CLINICA NEUROLOGICA - UNIVERSITA' DEGLI STUDI DI BRESCIA
UO Neurologia 2 - AZIENDA SOCIO SANITARIA TERRITORIALE degli SPEDALI CIVILI DI BRESCIA

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DIVISION IN WHICH RESEARCH WILL BE PERFORMED: Clinica Neurologica, Università degli Studi di Brescia – U.O. Neurologia 2, ASST Spedali Civili di Brescia.

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INTRODUCTION:
Transcranial stimulation with direct currents or transcranial direct current stimulation (tDCS) represents a neurophysiologic method of non-invasive modulation of the excitability of the central nervous system, which is having an increasing diffusion and

an ever-increasing range of potential therapeutic applications such as, for example, post-stroke rehabilitation, depression and pain, already established (Lefaucheur et al., 2017). This method, being non-invasive, low cost and simple to perform, has the potential to be widely used both as a research tool and as a tool to support rehabilitation treatment. A long-term effect of tDCS treatment on synaptic plasticity of the motor cortex, which depends on modulation of NMDA receptors and GABAergic systems, has been demonstrated (Nitsche et al., 2005).

The application of these techniques within the project of "Identification of a possible rehabilitative protocol through the use of direct current brain stimulation (tDCS) in patients affected by cerebellar ataxia of neurodegenerative origin." Approved by this Ethics Committee allowed to identify a clinical improvement also in patients affected by cerebellar ataxia subjected to cerebral transcranial electrical stimulation, also supported by neurophysiology data (Benussi et al., 2015a; Benussi et al., 2017).

This application could also be applied in amyotrophic lateral sclerosis (ALS), a progressive chronic neurodegenerative pathology in which there is an irreversible degeneration of the first and second motoneurons at the level of the motor cortex and of the spinal cord, respectively. ALS is characterized by muscle stiffness, muscle contractions and gradual progressive weakness secondary to muscular atrophy. This results in difficulty in speech, swallowing and, finally, breathing (Salameh et al., 2015). At present, there is no known cure for ALS. One drug, riluzole, can extend life expectancy by about two or three months (Miller et al., 2012).

For this purpose, cortico-spinal transcranial electrical stimulation techniques have been developed, in which a simultaneous stimulation of the cerebral cortex (in this case in the area of the scalp corresponding to the motor cortex of both sides) and of the spinal cord (in this case in the area of the back corresponding to the dorsal-lumbar spinal cord) is applied (Fiocchi et al., 2016; Parazzini et al., 2014).

In order to evaluate an effect of tDCS on the progression of the pathology, some transcranial magnetic stimulation techniques (TMS) will be used in addition to the clinical and life quality scales. In fact, TMS has been widely used in the study of some neurodegenerative diseases, in particular in Parkinson's disease and in dementia due to Alzheimer's disease. Over the years, numerous TMS protocols have been developed to obtain information on neurophysiological parameters with the aim of investigating both the pathogenesis and the evolution of some neurodegenerative diseases (Benussi et al., 2015b).

Short-interval intracortical inhibition (SICI) is currently used to investigate intracortical inhibitory circuits. From neuropharmacology studies, it has been hypothesized that SICI is dependent on the activity of $GABA_A$ receptors (Kujirai et al.,

1993). In this way, alterations of the intracortical inhibitory circuits have been identified in many neurological diseases, in particular in dystonia and in parkinsonism. The study of intracortical facilitation (ICF) is used to investigate the combined effect of secondary facilitation on inhibitory and excitatory mechanisms mediated by $GABA_A$ and NMDA receptors respectively (Ziemann et al. 1998).
In particular, in patients suffering from ALS, a deficiency of SICI and ICF has been observed, due to an imbalance in the mechanisms of inhibition and cortical facilitation mediated by GABAergic and glutamatergic interneurons (Menon et al., 2015). Based on these premises, treatment with tDCS is proposed in patients with ALS.

TARGETS:
The aim of the study is to evaluate the long-term effects of tDCS on clinical, biological and neurophysiological parameters in patients with ALS.
In particular, the clinical and quality of life scales currently used in clinical trials on ALS will be used (ALSFRS-R, ALSAQ-40, CBI, EQ-5D-5L, muscle strength assessed with the MRC scale). Moreover, SICI and ICF protocols will be evaluated through TMS.

PROCEDURES:
Subjects enrolled at the Neurological Clinic will be enrolled with the diagnosis of probable ALS, laboratory-supported, or defined according to the current clinical criteria (World Federation of Neurology Revised El Escorial criteria - Brooks et al., 2000).
Subjects will sign an informed consent and will be subjected to a neurological clinical evaluation using the clinical and quality of life scales currently used in clinical trials on ALS (ALSFRS-R, ALSAQ-40, CBI, EQ-5D -5L, muscle strength evaluated with the MRC scale in different muscle groups).
Clinical assessment: Neuromuscular impairment will be quantified by the five-point MRC scale megascore (shoulder abductors, elbow flexors and extensors, wrist flexors, thumb opponent, hip flexors, knee flexors and extensors, and ankle dorsiflexors and extensors on both sides for a total of 100). Good reliability and reproducibility for manual muscle testing in patients with ALS have previously been shown.
Clinical status will be evaluated with the ALSFRS-R, quality of life with the amyotrophic lateral sclerosis assessment questionnaire-40-item scale (ALSAQ-40), EQ-5D-5L and EQ-VAS, while caregiver burden will be evaluated with the caregiver burden inventory (CBI).

Through the use of TMS techniques, the effects of tDCS on neurophysiological parameters will be evaluated, in particular SICI and ICF.
Transcranial Magnetic Stimulation assessment: TMS will be performed with a figure-of-eight coil (each loop diameter 70 mm) connected to a Magstim Bistim2 system (Magstim Company, Oxford, UK). Motor evoked potentials (MEPs) will be recorded from the right first dorsal interosseous muscle (FDI) through surface Ag/AgCl electrodes placed in a belly-tendon montage and acquired using a Biopac MP-150 electromyograph (BIOPAC Systems Inc., Santa Barbara, CA, USA).
The TMS coil will be held tangentially over the scalp region corresponding to the primary hand motor area contralateral to the target muscle, with the coil handle pointed 45° posteriorly and laterally to the sagittal plane. The motor hot spot will be defined as the location where TMS consistently produced the largest MEP size at 120% of the resting motor threshold (rMT) in the target muscle and was marked with a felt tip pen on the scalp to ensure constant placement of the coil throughout the experiment. Resting motor threshold (rMT) will be defined as the minimal stimulus intensity needed to produce MEPs with an amplitude of at least 50 μV in 5 out of 10 consecutive trails during complete muscle relaxation, which will be controlled by visually checking the absence of EMG activity at high-gain amplification.
SICI and ICF will be studied at rest via a paired-pulse paradigm, delivered in a conditioning-test design, as previously reported. Briefly, the conditioning stimulus (CS) will be set at an intensity of 70% of the rMT, while the test stimulus (TS) will be adjusted to evoke a MEP approximately 1 mV peak-to-peak in the relaxed FDI. Different interstimulus intervals (ISIs) between the CS and TS will be employed to investigate preferentially both SICI (1, 2, 3 ms) and ICF (7, 10, 15 ms).
Ten stimuli will be delivered for each ISI and fourteen control MEPs in response to the TS alone will be recorded, for each paradigm, in all participants in a pseudorandomized sequence. The amplitude of the conditioning MEPs will be expressed as a ratio of the mean unconditioned response. The inter trial interval will be set at 5 sec (±10%). All protocols will be performed in a randomized order in all participants. Throughout the experiment, complete muscle relaxation will be monitored by audio-visual feedback where appropriate.
All clinical and, neurophysiological evaluations will be performed at baseline (T0), after 10 tDCS applications i.e. at two weeks (T1), at two months (T2) and at six months (T3).
After the first evaluation (T0), the patients will be divided into two groups randomly.
The first group will undergo a tDCS stimulation protocol, while the second group will undergo a placebo (sham) treatment. tDCS will be delivered by a battery-driven

constant current stimulator (HDCstim, Newronika, Milan, Italy) through a pair of saline-soaked (0.9% NaCl) surface sponge electrodes. The anodes will be placed on the scalp over the motor cortex area and the cathode over the spinal cervical enlargement (over C6) (see Fig. 1B). The electrodes will be secured using elastic gauzes and an electroconductive gel will be applied to electrodes to reduce contact impedance (<5 kΩ for all sessions). During real stimulation a constant current of 2 mA per each anodal electrode (4.0×6.5 cm2, current density 0.077 mA/cm2) and 4 mA per cathodal spinal electrode (5.0×7.5 cm2, current density 0.107 mA/cm2) will be applied for 20 minutes. For the sham condition, the electrode placement will be the same, but the electric current will be ramped-down 5 seconds after the beginning of the stimulation to make this condition indistinguishable from the experimental stimulation. To detect differences in the perception of the stimulation, patients will be asked whether they thought they were receiving real or sham stimulation at the end of the two-weeks' treatment.

INCLUSION CRITERIA for patients:

- Male or female patients diagnosed with probable ALS, laboratory-supported, or defined according to current clinical criteria (World Federation of Neurology Revised El Escorial criteria - Brooks et al., 2000).
- Age over 18 years;
- Disease duration ≤ 48 months;
- Score ≥ 2 at the item "swallowing" of the ALS Functional Rating Scale-Revised (ALSFRS-R);
- Score ≥ 1 at the item "walking" of the ALSFRS-R;
- Score ≥ 1 at the item "respiratory insufficiency" of the ALSFRS-R
- Signature of informed consent.

EXCLUSION CRITERIA for patients:

- Motor neuron diseases other than ALS;
- Diagnosis of dementia according to current clinical criteria;
- Severe head trauma in the past;
- Persons carrying static electrical stimulators (e.g. cardiac stimulators, nerve stimulators, auditory implants) that would not work or would be damaged by the magnetic field;
- Persons carrying special metallic foreign bodies (e.g. splinters, some prostheses, screws and nails) that could move if placed inside the magnetic field;
- People with a history of epilepsy ore seizures;

- History of ischemic stroke or hemorrhage;
- Severe comorbidity
- Given that the effects of TMS on the developing fetus are not known, pregnant women will be excluded from the study.

STUDY DESIGN:
This is a non-pharmacological, randomized double-blind controlled clinical trial. This study does not present sub studies. Patients will be randomized into two groups: each group will receive anodal bilateral motor cortex tDCS and cathodal spinal tDCS (real tDCS) or sham stimulation for 5 days/week for 2 weeks, in a 2:1 ratio respectively.

END POINTS:
The long-term effect of tDCS on clinical parameters (ALSFRS-R, ALSAQ-40, CBI, EQ-5D-5L, muscle strength assessed with the MRC scale in different muscle districts), and neurophysiological measures (SICI and ICF).
For efficacy analyses, the primary end point will be the defined as change from baseline in global MRC scores. Secondary end points will be defined as changes from baseline in the ALSFRS-R, ALSAQ-40, EQ-5D-5L, EQ-VAS, CBI and intracortical connectivity (SICI and ICF).

STATISTICAL PLAN:
Parametric analysis (t test) will be used to compare the demographic variables (e.g. age, duration of illness, education) of the various groups at the baseline. A *p*-value <0.05 will be considered significant.
In order to estimate the sample size, in order to obtain a significant difference in the primary outcome (Global MRC Score), we performed a "power analysis" based on the effects observed in a small group of patients (pilot study). The "effect size" f (V) was calculated by direct method. Taking into account from previous observations a "partial η^2" = 0.31, equal to an "effect size" f (V) = 0.67 and, considering alpha = 0.05 and the power (1-β) = 0.80, considering a variable as covariate, the sample size expected for two patient groups (real and sham) is 25 total subjects using a repeated-measure ANCOVA study. The proposed sample size of 30 patients should be adequate to achieve the main objective of the study and would allow monitoring for confounding factors or drop-out patients.
Considering the unneglectable number of drop-out patients, that characterize most trials in ALS, we performed intention-to-treat and per-protocol analysis to reduce any bias due to the possible unbalanced drop-out rates between treatment groups. For

patients with missing values, data were assigned using mixed effects models for repeated measures without any ad hoc imputation.
To assess the effect of tDCS treatment on clinical scores over time, we will use a two-way repeated measure ANCOVA with TIME (T0, T1, T2 and T3) as within-subject factors and TREATMENT (sham vs real stimulation) as between-subjects factor, and the baseline values of each score as covariate, to reduce possible effects of baseline impairment on clinical score changes over time.
To assess the effect of tDCS treatment on TMS parameters we will use a two-way mixed ANOVA with TIME (T0, T1, T2 and T3) as within-subject factors and TREATMENT (sham vs real stimulation) as between-subjects factor.
When a significant main effect was reached, *post hoc* tests with Bonferroni correction for multiple comparisons will be conducted to analyze group-differences at respective time points. Mauchly's test will be used to assess for assumption of sphericity, while Greenhouse–Geisser epsilon determination will be used to correct in case of sphericity violation. Spearman's rank-order correlations will be used to assess associations between the improvement in clinical scores and neurophysiological parameters. Statistical analyses will be performed using SPSS version 21 (SPSS, Inc., Chicago, IL, USA).

ETHICAL CONSIDERATIONS (RISK-BENEFIT):
tDCS is a non-invasive, low cost and simple non-invasive brain stimulation method that is having an increasing diffusion and an increasingly numerous spectra of potential therapeutic applications such as, for example, post-stroke rehabilitation, depression and pain, which have already been established. Its effectiveness in modulating the excitability of the motor cortex in humans has been demonstrated (Nitsche et al., 2005). We therefore hypothesize that tDCS can have a positive effect on the clinical and quality of life scales, and restoration of neurophysiological parameters.
Literature data show that the application of the method according to the current safety guidelines (Iyer et al., 2005; Nitsche et al., 2003; Bikson et al., 2016) has little relevant side effects, among which mild tingling sensation during or after stimulation, moderate fatigue, mild itching sensation at the electrode sites, mild burning or pain, headache, nervousness, nausea, blistering at the electrode application points in rarer cases.
As for the TMS method, it is a non-invasive brain stimulation that allows the study of neurophysiological parameters that can provide information on the pathophysiological mechanisms involved in neurodegenerative diseases.
Literature data show that the application of the method according to the current safety guidelines has minor side effects: a certain number of subjects participating in TMS

experiments (up to 20%) suffer from headaches or backaches, due to likely to an excessive muscle tension and a stiff position of the head and / or neck during TMS application. These effects are temporary and, in most cases, do not require any treatment.
With the parameters that we will use in this study, epileptic seizures have never been reported (Rossi et al., 2009). If an epileptic seizure occurs, this would occur during TMS, not later. However, this is an extremely rare complication. To prevent this, all TMS sessions will be performed in the presence of a neurologist and by personnel trained to give first aid and to immediately recognize the first signs of epileptic seizures. In addition, all TMS sessions will be carried out within the Neurology Unit, equipped with all the emergency material needed to deal with epileptic seizures and to prevent any damage to the patient (emergency trolley organized according to the treatment of neurological emergencies, antiepileptic drugs).
The occurrence of an epileptic seizure during a TMS experiment does not imply that seizures will reoccur in the future, nor that you should start taking a therapy for the prevention of seizures, nor that we should modify an eventual therapy already underway due to a seizure. The very few cases of TMS-induced seizures described in the literature no longer had episodes later, nor did they report health problems attributable to the event.
Since the TMS produces a magnetic field, people carrying static electric stimulators (e.g. cardiac stimulators, nerve stimulators, auditory implants) that would not work or would be damaged by the magnetic field cannot participate in the study. Also excluded from the examination are the carriers of particular metallic foreign bodies (for splinters, some prostheses, screws and nails) that could move if placed inside the magnetic field. Because the effects of TMS on the developing fetus are not known, pregnant women cannot participate in the study.

ADVERSE EVENT DETECTION:
The Principal Investigator will promptly inform the Local Ethics Committee of the Brescia Hospital of any adverse event incurred during the study.

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