved in their peripheral muscle dysfunction, increases in response to exercise. In this study, muscle oxidative stress was explored after quadriceps magnetic stimulation training. A randomized controlled study was conducted on very severe COPD patients, who underwent quadriceps magnetic stimulation training for 8 weeks. A control group was also studied. In both groups, *vastus lateralis* specimens were obtained before and after the 8-week period. Muscle protein carbonylation and nitration and antioxidant enzymes were determined using immunoblotting and proportions and sizes of type I and II fibres using immunohistochemistry. Compared to controls, magnetic stimulation muscle training did not modify redox balance, whilst inducing a significant increase in type I fibre sizes. In severe COPD patients, it is concluded that quadriceps magnetic stimulation training was a well-tolerated therapeutic intervention, which did not enhance muscle oxidative stress, while increasing the size of slow-twitch fibres.

Keywords: *Severe COPD, quadriceps magnetic stimulation training, oxidative stress, muscle structure.*

Introduction

Muscle dysfunction is a common systemic manifestation in patients with chronic obstructive pulmonary disease (COPD), leading to reduced exercise capacity, poor quality of life and increased mortality [1,2]. Exercise training of high intensity is currently an essential component of pulmonary rehabilitation programme s of COPD patients [3]. A previous study by our group [4], however, demonstrated that dogs subjected to high-intensity inspiratory loads for 2 weeks showed a significant increase in oxidant-induced protein modifications in their diaphragms, which was neutralized by concomitant treatment of the animals with the antioxidant N-acetyl-cysteine. In COPD patients, antioxidant therapy also attenuated exercise-induced muscle oxidative stress, partially restoring muscle function [5–7]. Indeed, oxidative stress, among other factors, is now considered to be a major player in the peripheral muscle dysfunction of COPD patients, both at rest and after exercise [5–11].

In patients with severe COPD and poor exercise capacity, mainly because of limited cardiopulmonary reserve, participation and compliance with exercise

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training programmes may be rather difficult. Interestingly, direct stimulation of the muscle nerves through externally applied electrical currents using electrical muscle stimulation was shown to have beneficial effects on muscle strength and performance in patients with disabling conditions such as major knee ligament injuries [12], prolonged immobilization periods after surgery [12], paraplegia [13], peripheral vascular disease [14] and refractory heart failure [15]. Moreover, a 6-week training period using neuromuscular electrical stimulation of the quadriceps muscle improved muscle function and exercise tolerance in severe COPD patients with incapacitating dyspnea and very limited exercise capacity [16]. Despite these advantages, the use of neuromuscular electrical stimulation for training purposes has some important drawbacks, since it induces skin pain with increasing intensities and is of poor reproducibility [17]. Alternatively, nerve stimulation can be ensured by means of magnetic stimulation, which does not induce skin pain and is more reproducible. Indeed, it has lately been proposed as an appropriate tool to be used in clinical settings for training purposes [17,18].

It remains, however, to be elucidated whether repetitive bouts of magnetic stimulation of the quadriceps muscle for several weeks enhances muscle oxidative stress in patients with severe COPD, who already exhibit increased oxidative stress in their peripheral muscles under resting conditions [9,10]. Accordingly, we present here a study in which the effects of reactive oxygen and nitrogen species (ROS and RNS, respectively) on muscle proteins and antioxidants were explored in the *vastus lateralis* of patients with severe COPD both before and after an 8-week training programme of magnetic stimulation of the quadriceps. Moreover, muscle fibre type composition was also determined in these patients.

Methods

Patients

Fifteen male patients with stable severe COPD were recruited on an out-patient basis. All individuals were Caucasian and were simultaneously participating in the project of the European Network for Investigating the Global Mechanisms of Muscle Abnormalities (ENIGMA) in COPD, specifically designed to investigate the mechanisms involved in muscle dysfunction in COPD. It is worth mentioning that none of the data shown in the current study has been used for the purposes of other studies. COPD diagnosis and classification was established on the basis of the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines [19]. All the patients were exclusively on inhaled medication (long acting-beta$_2$ agonists, anticholinergics and low-dose inhaled corticosteroids). Exclusion criteria included chronic respiratory failure, bronchial asthma, cardiovascular disease, limiting osteoarticular condition, chronic metabolic diseases, suspected para-neoplastic or myopathic syndromes and/or treatment with drugs known to alter muscle structure and/or function including oral corticosteroids. The Ethics Committees on Human Investigation at Cruces Hospital (Barakaldo, Basque Country, Spain) approved all experiments. Informed written consent was obtained from all patients.

Study design

This is a prospective randomized controlled study designed in accordance with both the ethical standards on human experimentation in our institutions and the World Medical Association guidelines (Helsinki Declaration of 1975, as revised in 1983) for research on human beings. All patients were randomized using a table of randomized numbers. Patients randomized to group treatment ($n = 10$) received magnetic stimulation for 8 weeks, while patients randomized to the control group ($n = 5$) were clinically followed for an 8-week control period. The follow-up of the control patients was supervised by the investigators responsible for the study at Cruces Hospital, consisting of three visits: one hospital appointment and two telephone calls conducted on a weekly basis throughout the 8-week period. All patients were strongly recommended to maintain their regular daily-life activities throughout the entire duration of the study. Both groups of patients were evaluated at baseline and after the magnetic stimulation or control periods and muscle specimens from the *vastus lateralis* were also obtained at these two time-points in both groups of patients.

Nutritional and functional assessment

Nutritional evaluation included body mass index (BMI) and determination of the fat-free mass index (FFMI) by bioelectrical impedance [20]. Forced spirometry was performed using standard procedures and reference values by Roca et al. [21] were used. General exercise capacity was assessed using the standard 6-min walking distance test [22].

Quadriceps muscle biopsies

Muscle samples from the *vastus lateralis* were always obtained from the middle portion of the thigh using surgical biopsy procedures (open biopsy technique). Biopsy sites were anaesthetized previously with 2% lidocaine and 1–1.5 cm skin incisions were made in each case. Muscle samples, 20–30 mg size on average, were all obtained below the *vastus lateralis* fascia through the same incision. The side for the baseline biopsy was chosen at random and the second biopsy was always obtained from the same anatomical site but in the contralateral *vastus lateralis* [23]. Muscle

specimens were immediately frozen in liquid nitrogen and stored at −80°C for further analysis or immersed in an alcohol-formol bath for 2 h to be thereafter embedded in paraffin.

Magnetic stimulation training protocol

After the initial evaluation, patients were randomized into two groups. In the trained patients, the quadriceps muscle was magnetically stimulated using a Medtronic Magpro electromagnet (Medtronic Denmark A/S, Copenhagen, Denmark) provided with a 60 mm refrigerated MCF 125 circular stimulating coil. In these patients, quadriceps muscles were stimulated in both lower limbs. The coil head was positioned on the superior third of the quadriceps muscle, centred over *rectus* and *vastus lateralis*, performing minor positional adjustments in order to determine the best spot in each lower limb. This position was then marked and used for the entire duration of the protocol, which consisted of the stimulation of both left and right quadriceps muscles for 15 min/day for 3 days/week for 8 weeks. The intensity of the magnetic stimulation was increased progressively from 40–70% of the maximal output of the stimulator, as tolerated. Consequently, and in order to avoid overheating of the coil, the frequency was reduced as the intensity progressively increased, ranging between 15–8 Hz.

Biological muscle studies

All the muscle biology analyses were conducted in the same laboratory, at IMIM-*Hospital del Mar*, in Barcelona. Both investigators and laboratory technicians in charge of the muscle sample manipulation (molecular biology techniques) were blinded throughout the entire duration of the experimental procedures.

Immunoblotting. The effects of oxidants on muscle proteins (reactive carbonyls and protein tyrosine nitration) were evaluated according to methodologies published elsewhere [10,24,25]. Immunoblotting experiments were specifically designed in such a way that muscle homogenates from control subjects ($n = 5$) and from treated patients ($n = 10$) both before and after the 8-week period of time were always run together and kept in the same order. The following antibodies were used to detect the different antigens and phenomena: anti-2,4-dinitrophenylhydrazone (DNP) moiety antibody (Oxyblot kit, Chemicon International Inc., Temecula, CA), anti-malondialdehyde (MDA) protein adducts antibody (Academy Bio-Medical Company, Inc., Houston, TX), anti-3-nitrotyrosine mouse monoclonal antibody (Upstate, Lake Placid, NY), anti-Mn-superoxide dismutase (SOD) antibody (StressGen, Victoria, BC, Canada) and anti-catalase antibody (Calbiochem, San Diego, CA).

Catalase activity assays. A commercially available catalase assay kit (Cayman Chemical Co., Ann Arbor, MI) was used according to the corresponding manufacturer's instructions to determine the activity of catalase enzyme in all muscle specimens. A plate reader with a 540 nm filter was used for quantification of the catalase activity. Total catalase activity was expressed as nmol/min/ml/mg. Samples were always run in triplicate and measured at the same time. Their corresponding activity was expressed as the mean value of the three measurements. The intra-assay coefficient of variation for catalase activity was 3.8%. The inter-assay coefficient of variation was 9.9% for the same activity.

Immunohistochemistry. Muscle morphometry in both patients and controls was assessed as previously described [23]. Monoclonal anti-myosin heavy chain-I (clone MHC, Biogenesis Inc., Poole, England, UK) and anti-myosin heavy chain-II antibodies (clone MY-32, Sigma, Saint Louis, MO) were used for immunohistochemical identification of type I and type II fibres, respectively. The cross-sectional area, mean least diameter and proportions of type I and type II fibres were assessed using a light microscope (Olympus, Series BX50F3, Olympus Optical Co., Hamburg, Germany) coupled with an image-digitizing camera (Pixera Studio, version 1.0.4, Pixera Corporation, Los Gatos, CA) and a morphometry program (NIH Image, version 1.60, Scion Corporation, Frederick, MD). At least 100 fibres were measured and counted in each muscle specimen [26].

Statistical analysis. Data are presented as median and interquartile range in the table, while they are presented as box and whisker plots in the figures. Mann-Whitney non-parametric test was used for comparisons between the two groups of patients at baseline. Moreover, Wilcoxon non-parametric paired test was employed to compare redox and fibre type variables in both groups of COPD patients at baseline and after the 8-week period. Spearman's coefficient was used to assess correlations between biological and physiological variables among the magnetic stimulated patients. A p-value of 0.05 or less was considered significant.

Results

Patient characteristics

Table I indicates the main characteristics of the study subjects at baseline. No significant differences in age, nutritional status as assessed by BMI and FFMI, lung function parameters or walking distance were observed between the two groups of COPD patients.

Table I. Main characteristics of study subjects at baseline.

	COPD patients	
	Control, $n=5$	Magnetic stimulation, $n=10$
Age (years)	68 (10)	59 (8), $p=0.206$
FEV_1 (% pred)	27 (6)	31 (10), $p=0.304$
FEV_1/FVC (%)	35 (26)	31 (10), $p=0.594$
BMI (kg/m^2)	27.0 (8.8)	26.4 (6.3), $p=0.440$
FFMI (kg/m^2)	18.4 (3.6)	19.0 (5.0), $p=0.999$
Six-minute walking distance (m)	422 (97)	440 (188), $p=0.606$

Data are presented as median and (interquartile range) and actual *p*-values.
SD, standard deviation; FEV_1, forced expiratory volume in 1 s; pred, predicted; FVC, forced vital capacity; BMI, body mass index; FFMI, fat-free mass index.

According to the GOLD guidelines all patients were classified as very severe (stage IV). All patients were able to complete the training sessions successfully. Magnetic stimulation was well tolerated by all the patients and no dropouts were registered. Indeed, a 5% increase in the magnetic stimulation intensity was achieved every three sessions in all the patients, without exhibiting any signs of discomfort or pain during the sessions. Patients did not have any exacerbation during the 8-week training programme and did not require the use of antibiotics, systemic glucocorticoids, or any change in their inhaled medication. Moreover, the trained patients exhibited a significant improvement of 25 m, on average [480 (215) m, median (interquartile range)], in walking distance compared to baseline, which was statistically significant ($p=0.008$).

Oxidative stress indices after magnetic stimulation

Several carbonylated and nitrated proteins were detected in the quadriceps of both groups of COPD patients at baseline and after the training period. In the control group of patients, muscle protein carbonylation and nitration (3-nitrotyrosine immunoreactivity) levels did not differ between baseline and after the control period (data not shown). Likewise, muscle protein carbonylation (reactive carbonyls and MDA-protein adducts) and nitration levels remained unmodified after the magnetic stimulation training compared to baseline (Figure 1, top, middle, and bottom panels, respectively). Within the group of patients undergoing the magnetic stimulation training, a significant inverse correlation was found between muscle protein carbonylation levels at baseline and the individual percentage of change of the walking distance ($r=-0.767$, $p=0.016$).

Antioxidant mechanisms after magnetic stimulation

The levels of the enzymes Mn-SOD and catalase (protein content and activity) did not significantly

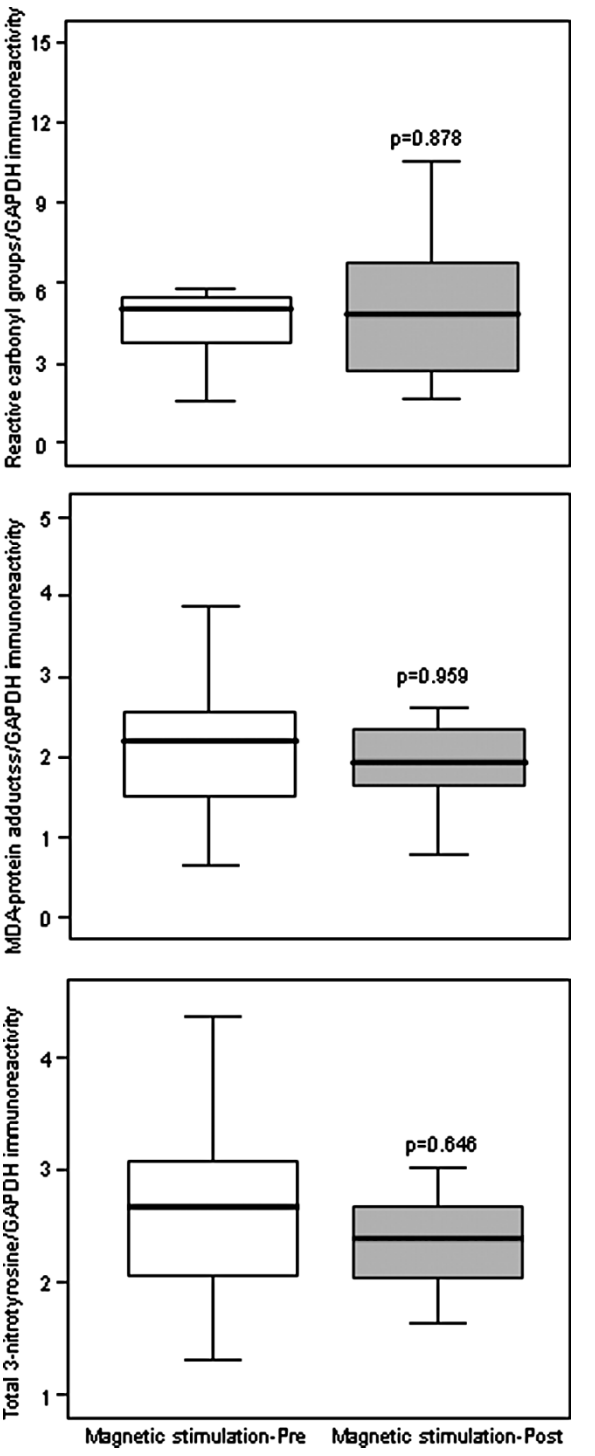

Figure 1. Optical densities in the box plots are expressed as the ratio of the optical densities of total reactive carbonyl groups, MDA-protein adducts and 3-nitrotyrosine immunoreactivity to those of GAPDH (top, middle and bottom panels, respectively). Standard box plots with median (25th and 75th percentiles) and whiskers (at minimum and maximum values) are depicted. No significant differences were found in total reactive carbonyls, MDA-protein adducts or protein tyrosine nitration in the quadriceps of the trained patients (median values 4.82, 1.92 and 2.39, respectively) compared to baseline values (median values 5.03, 2.19 and 2.67, respectively). Graphs (standard box plots with whiskers) corresponding to muscle reactive carbonyls, MDA-protein adducts and 3-nitrotyrosine immunoreactivity in the *vastus lateralis* of the trained COPD patients ↑.

differ in the quadriceps of the control patients after the 8-week control period with respect to baseline levels (data not shown). Likewise, magnetic stimulation training did not induce any significant change in the muscle content of the antioxidants Mn-SOD or catalase compared to baseline (Figure 2A and B). The levels of catalase activity were not modified after magnetic stimulation training (Figure 2C).

Muscle fibre type composition after magnetic stimulation

In the control patients, no significant modifications were observed in either the proportions or sizes of muscle fibres in the *vastus lateralis* after the 8-week control period compared to baseline (data not shown). Likewise, proportions of either type I or type II remained unchanged in the quadriceps muscle after the magnetic stimulation training (Figure 3A

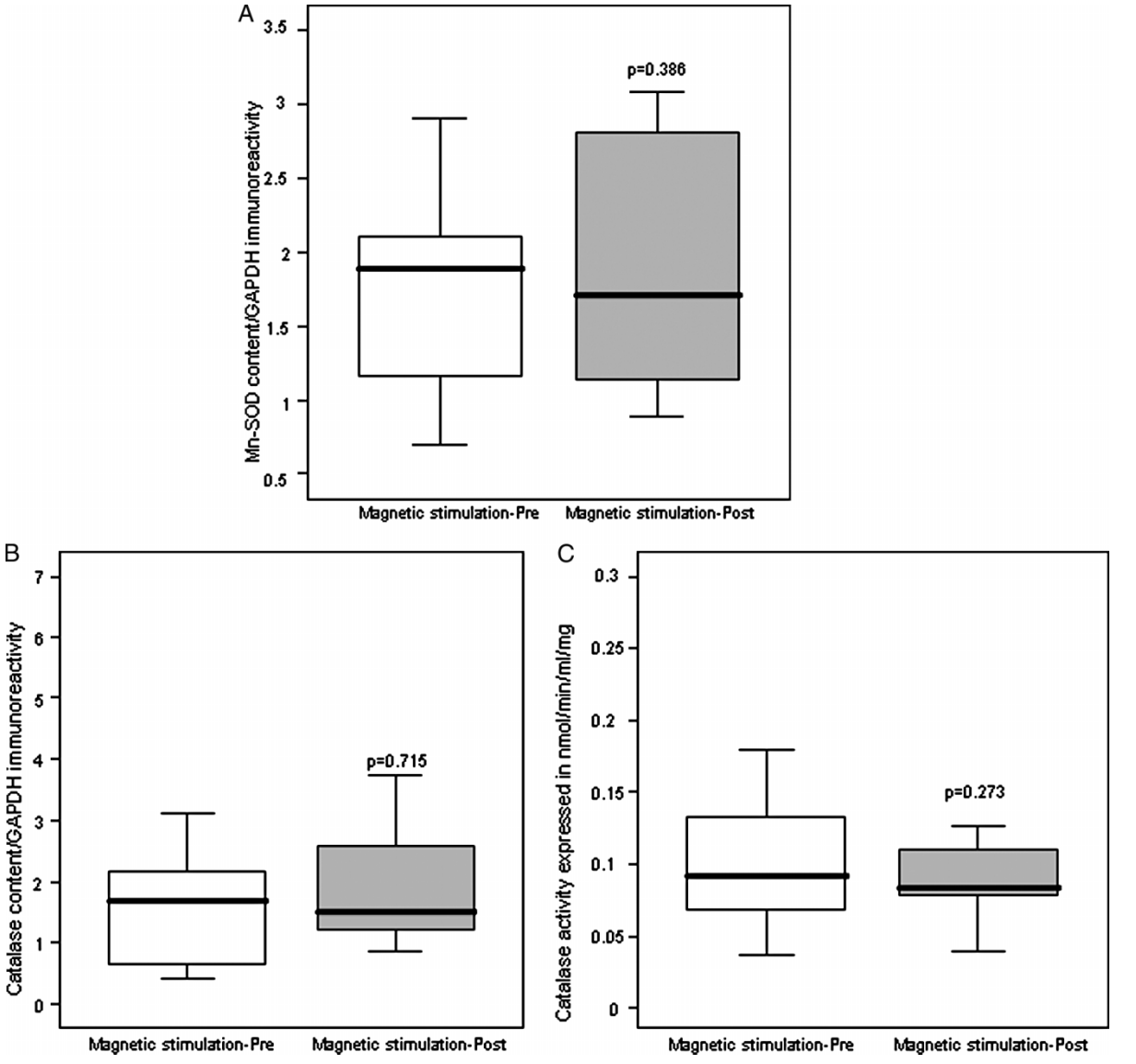

Figure 2. (A) Optical densities in the box plot are expressed as the ratio of the optical densities of Mn-SOD protein content to those of GAPDH. Standard box plot with median (25^{th} and 75^{th} percentiles) and whiskers (at minimum and maximum values) is depicted. No significant differences were found in Mn-SOD content in the quadriceps of the trained patients (median value 1.71) compared to baseline values (median value 1.88). (B) Optical densities in the box plot are expressed as the ratio of the optical densities of catalase protein content to those of GAPDH. Standard box plot with median (25^{th} and 75^{th} percentiles) and whiskers (at minimum and maximum values) is depicted. No significant differences were found in catalase content in the quadriceps of the trained patients (median value 1.50) compared to baseline values (median value 1.67). (C) Standard box plot with median (25^{th} and 75^{th} percentiles) and whiskers (at minimum and maximum values) of muscle catalase activity is depicted. No significant differences were found in catalase activity in the quadriceps of the trained patients (median value 0.083 nmol/min/ml/mg) compared to baseline values (median value 0.092 nmol/min/ml/mg). (A) Graphs (standard box plot with whiskers) corresponding to muscle Mn-SOD in the *vastus lateralis* of the trained COPD patients ↑. (B and C) Graphs (standard box plot with whiskers) corresponding to muscle catalase content and activity, respectively, in the *vastus lateralis* of the trained COPD patients ↑.

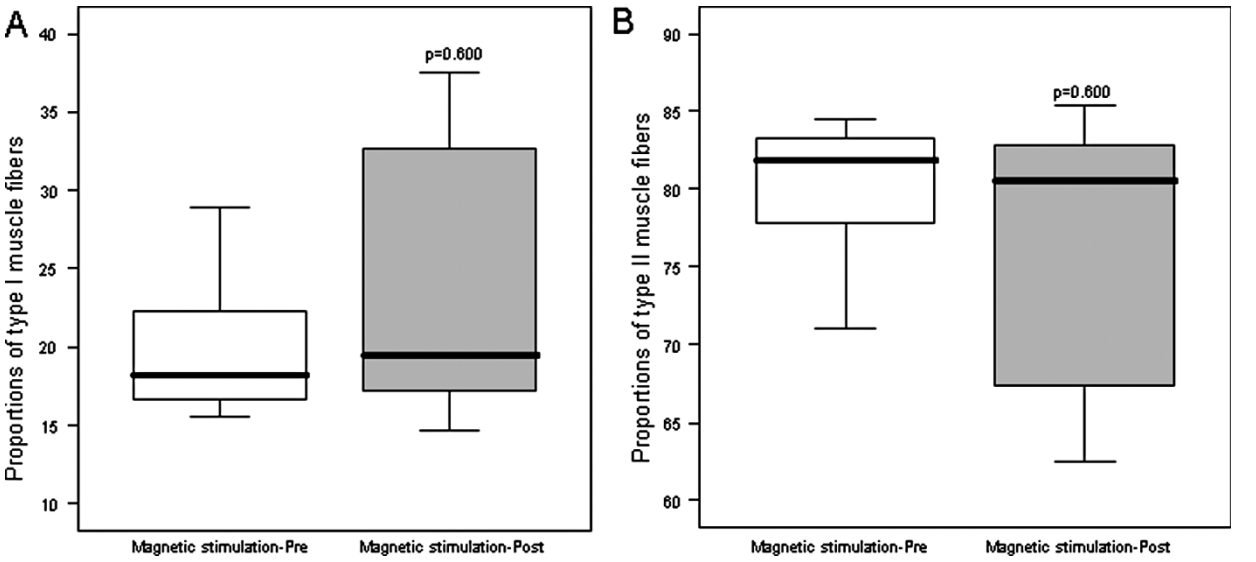

Figure 3. (A) Standard box plot with median (25th and 75th percentiles) and whiskers (at minimum and maximum values) of the proportions of type I muscle fibres is depicted. No significant differences were found in the proportions of type I fibres in the quadriceps of the trained patients (median value 19.44%) compared to baseline values (median value 18.12%). (B) Standard box plot with median (25th and 75th percentiles) and whiskers (at minimum and maximum values) of the proportions of type II muscle fibres is depicted. No significant differences were found in the proportions of type II fibres in the quadriceps of the trained patients (median value 80.55%) compared to baseline values (median value 81.81%). Graphs (standard box plot with whiskers) corresponding to the proportions of both type I and type II fibres, respectively, in the *vastus lateralis* of the trained COPD patients ↑.

and B, respectively). However, the size of type I fibres, but not that of type II fibres, was significantly greater in the *vastus lateralis* after magnetic stimulation training (Figure 4A and B, respectively).

Discussion

The main findings of this study are that in patients with severe COPD after an 8-week training protocol using magnetic stimulation: (1) walking distance significantly improved in the trained patients, (2) repetitive bouts of quadriceps magnetic stimuli for several weeks did not induce a significant increase in muscle oxidative stress and (3) the size of type I muscle fibres, but not that of type II fibres, was significantly increased after the magnetic stimulation training compared to baseline.

Magnetic stimulation as a muscle training modality

In severe COPD, transcutaneous electrical muscle stimulation of the lower extremities has recently been demonstrated to improve lower limb muscle function, maximal and endurance exercise capacity, walking distance and dyspnea [16,27]. The underlying molecular mechanisms for such clinical and physiological improvements remain to be elucidated. However, previous studies conducted on paraplegic patients [28] and rhesus monkeys [29] showed that electrical stimulation of the limb muscles induced an increase in the number of capillaries, in the muscle oxidative potential and in the size of both type I and type II muscle fibres; findings that, in turn, correlated with the clinical and functional improvements [28,29].

Alternatively, nerve stimulation can also be ensured by means of magnetic stimulation. In the clinical assessment of muscle strength, the advantages of using magnetic over electrical stimulation are mainly that it produces less discomfort to the patient, it is more reproducible and supramaximality is achieved in an easier and more reliable manner [17]. In fact, in the last decade, magnetic stimulation has been used as a non-volitional tool for measuring muscle strength in several clinical settings such as in neuromuscular and critically ill patients [17], in COPD [30–32], as well as for the measurement of quadriceps endurance in healthy subjects [32].

More recently, we have also demonstrated [18] that, in healthy individuals, the Medtronic Magpro electromagnet generates sufficient muscle contraction when applied to the quadriceps to be used for repetitive stimulation. In the current study, we provide the first evidence of the use of magnetic stimulation of the quadriceps muscle as a safe training strategy in patients with very severe COPD. Interestingly, this modality of muscle training was well tolerated by all the patients, who did not show any complaints about skin pain or discomfort during the sessions. In patients with severe COPD, the major advantage of this training modality over exercise training is the absence of ventilatory stress, which probably contributed to the excellent tolerance shown by the patients in this study. Although we acknowledge that exercise training has major advantages over passive training such as functional cardiorespiratory

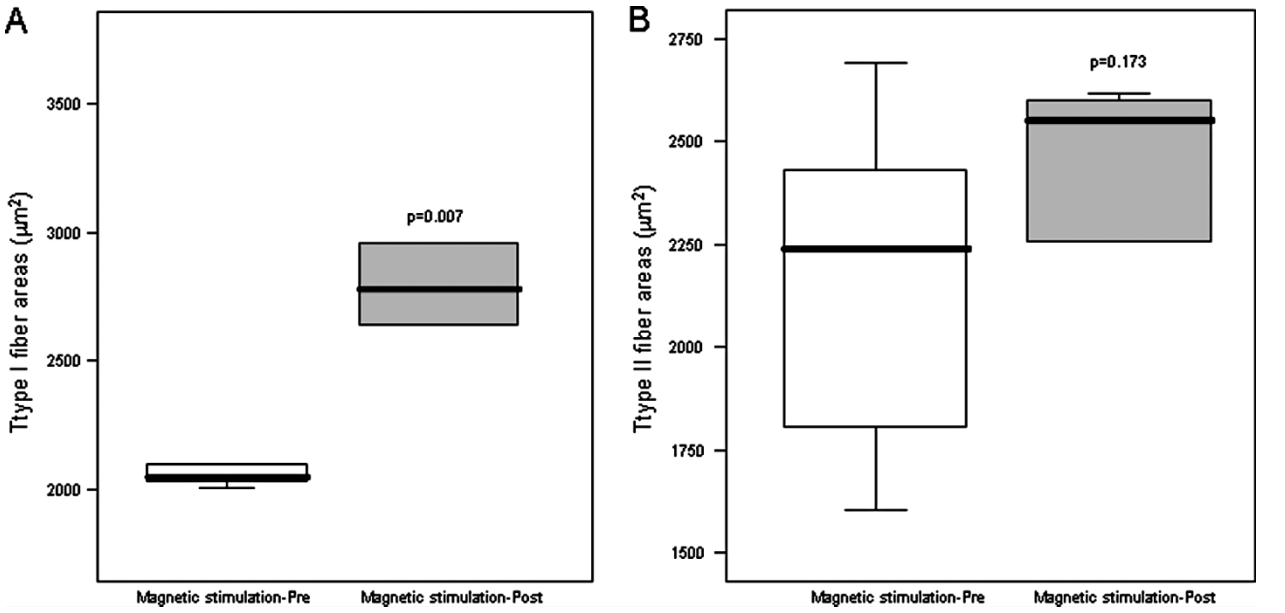

Figure 4. (A) Standard box plot with median (25th and 75th percentiles) and whiskers (at minimum and maximum values) of the sizes (cross-sectional areas) of type I muscle fibres is depicted. The size of type I fibres was significantly greater in the quadriceps of the trained patients (median value 2778.64 μm^2) compared to baseline values (median value 2044.43 μm^2). (B) Standard box plot with median (25th and 75th percentiles) and whiskers (at minimum and maximum values) of the sizes of type II muscle fibres is depicted. No significant differences were found in the sizes of type II fibres in the quadriceps of the trained patients (median value 2550.44 μm^2) compared to baseline values (median value 2238.05 μm^2). Graphs (standard box plot with whiskers) corresponding to the sizes of both type I and type II fibres, respectively, in the *vastus lateralis* of the trained COPD patients ↑.

improvements, non-volitional quadriceps magnetic stimulation for several weeks appears as a promising inexpensive modality of muscle training in patients with advanced COPD exhibiting limited exercise capacity.

Muscle redox balance

In this study we have measured several post-translational modifications of proteins induced by the presence of free oxygen radicals in the *vastus lateralis* muscles of very severe COPD patients after an 8-week protocol of magnetic stimulation training. On the grounds that strong muscle contractions lead to an imbalance between oxidant production and antioxidants in favour of the former, we hypothesized that a moderate increase in ROS production might take place in the *vastus lateralis* of very severe COPD patients after a prolonged period of repetitive bouts of magnetic stimulation. Indeed, recent data from our group (unpublished observations) indicate that a 3-week programme of high-intensity exercise enhances oxidative stress in the quadriceps of very severe COPD patients. In the present study, quadriceps magnetic stimulation training did not induce any significant change in either protein carbonylation or tyrosine nitration in the muscles of the trained patients compared to baseline. This suggests that short bouts of magnetic stimulation produce muscle contractions of sufficient intensity without enhancing oxidative stress within the limb muscles of our severe COPD patients. This is important, since patients with COPD exhibit increased muscle oxidative stress levels even at rest [5,9–11] and high-intensity inspiratory training for only 2 weeks was shown to significantly increase ROS production in the diaphragms of the trained dogs [4].

Magnetic stimulation training for 8 weeks did not induce any significant change in the content of the antioxidants Mn-SOD or catalase in the *vastus lateralis* of the trained COPD patients. Interestingly, these findings are consistent with those reported in previous studies, in which exercise training did not modify the antioxidant potential of the *vastus lateralis* of patients with severe COPD [33,34], while it induced a marked increase in muscle glutathione levels in healthy sedentary subjects [33–35]. In healthy humans, whether repeated quadriceps magnetic stimulation induces similar effects to severe COPD in terms of muscle oxidant production and antioxidant induction remains as yet unknown. It could be speculated, however, that in healthy individuals, muscle contractions of sufficient intensity would stimulate the induction of the antioxidant systems which would, in turn, counterbalance excessive oxidant production as shown to occur following exercise training [33,35]. In the current investigation, we prioritized the inclusion of a group of very severe COPD patients with limited exercise tolerance in order to explore whether this modality of muscle training was beneficial and well tolerated by these patients. Future studies in which larger groups of COPD patients and control subjects will be

included will shed light into the specific effects of magnetic stimulation on muscle redox balance also in healthy individuals.

Muscle structure

In the present study, the size of oxidative type I fibres, but not that of type II fibres, was significantly increased in the *vastus lateralis* muscles of the trained COPD patients. The magnetic stimulation muscle training, however, did not modify the proportions of either type of fibres in those muscles. This is in keeping with previous studies [28,29], where an increase in the sizes, but not in the distribution, of both oxidative and glycolytic fibres were shown after a few weeks of neuromuscular electrical stimulation of several muscles. Conversely, high-intensity exercise training of healthy subjects [36,37] and of COPD patients [23] induced a significant increase in both the sizes and proportions of all fibre types. Differences in the phenotypic outcome between passive muscle training and high-intensity exercise training probably rely on variations of the metabolic and/or mechanical stress induced by the intensity and/or duration of the training programmes. In line with this, in a study conducted on racehorses [38] undergoing different modalities of exercise training, muscle fibre-type shifts and hypertrophy were shown to be rather dependent on the intensity of the exercise training, whilst training duration was a major contributor to adaptation to muscle oxidative capacity and capillary growth [38]. On this basis, it could be concluded that in our study, muscle contractions generated by magnetic stimulation training for 8 weeks were of sufficient intensity to induce an increase in the sizes of the oxidative fibres, but not to significantly modify those of type II fibres or the fibre type distributions in the *vastus lateralis* of the severe COPD patients. Another important issue here would be to explore the long-term duration of the molecular and structural changes in the trained muscles as well as whether long-term intervention would still be beneficial in terms of exercise tolerance and quality of life. Future studies, in which different duration and intensity strategies of muscle magnetic stimulation will be used, will further clarify these questions.

On the other hand, the investigation of the underlying molecular mechanisms involved in muscle fibre size increase or hypertrophy has also been an important subject of research in the last decade using different models of training. For instance, hypertrophy appears to be the result of increased protein anabolism, especially of contractile proteins, together with a mild increase in protein breakdown [39]. In this regard, insulin-like growth factor (IGF)-1 genes, which code different isoforms of IGF-1 peptides or mechano-growth factor in human muscles, were shown to regulate muscle fibre regeneration and hypertrophy following mechanical overloading and damage [39,40]. More recently, it has been demonstrated that myonuclear addition via satellite cell recruitment is another mechanism of muscle hypertrophy in quadriceps of trained subjects [41]. Clearly, future research will shed light into the specific molecular mechanisms implicated in the magnetic stimulation-induced muscle fibre size increase.

Study limitations

One possible critique in our study is that the post-training muscle biopsy was taken from the contralateral side, which might have introduced a possible sampling error. In our opinion, a second open muscle biopsy should not be obtained from the same muscle at a time-point relatively close to the baseline biopsy as was the case in the current study (8 weeks). A series of molecular events, which may last for several weeks, related to muscle injury, inflammation and repair may occur after muscle sampling. Therefore, in order to avoid the interference of these molecular events with the study results, the post-training muscle biopsy was obtained from the contralateral *vastus lateralis* in all patients. However, in order to ensure as much as possible the exact same position of the contralateral post-training biopsies much care was taken in all the patients. Moreover, direct vision of the muscle through the open biopsy technique clearly diminishes the sampling error. This specific approach was already used in a previous study from our group [23], in which open biopsies were obtained from the two external intercostals and quadriceps muscles in patients undergoing inspiratory muscle training both before and after the training period. Interestingly, the proportions and sizes of the quadriceps fibres (control muscle) did not significantly differ between the baseline and contralateral post-training biopsy [23].

Another possible critique in this study is related to the interest of the results for the future applicability of magnetic stimulation training in different clinical settings such as in rehabilitation programmes of certain sub-groups of severe COPD patients. However, this recommendation should be taken cautiously for the following reasons: (1) this is a pilot study where early data are being disclosed and an alternative outcome would be possible in a larger study, (2) magnetic stimulation was used in the current study as a modality of passive muscle training, therefore, corresponding results should not be compared to those observed after exercise training programmes, especially in relation to expected improvements in exercise capacity and (3) the statistically significant improvement in the distance walked after quadriceps magnetic stimulation, might be considered not to be large enough to have a significant clinical impact on the patients [42]. In fact, we did not expect a large

improvement in the exercise capacity of our patients after specific magnetic stimulation training of the quadriceps muscle. We believe, however, that the absence of increased muscle oxidative stress after repetitive quadriceps magnetic stimulation, together with the improvements observed in the size of the oxidative fibres, may serve as the basis for the design of future studies, where magnetic stimulation may be used as a muscle training modality in a larger population of patients with advanced COPD.

Conclusions

We conclude from this study that magnetic stimulation training of the quadriceps was well tolerated by patients with severe COPD and limited exercise capacity. In these patients, the lack of an increase in muscle oxidative stress along with the improvements seen in muscle fibre structure following quadriceps magnetic stimulation for several weeks suggest that this safe training modality appears as a novel promising tool for the clinical management of this type of patients.

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