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A NEW EXPERIMENTAL MODEL OF MUSCLE PAIN IN HUMANS BASED ON SHORT-WAVE DIATHERMY

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Running head: Experimental muscle pain based on short-wave diathermy

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Significance: This study presents for the first time an experimental model to elicit sustained muscle pain based on short-wave diathermy. The main advantages of the model are its non-invasiveness, the possibility to control stimulation parameters in a reliable way, and the convenience of the time frame in which pain and hyperalgesia are developed.

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ABSTRACT

Background

Experimental models of pain in humans are crucial for understanding pain mechanisms. The most often used muscle pain models involve the injection of algesic substances, such as hypertonic saline solution or nerve growth factor, or the induction of delayed onset muscle soreness (DOMS) by an unaccustomed exercise routine. However, these models are either invasive or take substantial time to develop, and the elicited level of pain/soreness is difficult to control. To overcome these shortcomings, we propose to elicit muscle pain by a localized application of short-wave diathermy (SWD).

Methods

In this crossover study, SWD was administered to eighteen healthy volunteers to the wrist extensor muscle group, with a constant stimulation intensity and up to 4 minutes. Pressure pain threshold (PPT), pinprick sensitivity (PPS) and self-reported muscle soreness were assessed at baseline and at 0, 30 and 60 minutes after application of SWD.

Results

SWD evoked localized muscle pain/soreness in the wrist extensor muscle group and a decrease of PPT in the treated arm compared with the control arm that lasted for at least 60 minutes, reflecting ongoing hyperalgesia after SWD application. PPS was not significantly altered 30 to 60 min following SWD, suggesting a minimal contribution from skin tissue to sustained hyperalgesia.

Conclusions

SWD was able to elicit muscle soreness and hyperalgesia up to 60 min after its application. Thus, this new model represents a promising tool for investigating muscle pain in humans.

Key words (5 maximum): Short-wave diathermy, Experimental pain model, Hyperalgesia, Musculoskeletal pain

INTRODUCTION

Pain-related pathologies are associated with many concurrent physiological and psychophysical processes in patients, resulting in a large number of confounding factors in the evaluation of specific mechanisms behind pain (Raffaeli & Arnaudo, 2017). Thus, researchers have developed surrogate experimental pain models to be tested on healthy volunteers, facilitating the assessment of pain effects on the sensory-motor system (Graven-Nielsen, 2006; Le Bars et al., 2001). In general, experimental pain models should have the following desirable features: they should be established

in a short time, the stimuli that elicit pain should be reasonably controlled and the effects of the experiments should be fully reversible, short lasting and homogeneous across volunteers. However, to the best of our knowledge, there are no experimental pain models to date that fulfill all these conditions.

Experimental pain models use different stimulus modalities to elicit pain, including mechanical (Nie et al., 2005; Staud et al., 2003), chemical (Babenko et al., 1999; Svensson et al., 2003), thermal (Meh & Denišlič, 1994; Miron et al., 1989) or electrical stimuli (Curatolo et al., 2001; Laursen et al., 1997; Neziri et al., 2011), among others. For acute muscle pain, one of the most widely used models involves the administration of intramuscular injection of algesic substances like hypertonic saline solution into the muscle itself or its surrounding areas (Graven-Nielsen et al., 1997; Mista et al., 2015). These models require invasive procedures, elicit an uneven distribution of pain intensity and present a short time frames for the development and assessment of pain. Another commonly used model is delayed-onset muscle soreness (DOMS) elicited by unaccustomed eccentric exercise routine. DOMS has a slow development (24 to 48 h), the resulting level of pain/soreness is hard to control and depends on the subject's training status (Proske & Morgan, 2001). Thus, although these models are widely used, their limitations uphold the development of new alternatives.

An unexplored possibility is to elicit muscle pain by a localized application of short-wave diathermy (SWD). High frequency oscillations of non-ionizing electromagnetic fields in the radiofrequency (RF) range, around 27 MHz (short-wave), can heat deep tissues in a well-localized region (Draper et al., 1999; Goats, 1989; Shields et al., 2002). Deep tissue heating by means of RF is a safe and extensively used technique in palliative treatment of pain and as a healing agent in soft tissues (Guo et al., 2012; Yu & Peng, 2017). However, SWD has not been previously explored with intensities suitable for inducing sustained muscle pain.

The aim of the present study was to investigate whether SWD can elicit localized muscle pain/soreness on the wrist extensor muscle group. Pressure pain threshold (PPT), pinprick sensitivity (PPS) and self-reported muscle soreness were assessed in both the dominant and the non-dominant arm (acting as control) at baseline and at 0, 30 and 60 minutes after application of SWD, in order to test for long lasting effects.

METHODS

Participants

Nineteen healthy volunteers (7 females and 12 males, age: 29 ± 5 years, weight: 69.3 ± 13.8 kg, height: 171.1 ± 10.4 cm, mean ± standard deviation) were recruited for the study. One volunteer was

excluded after reporting a previous surgery in the non-dominant arm. Volunteers had no history of pain or neuromuscular disorders affecting the upper limb region. All volunteers received written and verbal description of the procedures and gave written inform consent. The study was approved by the Central Bioethics Committee for Biomedical Practice and Research, dependent from the Ministry of Health of Entre Rios (identifier: IS001890). Study preregistration, including original hypothesis, description of primary and secondary outcomes, and initial sample size consideration, was done at ClinicalTrials.gov (identifier: NCT03573219) and the Declaration of Helsinki was respected.

Sample size considerations

Sample size was derived taking into account the expected effect size that the model will have on the primary outcome (PPT). Since there is no existing information on the expected size of the difference in PPT due to the application of SWD, this value was approximated taking into account reference values of differences in PPT generated by other experimental models of pain, such as the injection of hypertonic saline solution or DOMS. In these cases, PPT is usually reduced between 10 and 30% during the effects of the model, so an average reduction of 20% compared to baseline was considered. Taking into account a probability of making a type I error (α) of 5%, a statistical power (1 - β) of 80%, and an estimated correlation between measures of 0.8, the sample size required to detect a decrease of 20% in PPT immediately after the administration of SWD was 16 volunteers. In order to account for an unexpectedly larger variation, 19 subjects were finally recruited.

Short-wave diathermy

SWD was administered using a CEC M-8 short-wave thermotherapy unit (CEC Electrónica S.R.L., Argentina) that delivers RF at a frequency of 27.12 MHz. The device has two rectangular capacitive applicators (18 x 12 cm), that were positioned below and above the dominant forearm, over the extensor carpi radialis brevis (ECRB) muscle. Coplanar application was performed using the continuous wave mode. SWD application has two main parameters that can be controlled: application time and stimulation intensity. It is clear that at least one of these parameters must be fixed to reduce the degrees of freedom of the model. During pilot experiments, we tested both possible configurations: fixed application time with variable stimulation intensity, and fixed stimulation intensity with variable application time. The most consistent results in terms of pain/soreness elicited after stimulation were obtained using a fixed stimulation intensity at a constant value (12 out of 20 on the thermotherapy unit scale) and applying SWD for as long as subjects could tolerate the stimulation (i.e. until subjects reached tolerance threshold for thermal pain), at which moment stimulation was immediately interrupted. In this way, we found that the selected intensity was adequate to develop sustained pain/soreness within a reasonable time frame.

Response profiles during SWD application

A computerized, custom-made Visual Analog Scale (VAS) was used to continuously track the response profile to thermal stimuli across subjects during the application of SWD. The scale range was from 0 to 100, where 0 represents no perception, 30 represents the pain threshold (defined here as the time to reach a painful sensation at the predefined stimulation intensity) and 100 represents the tolerance threshold (defined here as the time at which the pain sensation becomes intolerable). The scale was anchored according to response profiles observed during pilot experiments, in which the early parts of the response profiles were reported as clearly non-painful thermal sensations. Additionally, the first two parts of the McGill questionnaire (related to the location and quality of pain) were used to describe the sensation when tolerance threshold was reached (Melzack, 1975).

Pressure pain threshold assessment

Pressure pain thresholds (PPT) were assessed using a digital algometer (Somedic SenseLab AB, Sweden), directly over the ECRB muscle, using a 1 cm^2 round tip. Pressure was gradually increased from 0 kPa at a rate of approximately 30 kPa/s (maximal achievable pressure: 2000 kPa). PPT was defined as the pressure at which the mechanical sensation becomes painful. The assessment was repeated three time for each arm, alternating sides between measurements. The median value of the three assessments was used for further analysis (Bergin et al., 2015; Neziri, Scaramozzino et al., 2011). Changes in PPT are indicative of the development of mechanical hyperalgesia in the muscle.

Pinprick assessment

Pinprick stimuli were applied perpendicularly on the skin over the ECRB muscle using a pinprick stimulator, consisting on a needle with a 0.25 mm^2 tip calibrated to a weight of 50 g. The stimulus was repeated three times for each arm, randomizing the order of assessment for each trial. Volunteers scored pinprick sensation on a Numerical Rating Scale (NRS), where 0 represents no perception, 30 represents the pain threshold, and 100 represents the tolerance threshold. Pinprick stimuli were assessed to differentiate deep-tissue from cutaneous hyperalgesia.

Self-reported muscle pain/soreness

A modified self-report Likert scale was used to follow the temporal progression of muscle pain/soreness at 0, 30, and 60 minutes after SWD, with 0 defining a complete absence of soreness and 6 indicating severe soreness (see Table 1 for a full description of the muscle soreness scores). This scale was selected based on previous studies reporting the effects of experimental muscle pain models (Andersen et al., 2008; Bergin et al., 2015). Additionally, self-assessment was repeated 24 h after the experiment, in order to check for potential longer lasting effects of SWD.

Experimental protocol

Volunteers participated in a single experimental session. They were instructed to sit down comfortably with the arms extended and the palm in prone position. Baseline measures (PPT, PPS, and self-reported muscle pain/soreness) were performed at the beginning of the experiment. SWD was then applied to the wrist extensor muscle group of the dominant arm, due to its incidence and prevalence of dominance in clinical muscle pain (Vicenzino & Wright, 1996) (Fig. 1). Initially, low intensity SWD (4 out of 20 on the thermotherapy unit scale) was applied in order to localize the region to be treated without warming unwanted areas (such as wrist flexor muscles), according to verbal reports from the subjects. Afterwards, stimulation was stopped and restarted using the prefixed stimulation intensity (12 out of 20). Subjects were instructed to report the ongoing thermal sensation using the VAS scale during stimulation, until tolerance threshold for thermal pain was reached, stopping immediately after. PPT, PPS, and self-reported muscle pain/soreness were quantified immediately after SWD application, as well as 30 and 60 min later. The assessment was performed only three time points in order to avoid substantial habituation to mechanical stimulation.

Data analysis and statistics

Statistical analysis was performed using R v. 3.5.1 (R Core Team, 2018). A two-way repeated measures ANOVA with within-subject factors *time* (0, 30, 60 minutes after SWD) and *arm* (treated and control) was used to evaluate differences in PPT and PPS due to the application of SWD, calculated as percentage of change from baseline. Mauchly's test was carried out to verify the assumption of sphericity, and the Greenhouse-Geisser correction was applied for PPS data. A non-parametric Friedman test was employed to quantify the self-reported muscle pain/soreness after SWD with within-subject factor *time* (baseline and 0, 30, 60 minutes after SWD). Tukey's post hoc tests (in its parametric and non-parametric versions) were carried out when appropriate. For model characterization purposes, Spearman's rank-order correlations (ρ) were calculated between the following variables: change in PPT, PPS and self-reported muscle pain/soreness at 0, 30 and 60 min, and time to reach tolerance threshold during SWD application. Values are reported as mean ± standard deviation or median [interquartile range] depending on whether the underlying data was normally distributed or not. *P* values smaller than 0.05 were regarded as statistically significant.

RESULTS

Reported pain intensity and quality during SWD

The induced muscle pain increased with different profiles for each subject, reaching the tolerance threshold in 1.59 [1.47] minutes (Fig. 2A). The resulting spatial extension of pain matched the treated forearm region (Fig. 2B). At the peak of the induced thermal muscle pain (i.e. at tolerance level), 22% of the participants described the pain as hot, 61% as burning, 11% as scalding, and the remaining 6% as searing.

Self-reported muscle pain/soreness

Subjects reported no muscle pain/soreness at baseline. A main effect of time was found for the self-reported muscle pain/soreness scores (χ^2_3= 19.441, P < 0.001). Post hoc tests showed that scores were significantly higher after SWD compared to baseline for all time points (P values ranging from < 0.001 to 0.043), but they were not significantly different among them (P values ranging from 0.065 to 0.945). None of the subjects reported muscle pain/soreness 24 h after the experiment (Fig. 3).

Pressure pain thresholds

Absolute values for PPT for all assessment time points are shown in Table 2. A main effect of *arm* was found for the PPT change scores ($F_{1,17}$ = 8.897, η_p^2 = 0.34, P = 0.008; Fig. 4). The treated arm showed a significant decrease of PPT values compared to the control arm at all time points (P = 0.008), presenting an average difference of 13% between arms. No significant differences were neither found for *time* ($F_{2,34}$ = 1.593, η_p^2 = 0.086, P = 0.218) nor for the interaction ($F_{2,34}$ = 0.042, η_p^2 = 0.002, P = 0.958).

Pinprick sensitivity

Absolute values for PPS for all assessment time points are shown in Table 2. Data from one subject was excluded from the pinprick sensitivity analysis as an outlier (the reported value was over three times larger than the standard deviation). No significant differences were found for *arm* ($F_{1,17}$ = 1.069, η_p^2 = 0.059, P = 0.315) or *time* ($F_{1.14,19.45}$ = 0.400, η_p^2 = 0.031, P = 0.487). However, a significant interaction was found ($F_{2,32}$ = 3.802, η_p^2 = 0.192, P = 0.033; Fig. 5). The post-hoc analysis revealed that the treated arm showed a significant increase of the PPS compared with the control arm only immediately after SWD (P = 0.019).

Correlations between model outcomes

On a purely exploratory basis, we attempted to correlate the following variables: change in PPT, PPS and self-reported muscle pain/soreness at 0, 30 and 60 min, and time to reach tolerance threshold during SWD application. Besides the expected significant intra-modal correlations across time points,

no significant correlations were found between assessment modalities or between any given modality and the time to reach tolerance ($P > 0.05$ for all calculated Spearman's ρ).

DISCUSSION

Response profile during SWD application

Volunteers reported a rapid increase in thermal pain during SWD application, reaching the heat tolerance threshold in a couple of minutes in most cases. This behavior is likely associated with responses from polymodal afferent fibers that act as heat-sensitive receptors. The transduction of the thermal stimulus is performed by a subset of channel receptors within the muscle afferents that sense and signal within specific temperature ranges. These receptors, including the temperature-activated transient receptors (TRPV), not only detect temperature in innocuous range but also in the nociceptive range (Patapoutian et al., 2003). Once the thermal stimulus is transduced, group IV and, in less proportion, group III afferent fibers are associated with the transmission of the thermal stimulus from the muscle to the central nervous system (Graven-Nielsen et al., 2002; Raja et al., 2018). Although the temperature inside the muscle was not measured during the SWD, it is known that temperatures over 43 °C are very uncomfortable for humans (Draper et al., 2004). Therefore, even though it is hypothesized that the temperature inside the muscle was above 43 °C before the stimulation was stopped, application timespans of a few minutes (max. 4 min for this experiment) are not enough to induce permanent thermal damage on the muscle tissue (Ichinoseki-Sekine et al., 2007; Yarmolenko et al., 2011).

Mechanisms associated with hyperalgesia induced by SWD

Subjects reported muscle soreness and a decrease in PPT values immediately after SWD application compared to the control arm, that lasted for at least 60 min after the intervention, reflecting hyperalgesia in the treated region. It should be noted that five subjects showed a slight increase in PPT after SWD application. In all cases, however, the increase in PPT in the control arm was larger, probably reflecting habituation to mechanical stimulation, so the net difference between arms still showed an overall effect of SWD. Furthermore, four subjects did not report muscle soreness at any time point after SWD, although cross-referencing the data showed that all subjects but one showed a decrease in PPT.

The observed effect is most likely due to an inflammatory response of the neuromuscular system triggered by a fast temperature increase in the muscle, coupled with an incapability of the musculoskeletal tissue to dissipate heat at the same speed, resulting in the release of algesic substances and/or local tissue damage. However, further studies assessing inflammatory markers are required in order to confirm an ongoing inflammatory process and to gain deeper understanding

of the model effects in the muscle, since most inflammatory pain models are related to skin (Kilo et al., 1994; Pedersen & Kehlet, 1998; Sandkühler, 2009; Treede et al., 1992). In this regard, it is worth mentioning that although hyperalgesia has been observed when inducing thermal pain in skin tissue (Pedersen & Kehlet, 1998), PPS after SWD only hinted at short-lasting changes after application in the present study, suggesting that the contribution of the skin to ongoing hyperalgesia after a few minutes is minimal.

Several mechanisms may be involved in the initiation and prolongation of the inflammation in response to SWD. Among these mechanisms, neurogenic inflammation could account for the rapid development of hyperalgesia. This inflammation process is triggered by the axon reflex, which causes a release of pro-inflammatory neuropeptides from the afferent fibers (Chiu et al., 2012; Meggs, 1993). Different substances are involved in the process, including substance P and calcitonin gene-related peptide that are released both peripherally as well as in the dorsal spinal cord (Richardson & Vasko, 2002). These substances mediate neurogenic inflammation symptoms by interacting with muscular and connective tissue cells (Campos & Calixto, 2000). Another pathway that may be associated with the observed hyperalgesia is the release of pro-inflammatory substances by the musculoskeletal tissue itself (Welc et al., 2013). For instance, interleukin-6 (IL-6) might be particularly relevant, since it has been previously demonstrated that it is highly upregulated following a significant increase of heat in the muscle (Welc et al., 2013; Welc et al., 2013). This pro-inflammatory cytokine is synthesized in the initial stage of inflammation, and has also been suggested to play a role in the process of pathological pain (Tanaka et al., 2014; Zhang & An, 2007).

As mentioned before, mechanical hyperalgesia observed after SWD is presumably associated with the described peripheral responses at the beginning of the process. Nevertheless, central mechanisms cannot be excluded as a contributing modulatory factor. There is strong evidence that acute peripheral inflammation involves specific central mechanisms, for example, through hyperexcitability of dorsal horn nociceptive neurons (Andrew & Greenspan, 1999; Schaible, 2007; Treede et al., 1992) and through changes in the release of inflammatory mediators or vasodilation by the sympathetic and parasympathetic system (Waldburger & Firestein, 2010). In rats, subcutaneous inflammation causes an increase of spinal glia activity, constituting a direct evidence of a central change induced by an acute stimulation (Sweitzer et al., 1999). In human experimental inflammation of the skin, it has been suggested that central excitability remains increased once triggered even after a single stimulation, and it does not require an ongoing nociceptive input (Kilo et al., 1994). However, the contribution of central mechanisms to hyperalgesia remains unclear, and further studies are needed to elucidate the processes involved in muscle inflammation after SWD.

SWD as a muscle pain model

The experimental pain model presented in this study has several key features. First, it is based on an exogenous stimulation technique that does not require invasive procedures, unlike intramuscular injection of algesic substances that are invasive and demand additional precautions when used, including correct asepsis, the use of sterile and disposable substances, and experience in the injection technique to avoid damage to nerves or other structures (Bergin et al., 2015; Thomas Graven-Nielsen et al., 2001; Sjøogaard et al., 2000). In addition, the hyperalgesia observed after the stimulation presented similar profiles compared to those reported in longer-lasting muscle pain models using nerve growth factor (NGF) or DOMS, but without requiring a long development time (Bergin et al., 2015; Hedayatpour et al., 2008). Furthermore, muscle exercise induced pain triggers the release of algesic substances, including cytokinin and NGF, resulting in hyperalgesia and movement evoked pain for at least 24-48 hours after the intervention (Bergin et al., 2015; Murase et al., 2010; Tegeder et al., 2002). This does not correspond with the findings presented here using SWD, where there was an absence of discomfort or movement evoked pain when assessed after 24 h. In terms of the scientific and ecological validity of the model, it is worth stressing that even though the stimulus used to induce pain is thermal, the duration and nature of the effects observed suggest that the physiological mechanisms responsible for maintaining pain/soreness are of inflammatory origin. Finally, it should be noted that our goal is not to replace or criticize existing muscle pain/soreness models, but to develop an alternative or complementary model that is non-invasive and can be easily applied using inexpensive equipment commonly used for physical therapy, in which the stimulation parameters can be readily controlled. Furthermore, the model has a rapid development time and a reasonable duration, and was successfully established in almost all volunteers tested, which suggests good reliability.

Limitations and future work

Given the fact that there is no previous investigation about SWD as a pain model, the parameters of the stimulation were set after pilot experiments, and hence further work is required to fully describe the effects of changes in the parameters (e.g. SWD intensity, application time, type of applicator) in the model outcome. As described in the pre-registered protocol, we aimed at a 20% change in PPT compared to baseline, and the observed effect was slightly smaller (still detected likely due to the effect lasting longer than anticipated). Thus, we hypothesize that a larger and more homogeneous effect can be achieved using different parameters, for example using a higher stimulation intensity or by rekindling, since the observed PPT variability was still relatively high. In addition, it might be argued that PPT or PPS stimulate both skin and muscle, but changes in PPT and PPS did not show any significant correlation. Furthermore, it has been shown that the pinprick threshold is increased

whereas PPT values are not affected when the skin in anesthetized (Graven-Nielsen et al., 2004). Additionally, muscle tissue subjected to a sustained temperature of 42 °C over 30 minutes showed not effect in the force exerted in rats, although it drastically affected the contractile properties of the muscle, reducing tetanic and peak twitch tension (Locke & Celotti, 2014). Therefore, studies assessing muscle control and function during movement or a force task may have to take the changes in contractile properties into consideration in their conclusions. Finally, further experiments are required in order to confirm the physiological mechanisms behind the model, in order to better associate it with mechanisms found in physiopathological conditions.

CONCLUSION

This new model based on SWD represents a promising tool for investigating muscle pain/soreness in humans. The main advantages of the model are its non-invasiveness, the ability to control stimulation parameters, and the convenience of the time frame in which pain and hyperalgesia are developed.

CONFLICT OF INTEREST

None declared.

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AUTHOR CONTRIBUTIONS

All authors of this manuscript have discussed the results and commented on the manuscript.

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FIGURE LEGENDS

Fig. 1. (a) Short wave diathermy (SWD) was applied to the forearm until tolerance threshold was reached, while volunteers reported the response profile of thermal stimuli using an Analog Visual Scale (VAS). (b) Experimental procedure. Pressure pain threshold (PPT) and pinprick (PPS) were first measured at baseline. Afterwards, SWD was administered to the dominant forearm. A self-reported muscle pain/soreness assessment of the model effects was carried out, then PPT and PPS assessment was repeated after 0, 30, and 60 min.

Fig. 2. (a) Time course of the reported thermal induced muscle pain during SWD (0 represents the beginning of the stimulation), it can be noted that tolerance thermal threshold was reached with different speed across subjects. (b) pain chart drawings of the painful area at the tolerance threshold in the treated arm.

Fig. 3. Self-reported muscle soreness scores of the treated arm at baseline, 0, 30, 60 min, and 24 hs (see Table 1 for a description of the soreness scores).

Fig. 4. Individual (light numbered lines) and average (heavy line) PPT from control (left) and treated (right) arms at 0, 30, 60 minutes after intervention. Values are presented as percentage of change from baseline -i.e. before SWD. ** Treated arm showed a significant decrease in PPT compared with control arm for all time points ($P = 0.008$).

Fig. 5. Individual (light numbered lines) and average (heavy line) PPS scores from treated and control arm at 0, 30, 60 minutes after application of SWD. Values are presented as percentage of change from baseline -i.e. before SWD. * Treated arm showed a significant increase of the PPS compared with the control arm only immediately after SWD ($P = 0.019$).

TABLE LEGENDS

Table 1. Modified Likert scale of muscle pain/soreness.

Table 2. Absolute values for pressure pain thresholds (PPT) and pinprick sensitivity (PPS) before, immediately after, and 30 and 60 min after administration of short-wave diathermy (SWD).

Score	Description
0	a complete absence of soreness
1	a light soreness in the muscle felt only when touched/a vague ache
2	a moderate soreness felt only when touched/a slight persistent ache
3	a light muscle soreness when lifting objects or carrying objects
4	a light muscle soreness, stiffness or weakness when moving the wrist without gripping an object
5	a moderate muscle soreness, stiffness or weakness when moving the wrist
6	a severe muscle soreness, stiffness or weakness that limits my ability to move

		Baseline	**0 min**	**30 min**	**60 min**
PPT	**Treated**	177.61 ± 83.03	151.39 ± 66.43	148.55 ± 64.42	156.28 ± 58.35
	Control	164.33 ± 64.50	166.44 ± 78.76	162.44 ± 72.56	176.44 ± 92.29
PPS	**Treated**	30.83 ± 16.11	38.05 ± 16.64	36.11 ± 18.27	34.17 ± 16.38
	Control	29.83 ± 14.03	30.67 ± 14.36	31.67 ± 14.55	33.61 ± 14.53