Acute effects of high-frequency microfocal vibratory stimulation on the H reflex of the soleus muscle. A double-blind study in healthy subjects

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Summary

This study in healthy subjects examined the effects of a system delivering focal microvibrations at high frequency (Equistasi®) on tonic vibration stimulus (TVS)-induced inhibition of the soleus muscle H reflex. High-frequency microvibrations significantly increased the inhibitory effect of TVS on the H reflex for up to three minutes. Moreover, Equistasi® also significantly reduced alpha-motoneuron excitability, as indicated by the changes in the ratio between the maximum-amplitude H reflex (Hmax reflex) and the maximum-amplitude muscle response (Mmax response); this effect was due to reduction of the amplitude of the H reflex because the amplitude of muscle response remained unchanged. The present findings indicate that Equistasi® has a modulatory effect on proprioceptive reflex circuits. Therefore, Equistasi® might interfere with some mechanisms involved in both physiological and pathophysiological control of movement and of posture.

KEY WORDS: H reflex, high-frequency microvibrations, tonic vibratory stimulation.

Introduction

In neurophysiology it is an established concept that muscle tendon vibration determines a change in the discharge frequency of muscle spindle afferents. More specifically, it has been demonstrated that application of a high-frequency tonic vibratory stimulus (TVS) over a muscle tendon induces tonic activity of the muscle that is called the tonic vibration reflex (De Gail et al., 1966). A TVS over the soleus muscle tendon physiologically reduces the amplitude of the proprioceptive T and H reflexes (De Gail et al., 1966; Delwaide, 1973). The precise mechanisms responsible for this inhibitory effect are not completely understood, although it is likely that there are different contributory factors, such as post-synaptic mechanisms facilitating neurotransmitter depletion that leads to post-activation depression, and a supraspinal, presynaptic mechanism in which increased GABAergic transmission inhibits Ia afferents (De Gail et al., 1966; Delwaide, 1973; Hultborn et al., 1987).
The recent development of smart devices has been paralleled by important advances in applied nanotechnologies, a field that has been a focus of growing interest in various branches of medical and physiological research (Lue, 2007). Nanogenerators have been developed that are able to transform minimal thermal variations into mechanical energy by self-producing a focal vibration. The Equistasi® system (Equistasi S.r.l., Milan, Italy) provides an example of the generation of microvibration energy. Equistasi® is a vibrotactile device, based on vibrational technology, which self-produces a mechanical focal vibration with a non-constant frequency of about 9000 Hz and with a very low pressure of about 3-4 E^{-6} Pa. In order to gain further insight into the mechanisms underlying the role of high-frequency microvibrations in motoneuron activation, we explored the effects, on motoneuron excitability and proprioceptive reflex pathways, of the application of Equistasi® in healthy subjects. Specifically, we used high-frequency microvibrations applied to the soleus muscle, investigating their effect on alpha motoneuron excitability and on the modulatory inhibition of the H reflex.

Materials and methods

Subjects

The study included 19 heathy volunteers (9 males and 10 females), aged 28.4 ± 7.9 years. Informed consent

was obtained from all the participants, none of whom had a history of neurological disease. The research protocol was approved by the Ethics Committee of the C. Mondino National Neurological Institute and the study complied with the Declaration of Helsinki.

Electrophysiological investigations

All the subjects were investigated at about the same time (between 2 p.m. and 5 p.m.) in order to minimize the possibility of circadian fluctuations of the electrophysiological measures. The electrophysiological investigations were performed using a Synergy SYN5-C electromyograph (© Viasys Healthcare, Old Woking, Surrey, UK) connected to a BMST6 constant current stimulation unit (Biomedical Mangoni, Pisa, Italy). All the procedures were carried out in a soundproof room kept at a constant temperature (25°C).
Motor nerve conduction studies of the fibular nerve and sensory nerve conduction studies of the sural nerve were performed bilaterally in each subject to rule out the presence of subclinical signs of sensorimotor neuropathy.

H reflex

The H reflex was studied with the subject lying prone on a bed with the head in a neutral position. It was recorded from the soleus muscle with surface electrodes; the active electrode (cathode) was placed over the soleus muscle, two fingerbreadths below the junction between the lateral and the medial heads of the gastrocnemius muscle, while the reference electrode (anode) was placed over the Achilles tendon, 3 cm distal to the cathode. The ground electrode was applied over the calf, between the stimulating and the recording electrodes. The EMG signals of the H reflex were band-pass filtered between 10 Hz and 2 kHz. The sweep time for the EMG recordings was 10 ms/div. The tibial nerve was stimulated at the popliteal fossa using percutaneous bipolar electrodes, with the cathode placed proximal to the anode (inter-electrode distance of 2.5cm). The duration of the stimulus was 1 ms and the frequency of stimulation was 0.1 Hz. In order to elicit a maximum-amplitude H reflex (Hmax reflex) from the soleus muscle, tibial nerve stimulation was performed by increasing the stimulation intensity in steps of 1 mA until the Hmax reflex was reached. To elicit a compound muscle action potential (CMAP) of maximum amplitude from the soleus muscle, i.e. maximum-amplitude muscle response (Mmax response), the tibial nerve was stimulated by increasing the stimulation intensity beyond that needed to elicit the Hmax reflex, again in steps of 1 mA, until the Mmax response was obtained (Preston and Shapiro, 2005).
The TVS was delivered by applying a vibrator (Vibration Exciter Type 4809, Brüel and Kjaer, Skodsborgvej, Denmark), driven by monophasic rectangular pulses of 2-ms duration, 100 Hz frequency, over the Achilles tendon for 100 seconds. The strength of the vibration was monitored by means of a power amplifier and the amplitude of the TVS was constantly kept below 1.0 mm (De Gail, 1966; Preston and Shapiro, 2005). To evaluate motoneuron pool excitability of the soleus muscle at baseline, before application of the TVS, we examined the ratio between the Hmax reflex and Mmax response (Delwaide, 1984; Hagbarth et al., 1973). This ratio, denoted H1/Mmax, was obtained by calculating the average values of 10 consecutive Hmax reflexes (H1) and of 10 consecutive Mmax responses (Mmax). This intensity of stimulation was kept constant for the evaluation of the average of 10 Hmax reflexes, both at baseline (H1) and subsequently during application of the TVS (H2). The frequency of stimulation was always 0.1 Hz (1 stimulus every 10 seconds).
To evaluate the effects of TVS on the Hmax reflex a "vibratory index" (VI) was calculated using the following formula: (H2/H1)x100. This parameter is used to quantify the inhibitory effect of the TVS on the H reflex (Delwaide, 1973). Three minutes after delivery of the TVS, the Hmax reflex was again recorded as the average of 10 responses (H3) obtained with a 0.1 Hz stimulation frequency and constant intensity of stimulation. This was done in order to investigate the late effects of TVS. Therefore, a "vibratory index late effect" (VI late) was calculated as: (H2/H3)x100. This parameter was used to quantify longlasting effects of TVS on the H reflex. At this time, we also reassessed motoneuron pool excitability by again evaluating the Mmax response and by calculating the H3/Mmax ratio.
The parameters investigated in this study are summarized in table I.

Table I - The neurophysiological parameters considered in the study.

Abbrevations	Electrophysiological parameters
Hmax reflex	Maximum-amplitude H reflex
Mmax response	Maximum-amplitude muscle response (maximum-amplitude CMAP)
Mmax	Mean of 10 consecutive Mmax responses
H1	Mean of 10 maximum H-reflex amplitudes at baseline
H2	Mean of 10 maximum H-reflex amplitudes during TVS
H3	Mean of 10 maximum H-reflex amplitudes 3 minutes after TVS
H1/Mmax	Ratio between 10 consecutive Hmax reflexes and 10 consecutive Mmax responses at baseline
H3/Mmax	Ratio between 10 consecutive Hmax reflexes and 10 consecutive Mmax responses 3 minutes after TVS
Vibratory index (VI)	(H2/H1)x100
VI late - vibratory index late effect	(H2/H3)x100

Experimental paradigm and study design

The study was conducted according to a randomized, double-blind, placebo-controlled design (Fig. 1).
In particular, electrophysiological investigations were performed twice in all the subjects. First, all the subjects underwent baseline electrophysiological investigations without Equistasi® or placebo devices (T_0) and then they repeated these investigations while wearing Equistasi® or placebo devices (T_1). The subjects were randomly divided into two groups: the Equistasi® group (n=10 subjects) and the Placebo group (n=9 subjects). The Equistasi® and placebo devices were of the same shape and size and they were applied to the skin of the tendon of the triceps surae muscle 100 mm proximal to the lower edge of the heel three minutes before the electrophysiological investigations. Both the volunteers and the neurophysiologist were blind to the procedure.

Statistical analyses

The statistical tests were carried out using XSTAT - 2014 software (Addinsoft SARL, New York). We compared electrophysiological variables H1, H2, H3, H1/Max, H3/Mmax, VI, and VI late in the two groups under investigation (Equistasi® vs Placebo). For these variables we applied both inferential parametric (Student's t for paired samples or two-way ANOVA) and non-parametric (Wilkinson's) tests. Then we tested the null hypothesis H_0, in which the two means (before and after treatment) are the same, against an alternative hypothesis (H_1), in which the pre-treatment mean is significantly larger than the post-treatment mean: H_0: $\mu_1 = \mu_0$ vs H_1: $\mu_0 > \mu_1$. For all analyses the level of statistical significance was set at $p<0.05$.

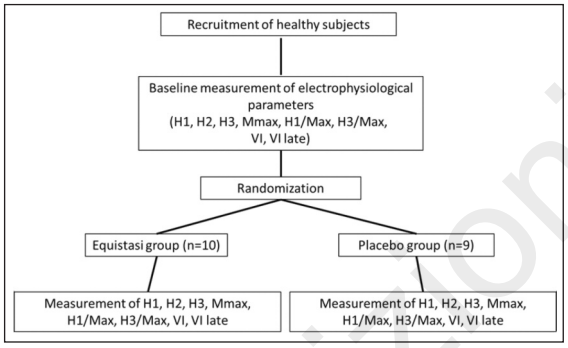

Figure 1 - Study design.

Results

Table II lists the electrophysiological parameters examined at T_0, while the larger set of electrophysiological parameters examined at T_0 and T_1 in the two groups (Equistasi® and Placebo) are shown in tables III and IV, respectively.

Intragroup comparisons

In the Equistasi® group we observed that both VI and VI late at T_1 were significantly lower as compared to the values obtained at T_0 (Fig.s 2 and 3). Furthermore, the decrease in the Hmax reflex observed at the end of TVS (H2) was statistically significant with respect to T_0. In addition, the H1/Mmax and the H3/Mmax values at T_1 were lower than the corresponding parameters obtained at T_0 (Table III and Fig. 4).
In the Placebo group we did not detect any significant difference in any parameter (H2, H3 and H3/Mmax) between T_0 and T_1 (Table IV, Fig.s 2, 3, and 4).

Table II - Demographic characteristics and baseline electrophysiological parameters of the subjects examined.

	Study population	Equistasi® group	Placebo group	p-value
Age (yrs)	28.4±7.9	28.1±8.2	28.8±7.6	ns
Sex (M/F)	9/10	4/6	5/4	ns
H1 (mV)	9.03±4.53	9.29±4.01	9.21±5.33	ns
Mmax (mV)	18.19±5.81	18.26±5.98	17.32±5.71	ns
H1/Mmax	0.49±0.21	0.49±0.16	0.53±0.24	ns

Abbreviations: H1=mean of 10 maximum H-reflex amplitudes at baseline; Mmax=mean of 10 consecutive Mmax (maximum-amplitude CMAP) responses; H1/Mmax=ratio between H1 and Mmax.

Table III - Electrophysiological parameters without the device (T_0) and while wearing device (T_1) in the Equistasi® group.

	T_0	T_1	p-value
H1 (mV)	9.29±4.01	8.7±4.7	ns
H2 (mV)	1.3±2.52	0.83±2.34	0.025
H3 (mV)	8.77±4.04	7.64±4.27	ns
Mmax (mV)	18.26±5.98	18.8±6.04	ns
H1/Mmax	0.49±0.16	0.44±0.19	0.036
H3/Mmax	0.47±0.19	0.40±0.19	0.042
VI	9.51±15.85	4.79±12.80	0.006
VI late	10.33±17.40	5.65±14.81	0.01

Abbreviations: H1=mean of 10 maximum H-reflex amplitudes at baseline; H2=mean of 10 maximum H-reflex amplitudes during TVS; H3=mean of 10 maximum H-reflex amplitudes 3 minutes after TVS; Mmax=mean of 10 consecutive Mmax (maximum-amplitude CMAP) responses; H1/Mmax=ratio between H1 and Mmax; H3/Mmax=ratio between H3 and Mmax; VI (Vibratory Index)=(H2/H1)x100; VI late (Vibratory Index late effect)=(H2/H3)x100.

Table IV - Electrophysiological parameters without the device (T_0) and while wearing device (T_1) in the Placebo group.

	T_0	T_1	p-value
H1 (mV)	9.21±5.34	8.38±5.25	ns
H2 (mV)	1.61±2.01	1.24±1.69	ns
H3 (mV)	8.70±5.07	8.01±5.18	ns
Mmax (mV)	17.32±5.71	17.49±5.45	ns
H1/Mmax	0.53±0.24	0.49±0.26	ns
H3/Mmax	0.50±.0.24	0.48±0.28	ns
VI	12.95±12.87	10.50±10.31	ns
VI late	13.49±13.55	10.84±10.36	ns

Abbreviations: H1=mean of 10 maximum H-reflex amplitudes at baseline; H2=mean of 10 maximum H-reflex amplitudes during TVS; H3=mean of 10 maximum H-reflex amplitudes 3 minutes after TVS; Mmax=mean of 10 consecutive Mmax (maximum-amplitude CMAP) responses; H1/Mmax=ratio between H1 and Mmax; H3/Mmax=ratio between H3 and Mmax; VI (Vibratory Index)=(H2/H1)x100; VI late (Vibratory Index late effect)=(H2/H3)x100.

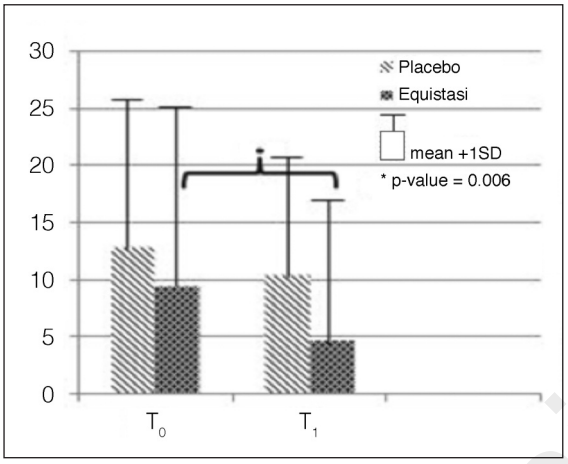

Figure 2 - Vibratory index (VI) in the Placebo and Equistasi® groups at baseline (T_0) and when investigated while wearing Equistasi® or Placebo devices (T_1).
A significant increase in the inhibitory effect on the VI was observed only in subjects wearing the Equistasi® device. VI = (H2/H1)x100 in which H2= mean of 10 maximum H-reflex amplitudes during TVS, H1= mean of 10 maximum H-reflex amplitudes at baseline.

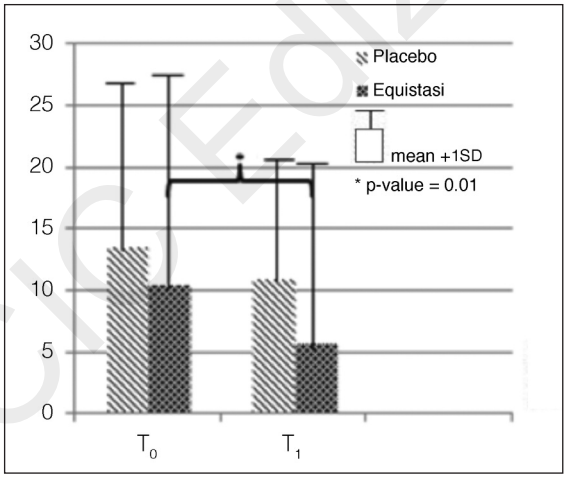

Figure 3 - Vibratory Index late (VI late) in the Placebo and Equistasi® groups at baseline (T_0) and when investigated while wearing Equistasi® or Placebo devices (T_1).
A significant increase in the inhibitory effect on the VI late was observed only in the subjects wearing the Equistasi® device. VI late = (H2/H3)x100; in which H2 = mean of 10 maximum H-reflex amplitudes during TVS, H3 = mean of 10 maximum H-reflex amplitudes 3 minutes after TVS.

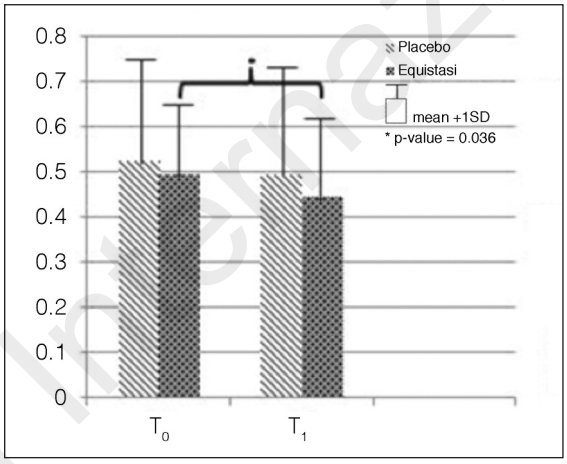

Figure 4 - H1/Mmax of Placebo and Equistasi® groups at baseline (T_0) and when investigated while wearing Equistasi® or Placebo devices (T_1).
A significant decrease of the H1/Mmax ratio was observed only in the subjects wearing the Equistasi® device. H1 = mean of 10 maximum H-reflex amplitudes at baseline; Mmax = mean of 10 consecutive Mmax (maximum-amplitude CMAP) responses.

The Mmax response did not differ significantly between T_0 and T_1 in either the Equistasi® group or the Placebo group (Tables III and IV).

Equistasi® group vs placebo group

At T_1, the inhibitory effect induced by TVS on the Hmax reflex was more marked in the Equistasi® group than in the Placebo group with a significant reduction of both VI and VI late recorded in the Equistasi® group (Fig.s 2 and 3).
Similarly, the decrease in H1/Mmax between T_0 and T_1 was significant in the Equistasi® group but not in the Placebo group (Fig. 4).
The Mmax response did not show significant differences at T_0 or at T_1 between the Equistasi® and Placebo groups.

Discussion

The results of the present study indicate that application of a microfocal vibratory stimulation at high

frequency over a muscle tendon can change motoneuron excitability under physiological conditions. Indeed, we showed that Equistasi® increased the H-reflex inhibition produced by TVS, while reducing alpha motoneuron excitability (significant reduction in H3/Mmax). This latter effect may be caused by the modulation, by Equistasi®, of the excitability of Ia proprioceptive afferents, because it is related to a reduction of the Hmax reflex in the absence of significant effects on the Mmax response. These results suggest that microfocal vibratory stimulation acts through a "busy-line" mechanism on Ia proprioceptive afferents. This mechanism was hypothesized by Hagbarth et al. (1973) as an explanation for the inhibitory effect of TVS on the H reflex. The persistence, three minutes after TVS application, of the significant inhibitory effect induced by Equistasi® suggests that Equistasi® may also modulate central nervous system activity through other mechanisms, i.e. reinforcement of pre-synaptic or post-synaptic inhibitions of the H reflex (De Gail et al., 1966; Delwaide, 1973; Hultborn et al., 1987; Delwaide, 1984).

On the other hand, the modulatory effect induced by Equistasi® on the H reflex might involve multiple proprioceptive pathways, not limited to Ia afferent circuits. In particular, several studies have demonstrated that vibratory stimuli can also excite proprioceptive type II afferents (Burke et al., 1976; Jankowska, 1992; Nardone and Schieppati, 1998; Bove et al., 2003). Vibratory stimulation of these afferents significantly changes some physiological parameters of the medium latency response of the stretch reflex during standing position (Bove, 2003; Eklund, 1972; Quoniam et al., 1995; Courtine et al., 2007). This effect seems to be much more evident after than during tendon vibration, and it could depend on "an interaction between peripheral and central drive on group II interneurons in order to produce sufficient EMG activity to maintain a given postural set" (Bove, 2003).

Moreover, other kinds of afferents may also play a role, since vibratory stimulation may also excite exteroceptive afferents, such as mechanoreceptors (Macefield, 2005). Indeed, the different types of mechanoreceptors preferentially encode different stimulus modalities, but all are capable of encoding mechanical vibration over a wide range of frequencies (Bolanowski and Verrillo, 1982; Johansson et al., 1982).

Vibration exercise is also known to increase muscle force and power. In particular, the effects of vibration exercise are similar to those of resistance training. In these cases, the modulation of proprioceptive circuits induced by Equistasi® could interfere with central motor activation and fatigue because "central fatigue" is regulated by reflex servo-mechanisms in which motoneuron discharges (expression of the motor output of the maximal muscle force) are also modulated in an inhibitory sense by proprioceptive circuits linked to the Ia and II afferents of muscle spindles (Gandevia, 2001; Bigland-Ritchie et al., 1986; Delecluse et al., 2003; Cardinale and Bosco, 2003).

Tendon vibration, too, might induce an excitatory effect on the motor cortex, as suggested by the increased amplitude of motor evoked potentials observed with transcranial magnetic stimulation applied over the motor cortex after muscle vibration (Kossev et al., 2001). Therefore, we can hypothesize that high-frequency microvibrations applied over muscle tendons may produce changes in motor cortex excitability with consequent facilitation of descending corticospinal motor pathways and excitatory supraspinal control over the spinal reflex activity, or that they may act directly over the spinal motoneuron pool. In the present study, microvibrations produced by Equistasi® do not appear to have directly influenced the excitability of peripheral motor axons or the excitability of spinal motoneurons. Their effect, instead, seems to have been produced though indirect mechanisms involving inhibitory modulation of Ia proprioceptive afferents. This is suggested by the fact that the Mmax response (i.e. the maximum motor evoked response obtained by direct stimulation of the motor axons of the nerve) was not affected by Equistasi®. Therefore, we suggest that the first hypothesis, namely that of direct modulation by Equistasi® of Ia proprioceptive afferents, is the most likely.

It is noteworthy that recent observations indicating that Equistasi® may also exert a restorative effect in some movement disorders, e.g. in the rehabilitation of postural instability in Parkinson's disease (Volpe et al., 2014), raise the possibility that the system might be used to address impaired control of movement.

In conclusion, our study indicates that focal microvibratory stimulation is able to modulate phasic proprioceptive reflexes in healthy subjects. More targeted studies are needed to assess whether microvibrations induced by Equistasi® can improve the physiological mechanisms involved in motor strength and fatigue, and to confirm the potential effect of this kind of stimulation as a means of improving, also, balance abnormalities and motor disorders.

References

Bigland-Ritchie BR, Dawson NJ, Johansson RS, et al (1986). Reflex origin for the slowing of motoneurone firing rates in fatigue of human voluntary contractions. J Physiol 379:451-459.

Bolanowski SJ Jr, Verrillo RT (1982). Temperature and criterion effects in a somatosensory subsystem: a neurophysiological and psychophysical study. J Neurophysiol 48:836-855.

Bove M, Nardone A, Schieppati M (2003). Effects of leg muscle tendon vibration on group Ia and group II reflex responses to stance perturbation in humans. J Physiol 550: 617-630.

Burke D, Hagbarth KE, Löfstedt L, et al (1976). The responses of human muscle spindle endings to vibration of non-contracting muscles. J Physiol 261: 673-693.

Cardinale M, Bosco C (2003). The use of vibration as an exercise intervention. Exerc Sport Sci Rev 31: 3-7.

Courtine G, De Nunzio AM, Schmid M, et al (2007). Stance and locomotion-dependent processing of vibration-induced proprioceptive inflow from multiple muscles in humans. J Neurophysiol 97: 772-779.

De Gail P, Lance JW, Neilson PD (1966). Differential effects on tonic and phasic reflex mechanisms produced by vibration of muscles in man. J Neurol Neurosurg Psychiatry 29: 1-11.

Delecluse C, Roelants M, Verschueren S (2003). Strength increase after whole-body vibration compared with resistence training. Med Sci Sports Exerc 35:1033-1041.

Delwaide PJ (1984). Contribution of human reflex studies to the understanding and management of the pyramidal syndrome. In: Shahani BT (Ed.) Electromyography in CNS Disorders: Central EMG. Boston, MA, Butterworths, pp. 77-109.

Delwaide PJ (1973). Human monosynaptic reflexes and presynaptic inhibition. An interpretation of spastic hyperreflexia. In: Desmedt JE (Ed) New Developments in Electromyography and Clinical Neurophysiology. Vol 3. Basel, Kager, pp. 505-522.

Eklund G (1972). General features of vibration-induced effects on balance. Ups J Med Sci 77: 112-124.

Gandevia SC (2001). Spinal and supraspinal factors in human fatigue. Physiol Rev 81:1725-1789.

Hagbarth KE, Wallin G, Löfstedt L (1973). Muscle spindle responses to stretch in normal and spastic subjects. Scand J Rehabil Med 5: 156-159.

Hultborn H, Meunier S, Pierrot-Deseilligny E, et al (1987). Changes in presynaptic inhibition of Ia fibres at the onset of voluntary contraction in man. J Physiol 389: 757-772.

Jankowska E (1992). Interneuronal relay in spinal pathways from proprioceptors. Prog Neurobiol 38: 335-378.

Johansson RS, Landström U, Lundström R (1982). Responses of mechanoreceptive afferent units in the glabrous skin of the human hand to sinusoidal skin displacements. Brain Res 244:17-25.

Kossev A, Siggelkow S, Kapels HH, et al (2001). Crossed effects of muscle vibration on motor-evoked. Clin Neurophysiol 112:453-456.

Lue JT (2007). Physical properties of nanomaterials. Encyclopedia of Nanoscience and Nanotechnology 10:1-46.

Macefield VG (2005). Physiological characteristics of low-threshold mechanoreceptors in joints, muscle and skin in human subjects. Clin Exp Pharmacol Physiol 32:135-144.

Nardone A, Schieppati M (1998). Medium-latency responses to muscle stretch in human lower limb: estimation of conduction velocity of group II fibers and central delay. Neurosci Lett 249: 29-32.

Preston DC, Shapiro BE (2005). Electromyography and Neuromuscular Disorders. Clinical-Electrophysiologic Correlations. 2nd edition. Philadelphia, Elsevier Butterworths-Heinemann.

Quoniam C, Hay L, Roll JP, et al (1995). Age effects on reflex and postural responses to propriomuscular inputs generated by tendon vibration. J Gerontol A Biol Sci Med Sci 50: B155-B165.

Volpe D, Giantin MG, Fasano A (2014). A wearable proprioceptive stabilizer (Equistasi®) for rehabilitation of postural instability in Parkinson's disease: a phase II randomized double-blind, double-dummy, controlled study. PLoS One 9: e112065.