Neuromuscular electrical stimulation prevents muscle disuse atrophy during leg immobilisation in humans

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Short title: NMES prevents short-term muscle disuse atrophy

Keywords: NMES, immobilisation, skeletal muscle, disuse atrophy

Clinical trial registration: NCT01513486

Word count: 5611

Abstract

Short periods of muscle disuse, due to illness or injury, result in substantial skeletal muscle atrophy. Recently we have shown that a single session of neuromuscular electrical stimulation (NMES) increases muscle protein synthesis rates. **Aim:** To investigate the capacity for daily NMES to attenuate muscle atrophy during short-term muscle disuse. **Methods**: Twenty-four healthy, young (23±1 y) males participated in the present study. Volunteers were subjected to 5 days of one-legged knee immobilisation with (NMES; *n*=12) or without (CON; *n*=12) supervised NMES sessions (40 min sessions, twice daily). Two days prior to and immediately after the immobilisation period, CT-scans and single leg one-Repetition Maximum (1RM) strength tests were performed to assess quadriceps muscle cross-sectional area (CSA) and leg muscle strength, respectively. Furthermore, muscle biopsies were taken to assess muscle fibre CSA, satellite cell content and mRNA and protein expression of selected genes. **Results:** In CON, immobilisation reduced quadriceps CSA by 3.5±0.5% ($P<0.0001$) and muscle strength by 9±2% ($P<0.05$). In contrast, no significant muscle loss was detected following immobilisation in NMES although strength declined by 7±3% ($P<0.05$). Muscle MAFbx and MuRF1 mRNA expression increased following immobilisation in CON ($P<0.001$ and $P=0.07$, respectively) whereas levels either declined ($P<0.01$) or did not change in NMES, respectively. Immobilisation led to an increase in muscle myostatin mRNA expression in CON ($P<0.05$) but remained unchanged in NMES. **Conclusion:** During short-term disuse, NMES represents an effective interventional strategy to prevent the loss of muscle mass, but it does not allow preservation of muscle strength. NMES during disuse may be of important clinical relevance in both health and disease.

Abbreviations

CT, Computed Tomography; CSA, Cross Sectional Area; DEXA, Dual Energy X-Ray Absorptiometry; FAK, Focal Adhesion Kinase; FOXO1, Forkhead box protein O1; FT, fibre typing; LAT1, Large Neutral Amino Acid Transporter 1; MAFBx, Muscle Atrophy F-box/Atrogen-1; mTOR, mammalian target of rapamycin; MuRF1, Muscle RING-finger protein-1; NMES, Neuromuscular Electrical Stimulation; PAT1, Proton-coupled amino acid transporter 1; PBS, phosphate-buffered saline; P70S6K, P70S6 kinase; RT, room temperature; SC, satellite cell; 1RM, 1-Repetition Maximum.

Introduction

Situations such as the recovery from illness or injury require otherwise healthy individuals to undergo short periods of bed-rest or limb immobilisation. Under these circumstances there is a rapid loss of skeletal muscle mass [1-3] that leads to reduced functional capacity [1-4], loss of muscle strength [5], impaired insulin sensitivity [6], a decline in basal metabolic rate [7, 8], and a concomitant increase in body fat mass [9-11]. As a consequence, the extent of disuse atrophy that occurs due to illness or injury has previously been identified as an important predictor of the duration of hospitalization and subsequent rehabilitation [12].

During periods of disuse, muscle atrophy occurs as a consequence of an imbalance between muscle protein synthesis and breakdown rates. Previous studies, employing either 10-14 days of bed rest [10, 13] or 2-6 weeks of limb immobilisation [2, 14-16] as models of disuse, have demonstrated impairments in both fasting and post-prandial muscle protein synthesis rates without any discernible changes in muscle protein breakdown [13, 17]. Maintaining a certain minimal level of physical activity during periods of muscle disuse can offset such impairments in post-absorptive or post-prandial muscle protein synthesis rates [11, 18] and, as such, attenuate muscle tissue loss [19, 20]. Unfortunately, in many clinical situations physical activity is temporarily not feasible or simply impossible and, thus, surrogates should be sought to alleviate muscle disuse atrophy.

Neuromuscular electrical stimulation (NMES) offers an attractive alternative way to allow muscle contraction, thereby acting as a surrogate for habitual physical activity during periods of muscle disuse due to illness or injury. Recently, we applied contemporary stable isotope methodology with repeated muscle biopsy sampling to demonstrate that a single session of NMES increases muscle protein synthesis rates for several hours *in vivo* in men [21]. Moreover, self-administered NMES has previously been shown to maintain muscle protein synthesis rates during long term recovery from tibia fracture [14], and clinically applied NMES has shown beneficial effects on skeletal muscle function in patients recovering from surgery [22, 23] or suffering from severe cardiac complications [24, 25]. However, to date, the capacity of supervised NMES as an interventional strategy to counteract the loss of muscle mass and strength during a short period of disuse remains to be established. This may be of important clinical relevance as the loss of muscle mass and strength during short periods of bed rest or immobilisation following illness or injury are believed to delay subsequent recovery and likely contribute substantially to the loss of muscle mass with aging [26, 27].

In the present study we investigate the efficacy of NMES as a means to attenuate skeletal muscle disuse atrophy. We hypothesized that a twice daily supervised NMES program could preserve skeletal muscle mass and attenuate the loss of muscle strength during a 5 day period of leg immobilisation. We assessed changes in muscle mass following 5 days of one-legged knee immobilisation using a full leg cast in 24 healthy young men with or without twice daily supervised NMES sessions. Muscle mass was assessed at a limb level using CT and DEXA scans, whereas muscle biopsies were obtained prior to and immediately after immobilisation to assess changes in muscle fibre type characteristics and relevant myocellular signalling.

Materials and Methods

Subjects

A total of 24 healthy young males (age: 23±1 y; body mass: 76±2 kg; body mass index [BMI] 22±1 kg/m^2) were included in the present study which was approved by the Medical Ethical Committee of the Maastricht University Medical Centre+ in accordance with the Declaration of Helsinki. Prior to the study, subjects completed a routine medical screening and general health questionnaire to ensure their suitability to take part. Exclusion criteria were: BMI below 18.5 or above 30 kg/m^2; any back, knee or shoulder complaints which may interfere with the use of crutches; type 2 diabetes mellitus (determined by HbA1c-values >7.0%); any family history of thrombosis; and/or severe cardiac problems. Furthermore, subjects who had performed structured and prolonged resistance type exercise training during the 6 months prior to the study were also excluded. All subjects were informed of the nature and possible risks of the experimental procedures, before their written informed consent was obtained. During screening, an estimation of one-repetition maximum (1RM) single leg knee extension strength (Technogym, Rotterdam, the Netherlands) was made using the multiple repetitions testing procedure [28].

Study design

After inclusion, subjects were randomly allocated into either the control (CON; *n*=12) or the neuromuscular electrical stimulation (NMES; *n*=12) group. The experimental protocol is depicted in **Figure 1.** Both groups underwent a 5 day period of muscle disuse induced via one-legged knee immobilisation by way of a full leg cast, either with (NMES group) or without (CON group) NMES performed twice daily under supervision at home. The leg to be immobilized was randomized and counter-balanced between left and right. On two separate test days, 48 h before and immediately after the immobilisation period, single slice computed tomography (CT) scans were performed at the mid-thigh of both legs, whole body dual energy x-ray absorptiometry (DEXA) scans were taken, leg volume was measured by anthropometry [29], a single muscle biopsy and venous blood sample were collected, and one-legged knee extension strength (1RM) was assessed.

Muscle mass and function

Forty eight h prior to, and immediately after the immobilisation period, subjects visited the laboratory in the fasted state for 2 identical test days (i.e. test days 1 and 2). During the test days, several measurements of muscle mass and function were performed. First, anatomical cross-sectional area (CSA) of the quadriceps muscle and whole thigh were assessed via a single slice CT scan (Philips Brilliance 64, Philips Medical Systems, Best, the Netherlands). The scanning characteristics were as follows: 120 kV, 300 mA, rotation time of 0.75 s, and a field of view of 500 mm. While the subjects were lying supine, legs extended and their feet secured, a 3 mm thick axial image was taken 15 cm proximal to the top of the patella. On test day 1, the precise scanning position was marked with semi-permanent ink for replication on test day 2. CT-scans were analysed for the CSA of the whole thigh muscle as well as the quadriceps by manual tracing using ImageJ software (version 1.46d, National Institute of Health, Maryland, USA)[30]. Thereafter, body composition and bone mineral content were measured via DEXA-scan (Hologic, Discovery A, QDR Series, Bradford, MA, USA). Whole-body and regional lean mass were determined using the system's software package Apex version 2.3. Leg volume of both legs was also assessed by anthropometry as described previously [29]. Maximal calf circumference of both legs was measured as part of the measurements to determine leg volume. Maximum strength was evaluated for each leg separately by one-repetition maximum (1RM) strength tests on a leg extension machine (Technogym, Rotterdam, the Netherlands). The estimations obtained during the screening visit were used to determine 1RM as described previously [31].

Blood and muscle sampling

During test day 1, fasting venous blood samples were collected to determine basal plasma glucose and insulin concentrations. Blood (10 mL) was collected into EDTA-containing tubes and directly centrifuged at 1,000g for 10 min at 4°C. Aliquots of plasma were immediately frozen in liquid nitrogen and stored at -80°C until further analysis. Plasma glucose concentrations (Glucose HK CP, ABX Diagnostics, ref. A11A01667, Montpellier, France) were analysed with a COBAS FARA semi-automatic analyser (Roche, Basel, Switzerland). Plasma insulin concentrations were determined by radioimmunoassay (Millipore, ref. HI-14K , Billerica, MA, USA). Additionally, during test day 1 and 2, a single muscle biopsy sample was collected from the leg previously selected for immobilisation. After local anaesthesia was induced, percutaneous needle biopsy samples were collected from the *vastus lateralis* muscle, approximately 15 cm above the patella [32]. Any visible non-muscle tissue was removed immediately, and part of the biopsy sample was embedded in Tissue-Tec (Sakura Finetek, Zoeterwoude, the Netherlands) before being frozen in liquid nitrogen-cooled isopentane, while another part was immediately frozen in liquid nitrogen. Muscle samples were subsequently stored at -80°C until further analyses.

Leg immobilisation

Forty eight h following test day 1, a full leg cast (randomized and counterbalanced for left and right legs) was applied in the plaster room of the Academic Hospital in Maastricht at 8:00 on the first day of the immobilisation period. The leg cast extended from ~5 cm above the ankle until ~25 cm above the patella (i.e. approximately halfway up the upper leg). The cast was set so the knee joint was placed at a ~30 degree angle of flexion to prevent subjects from performing weight-bearing activities with the casted leg. The immobilisation period always comprised 3 week days and 2 weekend days. Additionally, for subjects assigned to the NMES group, placement of the electrodes for NMES was determined prior to fitting the cast (described below) and a small ‘window’ (a rectangle of approximately 12 × 6 cm) was cut in the cast ~5 cm above the knee. Following the removal of this window, the section of cast was placed back from where it was removed and bandaged firmly in place. Subjects were given crutches and instructed on their correct usage before being provided with transportation home. Application of the cast signified the beginning of the immobilisation period which continued for 5 d, after which the cast was removed at 8.00 at the plaster room immediately prior to performing test day 2.

Neuromuscular electrical stimulation

For subjects allocated to the NMES group, two NMES sessions were performed each day at the subjects’ home for the duration of the 5 day immobilisation period (i.e. 10 sessions in total). Neuromuscular electrical stimulation sessions were performed in the morning (7.00-12.00) and afternoon (13.00-18.00), with a minimum of 4 h between sessions. During each session, with the subject lying supine with a pillow placed under the knee to obviate the flexion angle, the window was removed from the cast and electrodes were placed on the distal part at the muscle belly of the *m. rectus femoris* and the *m. vastus lateralis*, and at the inguinal area of both muscles. The position of the electrodes was re-marked each day with semi-permanent ink to ensure that location of the electrodes was not altered between sessions.

Stimulation was provided by an Enraf Nonius TensMed S84 stimulation device (Enraf Nonius, Rotterdam, the Netherlands) and 4, 2 mm-thick, self-adhesive electrodes (50 x 50 mm; Enraf Nonius), discharging biphasic symmetric rectangular-wave pulses. The NMES protocol consisted of a warm-up phase (5 min, 5 Hz, 250 μs), a stimulation period (30 min, 100 Hz, 400 μs, 5 s on (0.75 s rise, 3.5 s contraction, 0.75 s fall) and 10 s off), and a cooling-down phase (5 min, 5 Hz, 250 μs). Subjects set the intensity of the stimulation to a level at which full contractions of *m. quadriceps femoris* were visible and palpable, and the heel began to slightly lift. This protocol was based on our previous work [21] demonstrating an acute increase in muscle protein synthesis following a single bout of NMES and selected due to previous work using high-frequency (>60 Hz), high pulse duration (>250 μs) NMES [33, 34]. Researchers encouraged subjects to increase the intensity of the stimulation during each subsequent session to provide a 'progressive' stimulus.

Dietary intake

On the evening prior to both test days subjects received a standardized meal containing 2900 kJ providing 51 Energy% (En%) as carbohydrate, 32 En% as fat, and 17 En% as protein. Subjects completed weighted dietary intake records for the 5 day duration of the immobilisation period as well as on a separate consecutive 5 day occasion before the immobilisation period. The same 5 days of the week were selected for both recording periods. Dietary intake records were analysed with DieetInzicht software, based on NEVO table 2011.

Muscle analyses

The portion of the muscle biopsies frozen and mounted in Tissue-Tek was cut into 5μm thick cryosections using a cryostat at -20°C. Pre and post samples from one control and one NMES subject were mounted together on uncoated, pre-cleaned glass slides. Care was taken to correctly align the samples for cross-sectional fibre analyses. Muscle biopsies were stained for muscle fibre typing (FT) and satellite cell (SC) content as described in detail previously [35]. In short, slides were incubated with primary antibodies directed against myosin heavy chain (MHC)-I (A4.840, dilution 1:25; Developmental Studies Hybridoma Bank, Iowa City, IA), laminin (polyclonal rabbit anti-laminin, dilution 1:50; Sigma, Zwijndrecht, the Netherlands) and CD56 (dilution 1:40; BD Biosciences, San Jose, CA). The following appropriate secondary antibodies were applied: goat anti-mouse IgM AlexaFluor555, goat anti-rabbit IgG AlexaFluor647, and Streptavidin Alexa 488 (dilution 1:500, 1:400, and 1:200, respectively; Molecular Probes, Invitrogen, Breda, the Netherlands). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, 0.238 µM; Molecular Probes). Images were captured at 10x magnification with a fluorescent microscope equipped with an automatic stage, and analysed using ImageJ software (version 1.46r, National Institute of Health [30]). Mean numbers of 184±17 and 220±22 muscle fibres were analysed in the biopsy samples collected pre and post immobilisation, respectively.

The portion of the muscle biopsy sample immediately frozen in liquid nitrogen was used to determine mRNA and protein expression of several target genes as described previously [36]. In short, total RNA was isolated from 10-20 mg of frozen muscle tissue, which was then quantified spectrophotometrically. Thereafter, RNA purity was determined and cDNA synthesis was performed, and Taqman PCR was carried out as reported previously using 18S as a housekeeping gene [37]. Taqman primer/probe sets were obtained from Applied Biosystems (Foster City, USA) for the following genes of interest: mammalian target of rapamycin (mTOR), P70S6 kinase (P70S6K), myogenic factor 4 (myogenin), MyoD, myostatin, Atrogen-1/Muscle Atrophy F-box (MAFbx), Muscle RING-finger protein-1 (MuRF1), Forkhead box protein O1 (FOXO1), Focal Adhesion Kinase (FAK), large neutral amino acid transporter 1 (LAT1) and Proton-coupled amino acid transporter 1 (PAT1). C*t* values of the target genes were normalized to C*t* values of the internal control 18S, and final results were calculated as relative expression against the standard curve. Muscle samples (~40 mg) for Western blotting analyses were analysed as described previously [37]. The antibodies used in this study were anti Myostatin (52 kD; dilution 1:500; rabbit polyclonal IgG; Santa Cruz sc-6885-R), anti MyoD (37 kD; dilution 1:1000; rabbit polyclonal IgG; Santa Cruz sc-760), anti Myogenin (34 kD; dilution 1:500; rabbit polyclonal IgG; Santa Cruz sc-576) and anti α-actin (42 kD; dilution 1:160.000, mouse monoclonal IgM; Sigma A2172).

Statistics

All data are expressed as mean±standard error of the mean (SEM). Baseline characteristics between groups were compared by means of an independent samples t-test. Pre- versus post-intervention data were analysed using repeated-measures ANOVA with time (pre vs. post) as within-subjects factor and treatment (CON vs. NMES) as between-subjects factor. Pearson's Correlation Coefficient was used to test for significant correlations. For the muscle fibre analysis, fibre type (type I vs. type II) was added to the repeated-measures ANOVA as a within-subjects factor. In case of a significant interaction, paired t tests were performed to determine time effects within groups or within type I or II fibres and independent t tests for group differences in the pre- and post-intervention values. Statistical significance was set at $P<0.05$. All calculations were performed using SPSS version 20.0 (Chicago, IL, USA).

Results

Subjects

Subjects' characteristics are provided in **Table 1**. No differences between the control (CON) and neuromuscular electrical stimulation (NMES) group were observed for any of the parameters.

Dietary intake

During the 5 days of immobilisation the daily energy intake averaged 8.5±0.7 and 8.7±0.6 MJ per day in the CON and NMES group, respectively, with average daily protein intakes of 1.01±0.04 and 1.00±0.08 g/kg body weight/day. For both energy intake and protein intake, no significant interaction effects were found.

Neuromuscular electrical stimulation

The intensity of the NMES intervention for subjects in the NMES group averaged 20.8±1.6 mA during the first session and was progressively increased to 42.2±3.7 mA in the final session. The average NMES intensity across all sessions and all subjects was 30.6±2.2 mA.

Muscle mass

For quadriceps muscle CSA, a significant time*treatment interaction was observed in the immobilized leg (**Figure 2**; P<0.001**)**. Quadriceps CSA in the CON group had decreased by 3.5±0.5% (from 7504±342 to 7238±324 mm^2; P<0.001), whereas in the NMES group no significant decrease in quadriceps CSA was detected (from 7740±259 to 7675±254 mm^2: P=0.07). In agreement, a significant time*treatment interaction (P<0.001) was also observed for changes in

CSA of the whole-thigh muscle, which showed a 3.7±0.6% decrease in the CON group ($P<0.001$), with no changes in the NMES group (-0.5±0.4%: $P=0.192$). In the non-immobilized leg, quadriceps and thigh muscle CSA did not show any changes following 5 days of immobilisation in both the CON and NMES group.

In line with the data on muscle CSA, a significant time*treatment interaction was observed for leg lean mass ($P<0.05$). Subjects in the CON group lost on average 147±72 g of muscle tissue in the immobilized leg, representing 1.4±0.7% loss of leg muscle tissue ($P=0.066$). In contrast, the NMES group showed an increase of 209±82 g (1.9±0.7%) in the immobilized leg after 5 days of immobilisation ($P<0.05$). No changes over time in leg lean mass were detected in the non-immobilized leg of subjects in the CON and NMES group ($P>0.05$).

For leg volume and calf circumference, no changes over time (time effect, $P>0.05$) or between groups (interaction effect, $P>0.05$) were observed.

Scatter plots for correlations between NMES intensity and key outcome measures are presented as supplemental information in **Figure 5**. No significant correlations were found between the NMES intensity and delta quadriceps CSA (**Figure 5A**), delta muscle strength (**Figure 5B)**, and delta leg lean mass (**Figure 5C)**, respectively.

Muscle strength

For muscle strength, a significant main effect of time ($P=0.001$) was detected in the immobilized leg such that one-legged 1RM declined by 9.0±2.2% (from 77.9±3.9 to 71.1±4.1 kg) and 6.5±3.2% (from 78.3±4.5 to 72.9±4.4 kg) in the CON and NMES groups, respectively, with no differences between groups. Muscle strength in the non-immobilized leg increased in both groups (time effect,

$P<0.05$); from 78.8±4.4 to 81.5±4.9 kg in the CON group and from 76.9±3.1 to 81.9±3.4 kg in the NMES group.

Muscle fibre characteristics

Muscle fibre characteristics are displayed in **Table 2**. Before the intervention, no significant difference was observed in type I and type II muscle fibre CSA between groups. A significant time*treatment*fibre type interaction was observed for muscle fibre CSA ($P<0.001$). Separate analyses showed no significant change in both type I and type II muscle fibre CSA in the CON group after immobilisation. In contrast, we observed a significant increase in type II muscle fibre CSA in the NMES group over time (from 5885 ± 426 to 6412 ± 586 μm^2; $P<0.05$), whereas in type I fibres no time effect was observed ($P>0.05$). Fibre distribution showed no differences at baseline between groups, and did not change over time in both groups ($P>0.05$).

For myonuclear domain size, a significant time*treatment*fibre type interaction was observed ($P<0.05$) and an overall effect of fibre type ($P<0.001$), with larger myonuclear domain sizes in type II vs. type I fibres in both the CON and NMES group. No changes in type I myonuclear domain size were found ($P>0.05$), while a significant time*treatment interaction was observed in type II fibres ($P<0.05$) caused by a greater myonuclear domain in the NMES vs. CON group after immobilisation.

At baseline, no differences in SC content were observed between groups ($P>0.05$). In addition, no changes over time were found for type I and type II SC content expressed per muscle fibre, per millimetre squared, or as a percentage of the total number of myonuclei ($P>0.05$ for all three parameters).

mRNA and protein expression

Figure 3 and **4** display the relative expression in skeletal muscle mRNA of selected genes of interest in the CON and NMES group, two days prior to and immediately following 5 days of one-legged knee-immobilisation. No differences in mRNA expression of selected genes were observed between CON and NMES at baseline. For muscle myostatin mRNA expression, a significant time*treatment interaction was observed (**Figure 3A**; $P<0.05$). Separate analysis showed a 68% increase following immobilisation in the CON group ($P<0.05$), whereas a trend for a decline was observed in the NMES group ($P=0.075$). For muscle mRNA expression of MyoD (**Figure 3C)** and myogenin **(Figure 3E)** a significant increase was observed over time ($P<0.05$ and $P<0.01$, respectively), with no differences between groups.

A significant time*treatment interaction was observed for the mRNA expression of muscle MAFbx (**Figure 4A**; $P<0.001$) and MuRF1 (**Figure 4B**; $P<0.05$). MAFBx mRNA expression was upregulated in the CON group (48%; $P<0.001$), whereas in the NMES group a decline was observed (35%, $P<0.05$). MuRF1 mRNA expression tended to increase in the CON group (56%, $P=0.066$), while no change over time was observed in the NMES group ($P>0.05$). No significant changes occurred over time or between groups in the muscle mRNA expression of FOXO1 (**Figure 4C**), mTOR (**Figure 4E**) or FAK (**Figure 4D**). A significant time*treatment interaction was observed for the muscle mRNA expression of P70S6K (**Figure 4F**; $P<0.05$), with an 18% upregulation following immobilisation in the CON group ($P<0.01$), whereas no change was observed in the NMES group ($P>0.05$). Muscle mRNA expression of the amino acid transporters LAT1/SLC (**Figure 4G**) and PAT1 (**Figure 4H)** had significantly increased following immobilisation (both $P<0.05$), with no differences between groups.

Protein expression of myostatin, myoD and myogenin are presented in **Figure 3**. For both myostatin and MyoD, no changes in protein expression were observed (both *P*>0.05). Myogenin protein expression tended to increase following immobilisation (*P*=0.054) with no differences between groups (*P*=0.122 for time*treatment interaction).

Discussion

In the present study, we demonstrated that neuromuscular electrical stimulation (NMES) prevented skeletal muscle atrophy to occur during 5 days of one-legged knee immobilisation. However, NMES could not rescue the loss of muscle strength during this short period of disuse. Moreover, we report that the molecular changes associated with muscle disuse atrophy can largely be prevented by the daily application of NMES.

Skeletal muscle disuse leads to a loss of muscle mass and strength and is accompanied by numerous negative health consequences [1-4, 6-11]. Based on previous studies, the rate of muscle loss during experimental lower limb immobilisation is approximately 0.5% per day [27, 38]. However, this loss does not appear to be linear with higher rates of muscle loss occurring during the first few days of disuse [39]. In the present study we report that merely 5 days of one-legged knee immobilisation significantly decreased quadriceps muscle cross sectional area by 3.5% in a group of healthy young males (**Figure 2**; CON group), representing ~150 g of muscle tissue lost from the immobilized leg. When translating our observations of muscle loss in a single limb to a whole-body level, assuming that 60% of whole-body muscle loss occurs in the lower limbs, patients could lose as much as 1 kg of muscle tissue during 5 days of bed rest [5, 40]. This is consistent with previous studies investigating the impact of 10 days of bed-rest [13, 40]. Furthermore, the 5 days of leg immobilisation also resulted in a substantial 9.0±2.2% decline in leg strength. Clearly, these data demonstrate the impact of short periods of muscle disuse on muscle mass and strength and underline the clinical relevance to develop effective interventional strategies to attenuate muscle disuse atrophy and associated negative health consequences.

The use of NMES has been proposed as an interventional strategy to alleviate muscle loss in a variety of clinical conditions [14, 22-25]. Recently, we showed that a single NMES session stimulates muscle protein synthesis *in vivo* in men [21]. In the current study, we investigated whether the application of daily NMES could attenuate the loss of muscle mass during a short period of muscle disuse. Strikingly, the application of supervised NMES performed twice daily on the immobilized leg entirely prevented any disuse atrophy (**Figure 2**), with no measurable loss of muscle observed in the NMES group (-0.8±0.4%; $P>0.05$). Given the inherent variability of the measurement of muscle fibre size [41], we were unable to detect specific muscle fibre atrophy following only 5 days of disuse in the control group (**Table 2**). However, we did detect a small but significant increase in type II muscle fibre size following immobilisation in the group receiving NMES. These data suggest that high-frequency NMES may exert its protective effect on skeletal muscle disuse atrophy predominantly through the recruitment of type II muscle fibres. This is of significant relevance as muscle loss due to more prolonged disuse [42, 43] and/or aging [44, 45] has been attributed to specific type II muscle fibre atrophy [41]. It is important to view the present data in the context of the potential clinical benefits of applying NMES to preserve muscle mass during relatively short periods of muscle disuse. Previously, NMES has generally been applied during rehabilitation [46, 47], when muscle mass has already been lost and has to be regained. However, in the present study we clearly demonstrate the relevance of applying NMES *during* a period of disuse or bed rest to prevent muscle tissue loss.

Muscle disuse atrophy is generally accompanied by a substantial decline in muscle strength and impairments in functional capacity [1-3]. Previous studies performing one-legged knee immobilisation have reported a decline in muscle strength ranging from 0.4 [48] to 4.2% per day [49] with an average muscle strength loss of ~1.3% per day [27]. In the present study, we report that 5 days of limb immobilisation resulted in a 9.0% loss of leg muscle strength (representing an average daily loss of 1.8% per day). Consistent with earlier reports [4, 49, 50], we show a greater relative decline in muscle strength when compared to the loss of muscle mass. This is in agreement with previous suggestions that neuromuscular deconditioning during the early stages of training or disuse is mainly responsible for the rapid changes in muscle strength [51, 52]. This also explains why the decline in muscle strength in the control group was only partially rescued with NMES (-6.5±3.2%). We speculate that the application of NMES will likely further attenuate muscle strength loss during more prolonged periods of muscle disuse, when muscle mass loss becomes the key determinant of the decline in muscle strength. In agreement, previous work assessing the impact of prolonged NMES training has been shown to effectively increase muscle strength in healthy young subjects [33, 34], in CHD patients [53] and in patients suffering from septic shock [54].

Aside from assessing the impact of NMES on muscle mass and strength during a period of disuse, we also investigated some of the myocellular mechanisms that may be responsible for the NMES mediated prevention of muscle mass loss during immobilisation. Skeletal muscle satellite cells (SCs) are essential for repair, maintenance and growth of myofibres [55-57]. Moreover, we have previously reported that type II fibre specific atrophy associated with aging [58] and spinal cord injury [59] is also accompanied by a decline in SC content in these fibres. In the present study, we hypothesized that a better maintenance of muscle SC content in the NMES group contributes to the preservation of muscle mass. However, short term immobilisation did not alter SC content in either type I or II fibres in either the control or NMES group (**Table 2**). As such, the present data suggest that changes in SC content are not instrumental in the early development of disuse atrophy, nor the NMES mediated prevention of muscle loss. However, it cannot be ruled out that the rate of SC proliferation may be of more relevance during muscle atrophy (or NMES mediated prevention of muscle loss) observed over a more prolonged period of disuse [60]. Furthermore, we determined the mRNA and protein expression of key signalling proteins thought to be important in the regulation of muscle maintenance. Myostatin is regarded as a negative regulator of muscle mass *in vivo* [61, 62], primarily by inhibiting myogenesis [63, 64] via its inhibitory action on the myogenic regulatory factors [65], notably MyoD and myogenin [66, 67]. Consistent with the proposed role of myostatin, we report an increased mRNA expression in the CON group that was prevented in the NMES group (**Figure 3**). Moreover, the significant increase in the mRNA expression of MyoD and myogenin seemed to be larger in the NMES group but was observed in both groups, while this did not result in an increased muscle protein expression (**Figure 3**). Collectively these data are consistent with a role for myostatin in the NMES mediated maintenance of muscle mass during disuse.

Increased rates of muscle protein breakdown have been suggested to play a role in short term (<10 days) muscle disuse atrophy [27, 68]. Muscle protein breakdown in humans is thought to be regulated primarily by the ubiquitin-proteasome pathway, with key roles for the ubiquitin ligases MAFbx and MuRF1 [69, 70], and their upstream transcription factor FOXO1 [71]. In accordance, in the present study we report that both MAFbx and MuRF1 mRNA expression increase with immobilisation (**Figure 4**). Strikingly, these effects were prevented in the NMES group, suggesting that NMES may also help to preserve muscle mass during disuse by preventing an increase in muscle protein breakdown.

In the present study we applied NMES to the quadriceps only. This muscle group is particularly susceptible to muscle loss during whole body disuse [5] and is functionally important to allow proper performance of daily living activities. From a clinical perspective, it could be speculated that multiple muscle groups should be targeted with NMES to ensure muscle mass maintenance during whole body disuse. Although extending the use of NMES to multiple muscle groups could introduce practical constraints (e.g. skin irritation, antagonistic contractions, time constraints), optimizing such protocols will allow (more) effective clinical use of NMES. Given the role of skeletal muscle mass in metabolic homeostasis, muscle preservation during disuse would likely have a positive impact on preserving both metabolic health [72] and functional capacity.

The present study clearly demonstrates that merely a few days of disuse will lead to substantial loss of muscle mass and strength. Furthermore, NMES is identified as an effective interventional strategy to preserve muscle mass during such short periods of disuse. These data are of important clinical relevance as hospitalization following acute illness or injury is generally accompanied by a hospital stay of ~6 days [73]. The loss of muscle mass and strength during such short (successive) periods of muscle disuse impairs functional capacity and hinders the subsequent rehabilitation upon discharge. In fact, it is now much speculated that the development of sarcopenia in the older population is, at least partly, attributed to the muscle loss that is experienced during short, successive periods of muscle disuse due to illness or injury occurring over the latter 2-3 decades of our lifespan [26, 39]. The use of NMES could also be of particular relevance to other patient groups and populations suffering from muscle atrophy, such as athletes recovering from injury [74], mechanically ventilated patients [54], spinal cord injured subjects [59], and post-surgery patients [23]. Preventing or attenuating the loss of muscle mass and strength during limb immobilisation or bed rest likely minimizes the burden of muscle disuse, shortens hospital stay, and facilitates subsequent rehabilitation in both health and disease.

In conclusion, NMES represents an effective interventional strategy to prevent the loss of muscle mass *during* short periods of muscle disuse. This is likely attributed to a stimulation of muscle protein synthesis and suppression of muscle protein breakdown. NMES forms a feasible strategy to prevent muscle loss and support subsequent rehabilitation during short periods of muscle disuse due to illness or injury.

Acknowledgements

The authors greatly acknowledge the cooperation and enthusiasm of the staff in the casting room of the Academic Hospital Maastricht. Furthermore, the assistance of Mellanie Geijen during the data collection, and the technical assistance by Joan M.G. Senden and Antoine Zorenc are very much appreciated.

Conflict of interest

No conflicts of interest are declared by the authors.

Author contributions

The study was performed at Maastricht University, Maastricht, the Netherlands. M.L.D., B.T.W. and L.J.C.v.L. did the conception and design of the study; M.L.D., B.T.W., T.S. and C.L.P.O. performed the experiments; M.L.D. and B.T.W. analysed the data; M.L.D., B.T.W., T.S., L.B.V. and L.J.C.v.L. interpreted the results; M.L.D. drafted the manuscript; M.L.D., B.T.W., T.S., C.L.P.O., L.B.V. and L.J.C.v.L. edited and revised the manuscript. All authors approved the final version of the manuscript.

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Tables

Table 1: Subjects' characteristics

	CON (*n*=12)	NMES (*n*=12)
Age (y)	22 ± 1	23 ± 1
Body mass (kg)	74.4 ± 3.5	77.7 ± 2.2
Height (m)	1.84 ± 0.03	1.84 ± 0.02
BMI (kg/m^2)	21.9 ± 1.0	23.1 ± 0.7
Leg volume (L)	8.23 ± 0.50	8.15 ± 0.30
Glucose (mmol/L)	5.01 ± 0.11	5.08 ± 0.07
Insulin (mU/L)	8.77 ± 0.70	8.93 ± 1.01
HOMA-index	1.97 ± 0.18	2.03 ± 0.26
Glycated haemoglobin (%)	5.1 ± 0.1	5.4 ± 0.1

Values are means±SEM. Abbreviations: BMI, Body Mass Index; HOMA-index, Homeostatic Model Assessment Index [75]

Table 2: Muscle fibre characteristics

		CON		NMES	
	Fibre type	Pre	Post	Pre	Post
Muscle fibre CSA (μm^2)	I	5259 ± 328	5378 ± 392	5676 ± 424	5493 ± 430
	II	6680 ± 328 *	6316 ± 441	5885 ± 426	6412 ± 586 †
% Fibre (number)	I	43 ± 3	45 ± 4	52 ± 3	46 ± 3
	II	57 ± 3 *	55 ± 4	48 ± 3	54 ± 3
Nuclei per fibre	I	2.8 ± 0.2	2.9 ± 0.2	2.9 ± 0.2	2.7 ± 0.2
	II	3.3 ± 0.1	3.3 ± 0.2	2.9 ± 0.2	2.9 ± 0.2
Myonuclear domain (μm^2)	I	1910 ± 57	1848 ± 68	1944 ± 87	1997 ± 63
	II	2057 ± 103 *	1935 ± 79	2004 ± 89	2233 ± 83 #
Number of SCs per fibre	I	0.090 ± 0.007	0.109 ± 0.009	0.115 ± 0.011	0.106 ± 0.010
	II	0.072 ± 0.006	0.075 ± 0.007	0.075 ± 0.011 *	0.060 ± 0.007
Number of SCs per mm^2	I	17.3 ± 0.3	20.8 ± 1.7	20.8 ± 2.0	19.2 ± 1.4
	II	11.5 ± 1.4 *	12.5 ± 1.6	12.5 ± 1.4 *	9.6 ± 1.0
SCs/myonuclei (%)	I	3.3 ± 0.2	3.8 ± 0.3	4.0 ± 0.4	3.8 ± 0.3
	II	2.2 ± 0.2 *	2.4 ± 0.3	2.5 ± 0.3 *	2.1 ± 0.2

Data represent means±SEM. Abbreviations: CSA, Cross sectional area; SC, satellite cell; SCs/myonuclei (%), the number of SCs as a percentage of the total number of myonuclei (i.e. number of myonuclei + number of SCs). * Significantly different from type I fibre value (P<0.05). † Significantly different from pre value in NMES group. # Significantly different from CON post-immobilisation value (P<0.05)

Figure legends

Figure 1: Schematic representation of the experimental protocol. NMES = Neuromuscular electrical stimulation

Figure 2: Cross-sectional area (CSA) of *m. quadriceps femoris* in the CON and NMES group, before and after 5 days of one-legged knee immobilisation, as measured by single-slice CT scan. Data were analysed with a Repeated Measures ANOVA, and demonstrated a significant time*treatment interaction (P=0.001). Data are expressed as means±SEM. * P<0.05; significantly different when compared with pre-immobilisation values.

Figure 3: Skeletal muscle mRNA expression of myostatin, MyoD and myogenin in the CON and NMES group before and after 5 days of one-legged knee immobilisation. Data were analysed with a Repeated Measures ANOVA, and expressed as means±SEM. * P<0.05; significantly different when compared with pre-immobilisation values.

Figure 4: Skeletal muscle mRNA expression of selected genes of interest in the CON and NMES group before and after 5 days of one-legged knee immobilisation. Data were analysed with a Repeated Measures ANOVA. * P<0.05; significantly different when compared with pre-immobilisation values. Data are expressed as means±SEM. Abbreviations: MAFbx, Muscle Atrophy F-box; MuRF1, Muscle RING-finger protein-1; FOXO1, Forkhead box protein O1; FAK, Focal Adhesion Kinase; LAT1, large neutral amino acid transporter 1; PAT1, Proton-coupled amino acid transporter 1.