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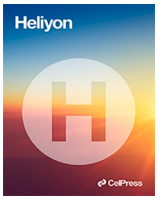

Research article

Frequency-dependent effects of superimposed NMES on spinal excitability in upper and lower limb muscles☆

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ABSTRACT

Superimposing neuromuscular electrical stimulation (NMES) on voluntary contractions has proven to be highly effective for improving muscle strength and performance. These improvements might involve specific adaptations occurring at cortical and spinal level. The effects of NMES on corticospinal activation seem to be frequency dependent and differ between upper and lower limb muscles. The aim of this study was to investigate acute responses in spinal excitability, as measured by H-reflex amplitude of flexor carpi radialis (FCR) and soleus (SOL) muscles, after NMES superimposed on voluntary contractions (NMES + ISO) at two different pulse frequencies (40 and 80 Hz). Conditions involved fifteen intermittent contractions at submaximal level. Before and after each condition, H-reflexes were elicited in FCR and SOL muscles.

H-reflex amplitudes increased in FCR and SOL following both NMES + ISO at 40 and 80 Hz. The potentiation of the H-reflex was greater following the 40 Hz condition compared to 80 Hz, although no differences between muscles emerged.

These findings indicated that superimposing NMES has an excitatory effect on spinal motoneurons in both upper and lower limb muscles with an overall greater response after low frequency NMES. Such facilitation could be associated to enhanced somatosensory stimuli conjunctly with higher supraspinal downward commands.

1. Introduction

The use of neuromuscular electrical stimulation (NMES) has gained substantial prominence in the last two decades due to its effectiveness in improving or restoring muscle function in both healthy individuals and those with injuries. Particularly, NMES superimposed on voluntary contractions has demonstrated the potential to enhance motor performance even more than voluntary exercise or passive NMES alone [1–5]. Additionally, superimposing NMES seems to facilitate motor-neuronal and corticospinal excitability, which, in turn, could underly specific neural adaptations that are associated with enhancements in muscle function [6–8].

However, the effects of NMES on corticospinal excitability and motor unit recruitment and activation seem to be frequency dependent [9,10]. In clinical settings, NMES is commonly administered at relatively low pulse frequencies (20–50 Hz), as the

☆ We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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smoothness and strength of the evoked contraction are thought to be improved [11], whereas the torque appears to decline rapidly when NMES is administered at higher frequencies [12–14]. There is some evidence that delivering low-frequency (30–50 Hz) NMES in mammals seems to have a strong influence on the recruitment of slow-twitch fatigue-resistant fibers [15,16] as well as promoting fast-to-slow conversion of the muscle fiber phenotype [17–19]. On the contrary, some authors reported that delivering NMES at higher frequencies (80–100 Hz) could lead to a preferential recruitment of fast-twitch fibers through increased temporal summation and more sustained depolarization [15,20]. However, some studies indicated that higher NMES frequencies can evoke muscle contractions involving a greater central contribution, whereas lower frequencies appear to excite more peripheral pathways through predominant recruitment of motor axons [7,21,22]. This might imply a further differentiation of motor unit recruitment in response to different stimulation frequencies.

Furthermore, there is evidence that cortical and spinal responses to NMES may considerably differ between upper and lower limbs [23]. Notably, the study of Mang et al. (2011) suggested that NMES facilitates corticospinal excitability in both target and non-target muscles when it is applied on the lower limb and increases excitability only in target muscle when it is applied on the upper limb, possibly due to the specificity of afferent projections to the cortex. However, there is still a limited knowledge on the differences in cortical and spinal plasticity in upper and lower limbs in response to NMES.

The Hoffmann reflex (H-reflex) has been considered a valid method for evaluating the adaptive plasticity of neural patterns at the spinal level in humans [24–26]. As a result, H-reflex has been commonly used to investigate the acute effects of different exercise modalities on spinal excitability, including NMES. Recent evidence exhibited that short bouts of NMES superimposed on voluntary contractions induced an acute potentiation of the soleus H-reflex, both when NMES was applied over the motor points of the triceps surae muscle [27–29] and over the posterior tibial nerve [30]. Moreover, a pilot study conducted by our research group confirmed this pattern of results in the upper limb muscles (see Supplementary Material). H-reflex is commonly measured, with high reliability, in the flexor carpi radialis muscle (FCR), and the soleus muscle (SOL) in the upper and lower limbs, respectively [26,31–33]. These two muscles activate rather differently during motor tasks. SOL activation primarily occurs during weight-bearing activities and is predominantly engaged in maintaining posture and propelling the body forward during locomotion. Conversely, FCR mainly activates during tasks requiring fine motor control and precise manipulation of objects. Moreover, due to these differences activation patterns and function, SOL and FCR seem to have different supraspinal control [34]. Furthermore, the FCR of mammals seems to have a higher distribution of fast twitch fibers than slow twitch ones [35–37]. According to Mizuno and colleagues' (1994) study on human biopsy samples, the human FCR has about 60 % of fast twitch fibers [38]. On the other hand, several authors indicated that the human SOL is composed for the most part of slow twitch fibers, with a percentage range of 70–80 % [39–42]. Therefore, there could be noticeable differences between H-reflexes elicited in the upper and lower limbs in response to different NMES protocols, and these could likely be attributable to distinct motoneuron's synaptic connectivity [26]. Nevertheless, to the best of the authors' knowledge, there are no studies that evaluated how different NMES pulse frequencies influence spinal reflex responses in upper and lower limbs.

In light of these considerations, the aim of this study was to assess the effects of NMES superimposed on voluntary contractions, applied with two different pulse frequencies (40 and 80 Hz), on H-reflex amplitude in the FCR and SOL muscles. We hypothesized that both SOL and FCR H-reflex amplitudes would increase after 40 and 80 Hz, based on the findings of previous studies [27,28,30,43]. Specifically, we expected that lower NMES frequency would lead to greater spinal excitability in muscles with high prevalence of slow twitch fibers, such as the SOL muscle. On the other hand, we expected that higher frequency would increase spinal excitability in muscles composed mainly of fast twitch fibers, such as FCR muscle.

Table 1
Characteristics of the participants. RT = resistance training; ET = endurance training.

Participants	Gender	Age	Weight (kg)	Height (m)	Physical activity	Frequency (weekly)
P01	Female	30	63	1.71	Circuit, RT	4
P02	Male	25	66	1.76	Boxing	3/4
P03	Male	30	82	1.82	Climbing, biking	3/4
P04	Male	25	67	1.75	Football, running	2/3
P05	Male	30	80	1.82	Volleyball	3/4
P06	Female	30	61	1.63	Aerobic, RT	2/3
P07	Male	26	65	1.73	RT	3
P08	Male	25	70	1.75	Tennis, ET	3
P09	Male	33	83	1.81	Climbing, RT	2/3
P10	Male	24	86	1.90	RT, aerobic	3/5
P11	Female	24	51	1.66	RT	2/3
P12	Male	26	70	1.75	RT	2
P13	Male	21	95	1.78	RT	3/4
P14	Female	19	60	1.65	RT	3
P15	Female	26	58	1.70	Climbing	3
P16	Male	28	70	1.73	Football	4/5
P17	Female	24	58	1.68	RT	3
P18	Female	28	59	1.58	Climbing	3/4
P19	Female	27	61	1.56	RT	3/4
P20	Male	31	82	1.82	Football, ET	4

Data are reported as indicated by the participants.

2. Materials and Methods

2.1. Participants

Twenty healthy volunteers (twelve males and eight females, mean ± SD age: 27 ± 3.5 years; mass: 69.4 ± 11.6 kg; height: 1.73 ± 0.08 m; BMI: 23.1 ± 2,4 kg/m^2), with no history of neurological or orthopedic disorders, volunteered to participate in the study. Individuals who engaged in physical activity for at least 2 times a week for at least 60 min per session were included in the study. The study was approved by the institutional ethics review board (IRB University of Rome "Foro Italico", CAR 131/2022, Approved on April 5th, 2022) and all participants gave informed written consent before participating. Participants' characteristics are summarized in Table 1.

2.2. Experimental design

A single group, repeated measures study design was adopted for this study which included four experimental conditions: (a) NMES superimposed on voluntary contractions (NMES + ISO) of the wrist flexor muscles, delivered with a pulse frequency of 40 Hz on the flexor carpi radialis muscle (FCR); (b) NMES superimposed on voluntary contractions of the wrist flexor muscles, delivered with a pulse frequency of 80 Hz on the FCR; (c) NMES superimposed on voluntary contractions of the plantar flexor muscles delivered with a pulse frequency of 40 Hz on the triceps surae muscle; (d) NMES superimposed on voluntary contractions of the plantar flexor muscles delivered with a pulse frequency of 80 Hz on the triceps surae muscle. Conditions were administered in a random order during a single experimental session, which lasted between 2.5 and 3 h. Each condition involved 15 intermittent contractions (6 s contraction/6 s rest) for a total duration of 4 min. Participants had a 15 min period of recovery between conditions [28,29]. The number and duration of contractions were selected in order to prevent development of muscle fatigue while at the same time modulating spinal excitability, according to previous studies' reports [27,28,44,45]. All the procedures were performed on each participant's dominant leg and arm. Leg dominance was determined as the limb preferred for hopping or kicking a ball [46] and the arm dominance was determined using the 10-item version of the Edinburgh Handedness Inventory [47]. Prior to the beginning of the experimental conditions, participants performed two maximal voluntary isometric contraction (MVIC) assessments per each limb, and an assessment of the H-reflex recruitment curve for the FCR and the SOL, respectively. Each exercise condition was preceded by assessments of a baseline (PRE) H-reflex and immediately followed by a post-treatment (POST) H-reflex assessment, and a repetition of the MVIC to determine whether muscle fatigue had arisen. The study protocol is illustrated in Fig. 1.

2.3. Surface electromyographic recordings (sEMG)

Surface electromyography (sEMG) was recorded at a sampling frequency of 2000 Hz, using a 64 EMG channel device with recording system with Wi-Fi communication (Sessantaquattro, OT Bioelettronica, Turin, Italy). As shown in Fig. 2b, for the upper limb, two self-adhesive electrodes (diameter 24 mm, Kendall Arbo H124SG, Neustadt/Donau, Florence, Germany) were placed on the one-third of the distance (~5–6 cm) on the diagonal line from the medial epicondyle of the humerus to the FCR muscle insertion, between

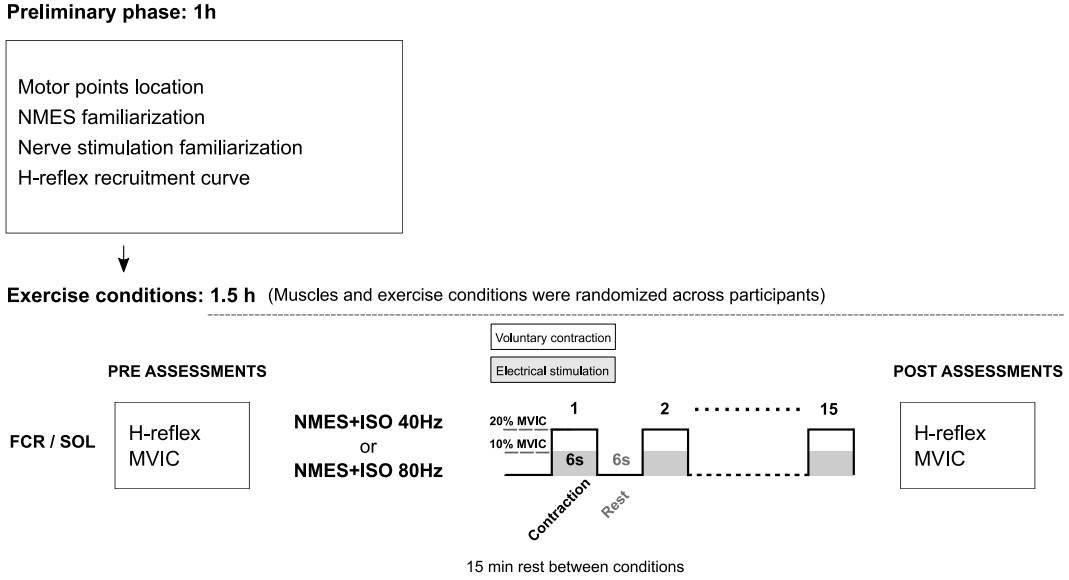

Fig. 1. Diagram of the experimental protocol. NMES + ISO neuromuscular stimulation superimposed on voluntary isometric contraction (randomly at 40 Hz or 80 Hz).

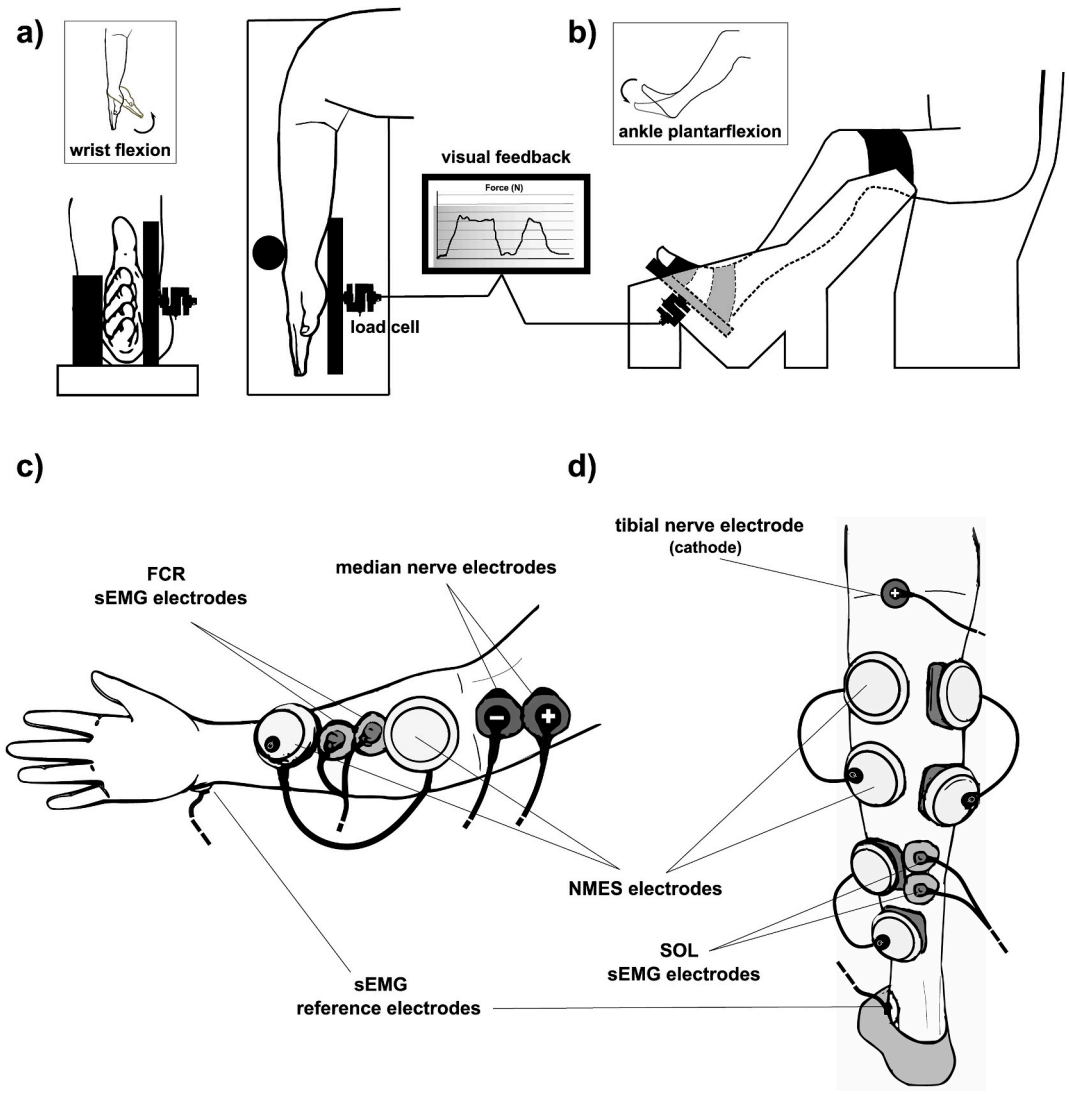

Fig. 2. a) Participant's arm/wrist and leg positioning on the dynamometer during the assessments of isometric wrist flexor and plantar flexor muscles force. b) Electrodes (recording and stimulating) positioning for the forearm muscles and calf muscles. Round pods represent the neuromuscular stimulator active wireless electrodes placed in a bipolar configuration on the FCR for the forearm muscles and on the GM, GL and SOL for the calf muscles. Bipolar sEMG electrodes were placed on the FCR and SOL muscles. For median nerve stimulation the cathode was placed proximally with respect to the cubital fossa and the medial epicondyle of the humerus, just above the elbow, under the curve of the biceps brachii muscle while the anode was placed about 2 cm below the cathode. For tibial nerve stimulation the cathode was placed in the popliteal fossa while the anode was placed above the patella (not shown in Fig. 2d).

the radial styloid and the II and III metacarpal bones, about 24 mm (center to center) apart [26]. For the lower limb, two pre-gelled, self-adhesive, electrodes (diameter 24 mm, Kendall Arbo H124SG, Neustadt/Donau, Florence, Germany) were placed about 24 mm apart on the soleus muscle (SOL), 2–3 cm below the gastrocnemii musculotendinous junction with Achille's tendon [25]. Before applying the surface electrodes, participants' skin was shaved and gently abraded with an abrasive paste to keep impedance below 5 kΩ and promote electrical signal transmission.

2.4. Maximum voluntary isometric contraction (MVIC)

A multi-joint isometric dynamometer (OT Bioelettronica, Turin, Italy) was used to assess the MVIC of the plantar flexor and wrist flexor muscles. For the assessment of isometric wrist flexor muscles force, participants sat comfortably on a chair, with the hip at 90° (0° = neutral hip position), the arm at 20° (0° = neutral arm position), the elbow at 120° (0° = full elbow flexion) and the wrist at 0° (0° = neutral wrist position) with the styloid process aligned with the axis of the dynamometer, the hand firmly secured to the device hand plate and the fingers at 0° (0° = neutral fingers position) (Fig. 2a). For the assessment of isometric plantar flexor muscles

force, participants sat on a chair, with the hip at 90° on the sagittal plane (0° = neutral hip position), the knee at 60° (0° = full knee extension) and the ankle at 0° of ankle plantar-dorsi flexion (0° = foot orthogonal to the shank axis) with the lateral malleolus aligned with the axis of the dynamometer, the foot firmly secured to the device foot plate and their trunk and knee fastened by instrumented belts [27] (Fig. 2b). After a 5 min period of warm-up and familiarization, during which participants performed 20 submaximal isometric contractions, the MVIC assessment consisted of a rapid increase to a maximum in the force exerted by the plantar flexor muscles and wrist flexor muscles. Participants had visual feedback of their performance on a computer screen and were verbally encouraged to promote their maximal isometric contraction and maintain it for at least 2 s before relaxing. Two attempts were performed, with each attempt being separated by 3 min rest to minimize fatigue. MVIC was chosen as the largest 500 ms average achieved within a force recording. The selected MVIC was then used to define a target isometric wrist flexor and plantar flexor muscles force as 20 % MVIC, which represents the constant force that participants were required to achieve during the two experimental conditions per limb. This force level was chosen based on previous research investigating changes in spinal activation following acute non-fatiguing NMES protocols [27,29,30,48].

2.5. Neuromuscular electrical stimulation (NMES)

A muscle stimulator (Chattanooga Wireless Professional, DJO Global, Vista, CA, USA) was used to deliver NMES over the FCR and the TS to evoke low intensity muscle contractions superimposed on voluntary isometric contractions. The stimulator produced a rectangular, balanced biphasic pulse and was safely handled and controlled by the investigator. Self-adhesive electrodes (50 × 50 mm, Compex Dura-Stick® Plus a Snap, DJO Global, Vista, CA, USA) with positive polarity were placed over the motor points of the FCR, and of the gastrocnemius lateralis (GL), gastrocnemius medialis (GM), and SOL (Fig. 2c and d). Motor points were identified at the beginning of the experimental session with a handheld anode ball electrode in accordance with the device user's guide. Four self-adhesive electrodes with negative polarity were placed on each muscle about 3 cm above the electrodes with positive polarity that were located on the motor points. NMES was delivered with a pulse frequency of 40 Hz and 80 Hz and a pulse width of 400 μs to effectively stimulate the TS and FCR while at the same time promoting the highest comfort during stimulation as reported in previous investigation [6,27,45]. Before the beginning of the experimental conditions, participants familiarized with the electrical stimuli, which were delivered at low current intensity, for about 15 min. During the experimental conditions, current pulse intensity was set to generate half of the force target (10 % MVIC) and participants were asked to voluntarily contract their plantar flexor and wrist flexor muscles to achieve the full force target of 20 % MVIC. Participants were asked to relax their plantar flexor and wrist flexor muscles before the first and after the tenth contraction, while the experimenter adjusted the pulse intensity to ensure that the force produced corresponded to half of the force target throughout the 15 contractions. If participants reported any signs of pain or discomfort, the application of NMES was immediately interrupted. For the FCR muscle, the NMES + ISO current intensity was 12.8 mA (range 7.6–19.3 mA) and 12.6 mA (range 7.6–17.8 mA) for the 40 Hz and 80 Hz conditions, respectively. For the SOL muscle, current intensity was 16.7 mA (range 10.7–24 mA) and 16.8 mA (range 10–25.2 mA) for the 40 Hz and 80 Hz conditions, respectively.

2.6. Hoffmann reflex (H-reflex) and motor wave (M-wave) recording

Single rectangular biphasic pulses, with a duration of 1 ms, were delivered to the median and the tibial nerve, respectively, using a constant voltage electrical stimulator (Digitimer DS7A, Hertfordshire, AL7 3BE, England, UK). For the FCR, the optimal stimulation site was located, using a hand-held anode ball electrode, proximally with respect to the cubital fossa and the medial epicondyle of the humerus, just above the elbow, under the curve of the biceps brachii muscle [26]. A self-adhesive cathode (diameter 24 mm, Spes Medica, Genova, Italy) was placed in the selected stimulation site and firmly secured with medical adhesive tape while the anode was placed distally from the cathode, about 2 cm below (Fig. 2c and d). The optimal stimulation site for the SOL was located, using a hand-held anode ball electrode, in the popliteal fossa. As described in the study by Borzuola et al. (2020), a self-adhesive cathode (diameter 24 mm, Spes Medica, Genova, Italy) was placed in the selected stimulation site and firmly secured with medical adhesive tape while the anode (50 × 50 mm, Compex Dura-Stick® Plus a Snap, DJO Global, Vista, CA, USA) was secured anteriorly on the knee above the patella (Fig. 2d). The FCR and SOL H-reflex recruitment curves were then obtained according to previously adopted procedures [27,49]. Briefly, low intensity single electrical stimuli, at increasing intensity (1 mA steps), were delivered to the median nerve for the FCR, and the popliteal fossa for the SOL, from the smallest detectable H-reflex until maximal motor wave (M-max) was achieved. Each stimulus induced an involuntary contraction of the FCR and the SOL, which was recorded via sEMG and visually monitored by the investigator immediately after the stimulus. Peak-to-peak analysis of the sEMG trace was used to define the amplitude of H-reflexes and M-waves. Each stimulus was delivered unevenly spaced from 4 to 6 s, to prevent participants from expecting the following stimulus and to reduce any effect from post activation depression [50]. The test reflex stimulus intensity was determined to obtain an H-reflex on the ascending limb of the recruitment curve with a peak-to-peak amplitude lying between 80 and 85 % of the maximal H-reflex (H-max), as detailed in previous research [26,51]. A small test M-wave (M-test), corresponding to the test H-reflex (H-test), was selected, and monitored throughout the entire experiment in order to control the stimulus consistency and repeatability before and after each condition. If the evoked H-reflexes showed an M-wave with an amplitude within a range of ±5 % of the selected M-test, the measure was accepted and kept for further analysis. We collected a minimum of ten acceptable H-reflexes during each neuromechanical assessment before and after each condition. Two M-max responses were also recorded after the test H-reflexes. All H-reflex and M-wave amplitudes were normalized to the M-max amplitude, averaged within trials, and used for off-line analysis.

2.7. Data analysis and statistics

The sample size was determined a priori based on a statistical power analysis (G*Power v 3.1.9.4; $\alpha = 0.05$, power = 0.90, effect size = 0.45) for repeated-measure ANOVA [52] in agreement with previous studies investigating spinal reflex modulation following acute exercise [27,30,53]. All acquired data were analyzed using a custom Matlab code (Matlab 2018b, Mathworks Inc., Natick, MA, USA). For each stimulation, sEMG traces of FCR and SOL were checked to determine if any pre-activation had occurred before the reflex measure. sEMG traces that revealed pre-activation of either FCR or SOL were removed from data analysis and thus, the corresponding H-reflex measure. Pre-activation was analyzed by evaluating the RMS of the sEMG trace in the 100 ms prior to the H-reflex stimulus artefact. Statistical analysis was performed using IBM SPSS 24.0 (IBM Corp., Armonk, NY, USA). The Shapiro-Wilk test and the Mauchly test were used to check the data for normality and sphericity, respectively. A 2 × 2 × 2 repeated measures analysis of variance (ANOVA) was used to evaluate statistical difference in H-reflex, M-wave and MVIC measures between different NMES + ISO Frequencies (40 Hz, 80 Hz), between Muscles (FCR, SOL) and over Time (PRE, POST) as within-participants factors. When a significant

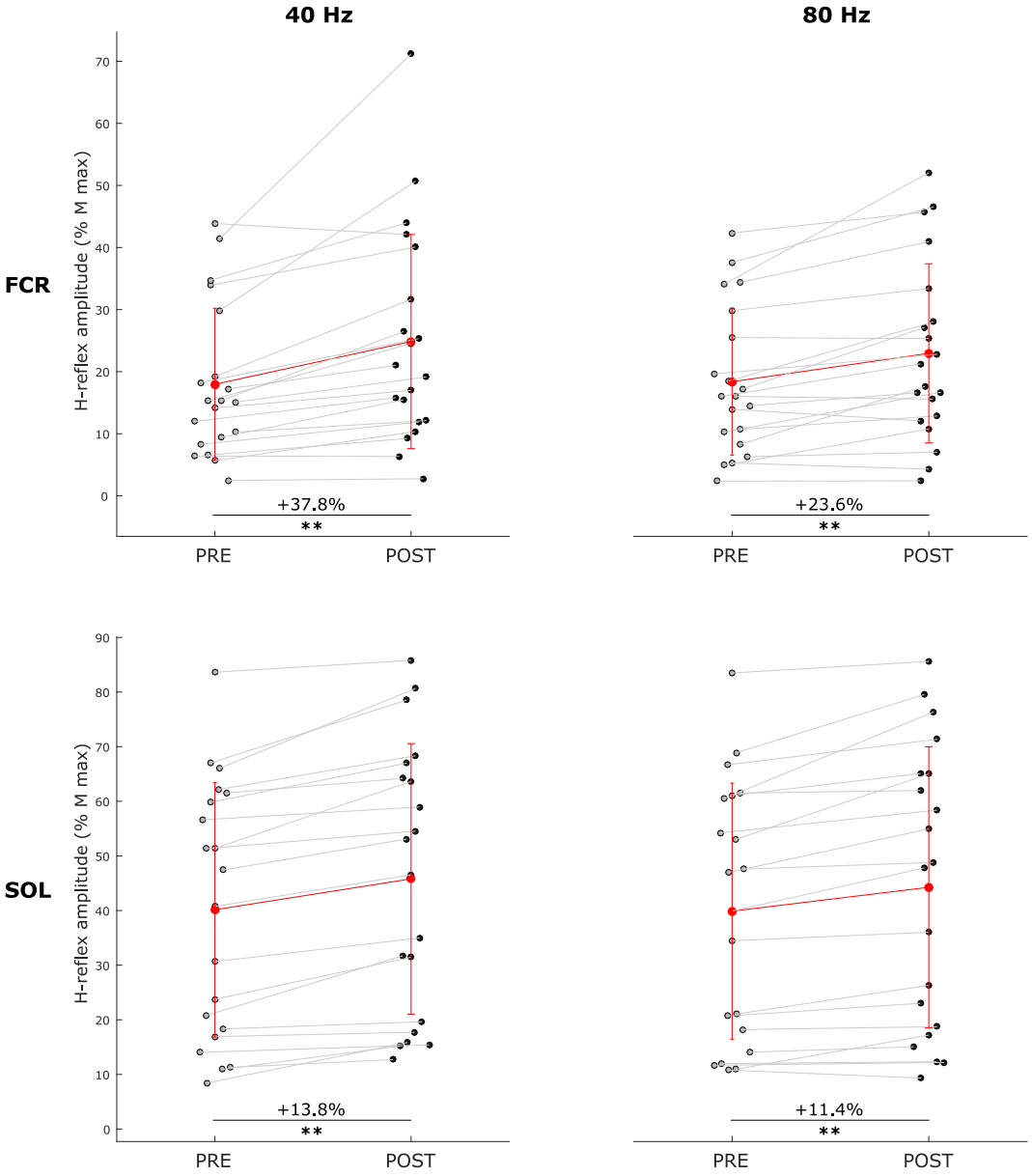

Fig. 3. Amplitude of the FCR and SOL H-reflex normalized to M-max before (PRE) and after (POST) NMES superimposed on voluntary isometric contractions of FCR and ankle plantar flexor muscles at 40 Hz (left) and 80 Hz (right) NMES frequency. Red circles and error bars indicate means ± standard deviation. Individual participant data are presented as grey dots for PRE and POST conditions measurements (n = 20). **Significant difference between PRE and POST($p < 0.01$).

main effect or interaction was found, a post-Hoc analysis was performed to detect significant differences between PRE and POST measures and frequencies. A Bonferroni correction was applied for multiple comparisons with a level of significance set at 0.05.

3. Results

All recorded data showed a normal distribution and the Mauchly test confirmed that the assumption of sphericity was not violated for any of the variables that were analyzed. The repeated measures ANOVA showed a significant main effect of Time ($F = 46.33$, $\eta p^2 = 0.71$, $p < 0.001$) and Muscle ($F = 20.38$, $\eta p^2 = 0.52$, $p < 0.001$) on the normalized H-reflex amplitude. Post-Hoc analysis showed a significant increase in the POST H-reflex amplitude after both the 40 Hz (FCR: +37.8 %, $p = 0.001$; SOL: +13.8 %, $p < 0.001$) and the 80 Hz (FCR: +23.6 %, $p = 0.001$; SOL: +11.4 %) compared to the PRE assessments (Fig. 3). On average, H-reflex amplitudes were considerably greater in the SOL (0.42 % M-max) compared to the FCR muscle (0.21 % M-max). There was no significant main effect of Frequency ($p = 0.15$) but a significant Frequency*Time interaction ($F = 4.42$, $\eta p^2 = 0.19$, $p = 0.049$) on the normalized H-reflex

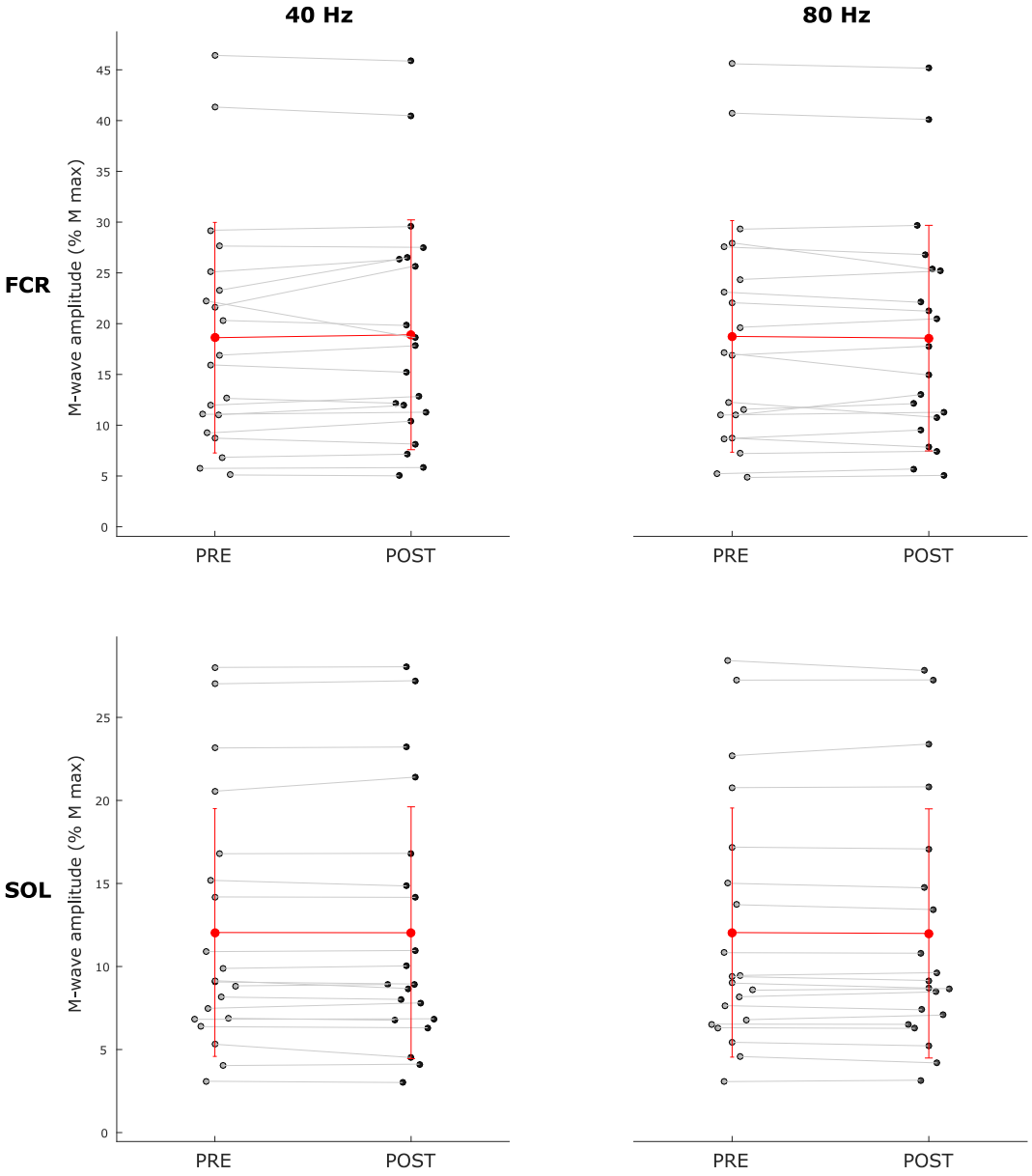

Fig. 4. Amplitude of the FCR and SOL M-wave normalized to M-max before (PRE) and after (POST) NMES superimposed on voluntary isometric contractions of FCR and ankle plantar flexor muscles at 40 Hz (left) and 80 Hz (right) NMES frequency. Red circles and error bars indicate means ± standard deviation. Individual participant data are presented as grey dots for Pre and Post measurements (n = 20). **Significant difference between PRE40Hz and POST40Hz for both muscle ($p < 0.01$).

amplitude. This suggests that the increase in H-reflex between PRE and POST assessment is frequency dependent. Post-hoc analysis indicated a greater increase in H-reflex amplitude following 40 Hz compared to 80 Hz when both muscles were considered together. However, the not significant Muscle*Frequency ($p = 0.88$), Muscle*Time ($p = 0.85$) and Muscle*Frequency*Time ($p = 0.42$) interactions indicate that the effect of NMES frequency on H-reflex is not muscle-specific, namely that the H-reflex change over time between the two frequencies is similar for both muscles.

We found no concomitant difference in the normalized test M-wave corresponding to the test H-reflexes for both FCR and SOL (Fig. 4; Table 2). Indeed, statistical analysis showed no main effects of Time ($p = 0.78$), Muscle ($p = 0.06$) and Frequency ($p = 0.838$) nor significant two-way or three-way interactions. We also found no main effect of Time ($p = 0.84$) and Frequency ($p = 0.94$) nor significant two-way or three-way interactions on MVIC values of both wrist flexor and plantar flexor muscles. As expected, MVIC values were significantly different from FCR and SOL as indicated by the significant main effect of Muscle ($F = 50.33$, $\eta p^2 = 0.74$, $p < 0.00$). Data are reported in Table 2.

4. Discussion

The main finding of this study was that superimposing NMES on isometric voluntary contractions using two pulse frequencies (40 and 80 Hz) induced an acute potentiation in the post-intervention (POST) H-reflex amplitude compared to the pre-intervention (PRE) values both in the SOL and FCR muscles. This finding was in accordance with our hypothesis and with previous studies investigating spinal excitability in response to NMES superimposed on voluntary contractions in both upper and lower limbs [27,28,30,43]. Interestingly, our findings indicated that the 40 Hz frequency led to a greater H-reflex potentiation compared to the 80 Hz.

Several authors indicated that the increase in H-reflex amplitude after NMES superimposed on voluntary contractions could be associated to a higher excitability of the stretch-reflex pathways, which could translate into greater force generation capacity [27,28,30]. The acute potentiation of the H-reflex after superimposed NMES might be explained by changes in presynaptic mechanisms, which are primarily responsible for H-reflex modulation [26]. Among these mechanisms, presynaptic inhibition (PSI) is considered one of the most important spinal regulatory networks [53] as it alters the efficacy of the transmission between Ia-afferents and α-motoneurons by regulating neurotransmitter release in the synaptic cleft [54]. Therefore, a reduced PSI onto Ia-afferent terminals has been suggested as a potential contributor to the enhanced spinal excitability as previously shown in young healthy adults after the application of the NMES [55]. Furthermore, compared to voluntary training or electrical stimulation alone, some authors highlighted that superimposing NMES on voluntary contractions can increase corticospinal excitability and plasticity, when applied to lower limb [28,56], or hand muscles [57]. This indicates that neuroplastic changes occurring at the supraspinal level could be markedly involved in the modulation of H-reflex when NMES is superimposed on voluntary contraction. Indeed, the intentional voluntary drive can effectively integrate afferent inputs from the peripheral part of the body, thereby rearranging the intracortical inhibitory circuits during motor adaptation learning, likely inducing alterations of motoneuronal excitability [58].

Regarding the effect of different NMES frequencies on spinal excitability, we expected a greater excitatory effect on the H-reflex amplitude in the FCR muscle after the 80 Hz NMES compared to the 40 Hz NMES, while the opposite trend was hypothesized for H-reflexes in the SOL. However, despite we found a greater excitatory effect of the 40 Hz frequency compared to the 80 Hz, there were no differences between the two muscles, suggesting a similar H-reflex modulation in response to each frequency. Such similar behavior of the two muscles in response to NMES is in line with the work of Mang et al. who reported that the magnitude of the facilitation of corticospinal excitability was similar between hand and leg muscles following a 100 Hz NMES intervention [23]. Some authors suggested that, during intermittent NMES application, there is no preferential recruitment of type I or type II muscle fibers independently whether low or high NMES frequency is used [59,60]. This could explain why, even when considering two muscles with a distinct muscle fiber composition such as FCR and SOL, NMES might have had a similar impact on muscle fiber recruitment.

Although the preferential recruitment of sensory over motor axons is strictly related to the width of the stimulation pulse, there are several studies showing that high stimulation frequencies (up to 80–100 Hz) promote an increased central contribution by a preferential recruitment of sensory axons [61–63] whereas lower stimulation frequencies appear to predominantly activate motor axons [61]. Based on this evidence, the results of the present study appear even more surprising as one could expect an increased activity in the spinal circuit after the administration of NMES at higher frequency. Nevertheless, there are some neurophysiological mechanisms that can explain this trend. First, higher stimulation frequencies might have led to an increase in post-activation depression as a consequence of a marked reduction in neurotransmitter release from synaptic afferents that have been repeatedly activated at high rate as in 80 Hz NMES [48,55]. This could have been less pronounced at 40 Hz, during which, conversely, a more effective temporal summation of excitatory postsynaptic potentials might have occurred, inducing greater overall excitatory effect on motoneurons in

Table 2
Test M-waves normalized by M-max of the SOL and of the FCR and MVIC of the wrist flexor muscles and ankle plantar flexor muscles before (PRE) and after (POST) each condition.

	M-wave (% M-max)	PRE 40Hz	POST 40Hz	PRE 80Hz	POST 80Hz
M-wave (% M-max)	FCR	17.1 ± 11.6	17.2 ± 11.4	17.2 ± 11.7	17 ± 11.4
	SOL	12.1 ± 7.4	12 ± 7.5	12.1 ± 7.5	12 ± 7.5
MVIC (N)	Wrist flexors muscles	160.7 ± 77.2	163.4 ± 66.5	167.9 ± 60.9	165.8 ± 62.7
	Plantar flexors muscles	462.2 ± 174.7	463 ± 177.1	459.7 ± 161	451.3 ± 168

Data are presented as group means ± standard deviation.

both muscles. Second, as the force target level was achieved using the same current intensity at both low and high stimulation frequencies, it could be argued that high frequency NMES might have engaged spinal inhibitory mechanisms more robustly than low-frequency stimulation. For instance, inhibitory interneurons in the spinal cord, such as Renshaw cells, could have been greatly activated by the antidromic volley generated by high-frequency stimulation, leading to increased recurrent inhibition of motoneurons which attenuated the increase in H-reflex amplitude [64]. However, although the effect of NMES frequency on spinal excitability resulted similar in FCR and SOL muscles, the neurophysiological mechanisms involved in H-reflex modulation could still differ given the distinct cortico-motoneuronal connectivity between lower and upper limb muscles. In the FCR, the large cortico-motoneuronal connectivity that was documented in previous studies [65,66] could imply that changes at the supraspinal level may play a key role in modulating FCR H-reflexes following NMES + ISO. Conversely, motoneurons of ankle plantar flexor muscles seem to have weak cortico-motoneuronal connections and limited supraspinal control [34] compared to wrist flexor muscles [67], suggesting that H-reflex modulation following NMES + ISO might be primarily associated with changes in spinal mechanisms rather than cortical plasticity. However, a more in-depth analysis of these neural mechanisms, and their role in modulating cortico-spinal responses in upper and lower limbs, is essential to fully understand how they are affected by different NMES settings.

There were a few limitations to the present study. First, we recruited healthy active participants with different training/activity backgrounds. This training-type heterogeneity could have affected the H-reflex results due to the different reflex sensitivity of participants performing either explosive or endurance activity [68]. As reported in previous works, H-reflex variability could be related to difference in motor unit type [69], intrinsic genetic endowment, and muscle fiber composition [24,26,68]. Future studies are warranted to address this issue by stratifying participants into more homogeneous and balanced subgroups based on the level and type of physical activity performed. Moreover, as the present study indicated that superimposing NMES promotes neuroplasticity at the spinal cord level, a future direction could involve applying this intervention, also by way of functional electrical stimulation (FES), in older adults or neurological patients to investigate the resulting neurophysiological adaptations. Another possible limitation could be related to the NMES pulse width used in this study. As previously described, there is evidence that sensory afferent fibers could be predominantly excited when NMES is delivered with high pulse width (>800 μs) [22,70,71], thus evoking a greater central contribution that results in higher reflex responses. However, the majority of the NMES devices used in clinical and training environments, including the one used in the present study, only allow to modulate pulse width between 100 μs and 500 μs. These parameters produce contractions predominantly via peripheral pathways due to a preferential activation of motor axons and a greater antidromic transmission along them [72]. Our findings indicate that NMES delivered at 400 μs induced a facilitation of the reflex pathways in the FCR and SOL muscles, although we do not exclude that further differences in H-reflex response may emerge when NMES is applied with larger pulse widths, particularly in muscle with prevalent spinal control, such as the SOL.

A potential limitation of the study lies in the fact that, while for the ankle plantar flexors all muscles of the triceps surae were stimulated, for the wrist flexor muscles, only the FCR muscle was stimulated. This was chosen because the FCR is the main contributor to wrist flexion and could be easily isolated and stimulated without incurring in the activation of other adjacent muscles. The MVIC of the wrist flexor muscles, however, is obtained through the contribution of multiple muscles, not only the FCR. This may have had some impact when NMES current intensity was adjusted to achieve half of the target level (10 % MVIC) for the FCR although the average current intensity required to achieve this force target level was rather low for the FCR muscle (range 7.6–17.8 mA), compared to the other stimulated muscles, and did not elicit pain or discomfort.

In this study, an assessment of the PSI was not included, although several researchers have identified presynaptic mechanisms as one of the principal responsible for neural adaptations at the spinal level [51,53]. Therefore, further studies are warranted to explore potential modifications of the PSI in relation to the increase in spinal excitability following NMES superimposed on voluntary contraction. Moreover, our study primarily focused on assessing changes in H-reflex responses without evaluating cortical and corticospinal mechanisms, thus limiting the breadth of our findings regarding overall neuromuscular excitability. To address this concern, future studies could incorporate additional neurophysiological assessments which include neuroimaging techniques such as transcranial magnetic stimulation or high-resolution electroencephalography to provide a more comprehensive evaluation of cortical and corticospinal adaptations.

In conclusion, our findings indicated that NMES superimposed on voluntary isometric contractions of the wrist flexor and ankle plantar flexor muscles induced an acute potentiation of the FCR and SOL H-reflexes, respectively, both at low and high stimulation frequency. This may stem from increased somatosensory stimuli induced by NMES which, in conjunction with supraspinal downward commands, could enhance the excitation of spinal motoneuronal pools. Although the lower NMES frequency appears to induce a greater reflex potentiation this was not different between FCR and SOL. This suggests that the modulation of NMES frequency may not preferentially target specific muscle fibers, resulting in similar reflex facilitation in both upper and lower limb muscles. However, given the distinct cortico-motoneuronal connectivity between the two muscles, further investigation is warranted to evaluate which spinal and supraspinal mechanisms are involved in the reflex modulation. The findings of the current study offer new perspectives on exercise-induced adjustment in spinal excitability, which could be valuable in designing tailored rehabilitation and training protocols. A deeper understanding of the neurophysiological changes in response to NMES intervention holds significant relevance in both training and clinical environments, thereby promoting its further development and application.

CRediT authorship contribution statement

Riccardo Borzuola: Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Valerio Caricati:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Martina Parrella:** Writing – review & editing, Writing – original draft,

Methodology, Investigation, Conceptualization. **Martina Scalia:** Writing – review & editing, Methodology, Investigation. **Andrea Macaluso:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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