Supplementary data

Skeletal muscle effects of electrostimulation on inpatients after COPD exacerbation

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MATERIALS AND METHODS

Study design and randomization procedure

Consecutive patients with COPD (forced expiratory volume in one second (FEV_1)/forced vital capacity (FVC) <70%) admitted to the Intensive Care Unit (ICU) of Gui Chauliac hospital for an exacerbation were included in this study. Patients were assigned either to the control (Sham) or to the experimental group (NMES). All patients were evaluated four times (at inclusion and after 2, 4 and 6 weeks of program). Intermediate assessments consisted of anthropometric measurements, maximal voluntary contraction, 6-min walk distance test and dyspnoea evaluated with the Medical Research Council Dyspnoea Scale (MRC). The first and last evaluations consisted of all the intermediate assessments as well as of vastus lateralis muscle biopsy. Patients were randomized after baseline testing in order not to bias the initial assessment by the knowledge of the group allocation. Randomization was performed in block sizes of 5, using blinded sealed envelopes prepared by an independent secretary. The program started after baseline evaluation in the ICU and continued in an inpatient unit (Clinique Souffle Vallonie, France). Patients were blinded to the intervention groups and tests were performed by researchers who were not blinded to the groups.

Sample Size and Power

Given the novelty of the study, we could not determine in advance the exact number of subjects required to carry out the project. Initially, we wanted to recruit about 30 patients with COPD exacerbations but we had to limit our trial to 17 randomized patients due to recruitment difficulties. However, the retrospectively calculated statistical power of the primary outcome (quadriceps force) was of 96% and significant results were found despite the relatively small population.

Neuromuscular electrical stimulation protocol

The quadriceps and hamstring muscles of both legs were stimulated simultaneously by transcutaneous neuromuscular electrical stimulation for a total of 1 h per day, for 6 weeks (5 days per week, a total of 30h). A programmable stimulator (Phenix S8 VIVALTIS, France) was used. The current was applied through self-adhering square surface electrodes (50mmx50mm Compex®, Technology "thousand threads", French). Electrodes were placed bilaterally on the quadriceps muscles, three 5 cm away from the inguinal fold and three 3 cm proximal to the upper border of the patella. Similarly, hamstring muscles were stimulated with one electrode placed 10 cm away from the sciatic tuberosity and 5 cm proximal to the knee joint fold. Biphasic symmetric, constant current impulses with a pulse width of 400 μs and a frequency of 35 Hz were used. Impulse trains were delivered for 6 s with a pause of 12 s. Each session started with a 5 min stimulation at 8 Hz pulse width 400 μs (warm-up period) and ended by 5 min at 2 Hz pulse width 400 μs (recovery). In the NMES group, the applied stimulation had to induce a visible contraction and was set at the maximal intensity tolerated by the patient as described in other similar papers [1-4]. The Sham group was exposed to the same regimen, but the intensity of stimulation did not lead to visible or palpable contractions. Patients were advised to lie down during the session.

Clinical and functional evaluations

Anthropometry and Pulmonary Function Tests: Body height and weight were determined. The body mass index (BMI) was calculated as the ratio of weight to height in square meters. Spirometry was performed in accordance with the recommended techniques detailed in the American Thoracic Society Guidelines [5]. The primary parameters of assessment were forced expiratory volume in one second (FEV_1), forced vital capacity (FVC) and FEV_1/FVC ratio. The results of the pulmonary function tests were compared to the normal values reported by Quanjer *et al* [6].

Muscle strength assessment: We assessed the maximum voluntary contraction (MVC) of the quadriceps of each leg using an exercise bench (Genin Médical, France). MVC was measured with a dynamometer (Salter, Made 235, Angl). Patients performed three brief (4 s) maximal contractions, each separated by 1 min of rest. If the maximum value of strength maintained for 3 s, as read on the dynamometer, was reproducible (<5% of variability between values), the highest value of the three contractions was defined as the MVC [7].

Six-minute walk test: This test was used to assess the functional exercise capacity according to the American Thoracic Society criteria [8]. Patients were asked to walk at their own maximal rate without running for 6 minutes. Patients were not encouraged during the test and could stop and restart. At baseline, patients completed at least two training walks to account for the learning effect [9]. The distance walked during the test was reported with the help of the Troosters' equation [10] to be expressed as a percentage of the predicted value. This methodology is habitually used by our group [9, 11]. Symptoms of dyspnoea were assessed using the Medical Research Council (MRC) dyspnoea scale [12].

Muscle biopsy

Before and after the program, a muscle biopsy of the lateral part of the vastus lateralis was performed under local anaesthesia using the needle biopsy technique [13] Specimens were then dissected free of visible connective tissue and fat and the muscle tissue was immediately frozen in isopentane, cooled to freezing point with liquid nitrogen and stored at -80°C until analysis.

Muscle biopsy analysis

Muscle oxidative stress

Oxidative stress markers were assessed by protein oxidation and muscle lipid peroxidation. Protein oxidation was assessed by measuring protein carbonyl formation with the OxyBlot™ Protein Oxidation Detection Kit (S7150, Millipore) in which the carbonyl groups in the protein side chains are derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reacting with 2,4-dinitrophenylhydrazine (DNPH). Briefly, 15 µg of muscle proteins were used for each derivatization reaction. Proteins were denatured by addition of 12% sodium dodecyl sulphate. Samples were subsequently derivatized by adding 10 µl of 1X DNPH solution and incubated for 15 minutes. Finally, 7.5 µl of neutralization solution and 2-mercaptoethanol were added to the sample mixture. The DNP-derivatized protein samples were detected by immunoblotting [14-16].

MHC oxidation was assessed by measuring protein carbonyl formation with the OxyBlot™ kit. Firstly, MHC was extracted. Frozen muscle samples were homogenized in a buffer containing 210 mM sucrose, 30 mM HEPES, 2 mM EGTA, 40 mM NaCl, 5 mM EDTA pH 7.4 and 1% protease inhibitor cocktail. Samples were centrifuged and pellets resuspended in a second buffer containing 175 mM KCl, 5 mM EDTA, 20 mM Tris and 0.5%, Triton X-100, pH 6.8. After a new centrifugation, pellets were resuspended twice in a third buffer containing 150 mM KCl, 5 mM EDTA and 20 mM Tris, pH 7 [13]. After extraction of myosin, samples were then derivatized as for total proteins.

The most cytotoxic product of lipid peroxidation, 4-hydroxy-2-nonenal (4-HNE), was also evaluated by immunoblotting [16]. Thiobarbituric acid reactive substances (TBARs) were used as markers of muscle lipid peroxidation and were determined by spectrophotometry using the method described by Ohkawa *et al* [17]. Results were expressed in nM/µg protein.

Protein extraction: Frozen muscle samples were homogenized in a buffer containing 100 mM potassium phosphate buffer with 10 mM EDTA, 0.05% bovine serum albumin (BSA),

0.13mM butylated hydroxytoluene, 0.13 mM desferrioxamine (pH 7.4) and 1% protease/phosphatase inhibitors. The total muscle protein level in each sample was determined with the Bradford technique in triplicate and with BSA as standard (500-006, BioRad, France).

Immunoblotting: Crude muscle homogenates were separated on 7-12% polyacrylamide gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (PVDF). PVDF membranes were stained with Ponceau S for protein visualization to confirm equal sample loading in all lanes. Membranes were blocked with 5% non-fat powered milk in phosphate-buffered saline (PBS) 0.05% Tween (PBS-T), and incubated with monoclonal anti-DNP (OxyBlot^{TM} KitS7150, Millipore, Molsheim, France, dilution 1/150), anti-4-Hydroxy-2-Nonenal (4-HNE) (sc-130083, Santa Cruz Biotechnology, France, dilution 1:1000), anti-Myosin Heavy Chain (05-716, Millipore, Molsheim, France, dilution 1:5000), anti-Zn/Cu-SOD (SOD-101E, Euromedex, Strasbourg, France, dilution 1:1000), anti-Catalase (LFMA0010, Euromedex, Strasbourg, France, dilution 1:2000), anti-Glutathione Reductase (LFPA0056, Euromedex, Strasbourg, France, dilution 1:1000), and anti-Glyceraldehyde-3-Phosphate Dehydrogenase antibody (GAPDH) (ab9385, Abcam, Paris, France, dilution 1:1000) in blocking solution at room temperature with gentle shaking for 1 hour. Membranes were then washed in PBS-T. Immunolabelling was detected with horseradish peroxidase (HRP)-conjugated secondary antibodies and a chemiluminescence kit. Blots were scanned and the optical densities (OD) of specific proteins were quantified with ImageJ. Given the small size of muscle biopsies, we could not determine the levels of all proteins of interest in all patients.

Muscle structure

Part of each frozen biopsy was placed in a drop of Tissue-tek® (OCT compound) on a piece of cork with the fibre orientation perpendicular to the plane of the cork. Serial cryostat transversal sections (10 μm) were cut using a cryostat microtome at -20°C and mounted on slides which were stored at -20°C until analysis. Consecutive slides (each carrying two sections) per biopsy were used for fibre type characterization. At least 100 fibres that were present on all slides were numbered and analysed. Muscle fibre composition was determined by immunohistochemical staining using the monoclonal antibodies anti-type I MHC (A4.951-c, 1:1000), anti-type IIA MHC (2F7-c, 1:400), anti-type IIX MHC (6H1-c, 1:10) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa, USA). Following incubation with the primary antibody, sections were incubated with a secondary antibody. Sections were visualized using a fluorescence microscope. Fibres were classified as hybrid when they expressed two or more types of MHC [18, 19]. The number and size of fibres were identified in each slice using a micro-vision image analysis system (Histolab 6.1.0, Microvision Instruments).

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Legends to Supplementary Figures

Fig. 1S **Flowchart of the study**. Sham: patients who had sham neuromuscular electrostimulation. NMES: patients who had effective neuromuscular electrostimulation.

Fig. 2S

Individual values of MVC (A) and 6-minute walk test (B) at inclusion and after 2, 4 and 6 weeks of trial in the Sham (discontinuous line) and NMES group (continuous line). (A) During the first two weeks of program, the quadriceps strength increased only in the NMES group. The strength continued to ameliorate in the NMES group up to the end of the 6-week program. (B) During the first two weeks of the program, the distance covered during the six-minute walk test improved only in the NMES group and not in the Sham group. Between the 2^{nd} and 4^{th} week, the distance improved in the NMES group (median 24.5 [20 to 70] m), whereas it remained unchanged in the Sham group (median 0 [0 to 10] m). Only after the 4^{th} week the covered distance slightly improved also in the Sham group (median 37 [15 to 70] m), whereas it increased significantly in the NMES group (median 30 [16 to 47.5] m).

Fig. 3

(A) Individual effects of the 6-week neuromuscular electrostimulation program on protein carbonylation in quadriceps from Sham patients (n=6, discontinuous line) and NMES patients (n=9, continuous line). At baseline, total protein carbonylation levels were comparable in the two groups. At the end of the 6-week neuromuscular electrostimulation program total muscle protein carbonylation was significantly decreased in the NMES group when compared to the baseline values, whereas it remained unchanged in the Sham group. (B) Effect of the 6-week neuromuscular electrostimulation program on MHC carbonylation in quadriceps of Sham (n=5) and NMES patients (n=8). At baseline, MHC carbonylation levels were comparable in

the two groups. At the end of the 6-week neuromuscular electrostimulation program, MHC carbonylation was significantly decreased in the NMES group, whereas it remained unchanged in the Sham group.

Fig. 4

(A) Representative quantification of the change in the level of 4-HNE protein adducts between inclusion and end of the program. Data are described using standard box plots with medians (25th and 75th percentiles). No significant differences were found. (B) Changes in the level of TBARs in the Sham and NMES groups at the end of the trial. Standard box plots with medians (25th and 75th percentiles) are depicted. No significant differences were found.