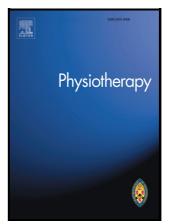
Changes in electromyographic activity of latent trigger points after a dry needling intervention: a randomised controlled trialelectromyographic activity after dry needling

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Title: Changes in electromyographic activity of latent trigger points after a dry needling intervention: a randomised controlled trial

Running head: electromyographic activity after dry needling

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Ethical Approval and clinical trial registration

The study was approved by the General Hospital Complex in Toledo (number-507, dated:16/04/2020) and the clinical trial was registered with ClinicalTrials.gov (NCT04684784). All participants gave written informed consent prior to their inclusion.

Author contributions

All authors contributed to data interpretation, drafting of the manuscript, and final approval of the manuscript. JSI, FJ and JAV contributed to the study design and oversight. ABS and PEG conducted blinded assessments of clinical outcomes. JSI performed the interventions. JAV and FJ conducted data analysis.

Declaration of conflicting interests

All authors report no conflicts of interest.

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Contribution of the paper:

1. In a healthy volunteer, dry needling is effective for reducing electromyography activity in latent trigger point

2. In a healthy volunteer, dry needling is effective for reducing muscle fatigue in latent trigger point

3. In a healthy volunteer, dry needling is effective in increasing pressure pain threshold in latent trigger point

Abstract

Objective

To analyse the effects of dry needling (DN) in upper trapezius latent trigger points (LTrPs) on pressure pain threshold (PPT) and surface electromyography (sEMG).

Design

Randomized, double-blind, placebo controlled clinical trial.

Settings

Sports Rehabilitation Laboratory, University of Castilla-La Mancha.

Participants

Forty-six participants (18-35 years old) with LTrP in the upper trapezius were divided into two groups: DN-group and Sham-DN-group.

Interventions

In the DN-group, the needle was inserted 10-times through the skin, and it was manipulated up and down using a "fast in and out" technique. In the Sham-DN-group, non-penetrating needles were used.

Main outcome measures

PPT, sEMG at rest, and sEMG in isometric contraction of the LTrP of the upper trapezius muscle were evaluated at baseline, 30 min after treatment, and after 24 hours, and 72 hours of follow-up.

Results

The mean change in sEMG at rest between baseline and 30 min was -0.38 (0.38) %refRMS for the DN group and -0.05 (0.31) %refRMS for the Sham-DN group (mean difference -0.34, 95% confidence interval (CI) of the difference: -0.54 to -0.13), and between baseline and 24 hours was -0.35 (0.35) %refRMS for the DN group and -0.06 (0.58) %refRMS for the Sham-DN group (mean difference -0.29, 95% CI: -0.57 to -0.01). In addition, the DN-group showed higher values of PPT than the Sham-DN group at 72 hours (5.22 (1.23) to 4.65 (1.03) kg/cm²; p<0.05).

Conclusions

A single session of DN intervention was effective in reducing the electromyographic activity, muscle fatigue and pain of the upper trapezius muscle in LTrP.

Registration

NCT046847XX

Keywords: Dry needling, latent trigger point, electromyography, upper trapezius

1. Introduction

Trigger points (TrPs) are defined as "a hyperirritable spot within a taut band of skeletal muscle that is painful on compression, stretch, overload, or contraction of the tissue which usually responds with a referred pain that is perceived distant from the spot".[1] TrPs can be classified as active or latent. Active-TrPs are those causing clinical complaints and their referred pain reproduces the symptoms of the patient; Latent-TrPs (LTrPs) can cause motor dysfunction (stiffness, restricted range of motion, fatigability) but not spontaneous sensory symptoms, unless they are stimulated.[1, 2] Active-TrPs and LTrPs show higher concentrations of the biochemical mediators associated with muscle pain and inflammation (calcitonin gene related peptide and substance P) than non-TrPs,[3] implying increased stiffness or delayed muscle onset soreness in patients with LTrPs.[4]

Several experiments have provided evidence that LTrPs may disturb normal patterns of motor recruitment and movement efficiency[2] and accelerate muscle fatigability with simultaneous overloading of the active motor units close to a LTrP, contributing to impaired muscle recruitment timing when performing active joint movement.[5] Previous studies which employed electromyography to analyse muscle electromyographic activity showed abnormal end plate activity, which is often reported

as spontaneous electrical activity, near to TrPs.[5, 6] This spontaneous electrical activity is higher in Active-TrPs and LTrPs, and absent in normal muscle tissue.[7] In addition, positive correlations between spontaneous electrical activity and pain intensity, pressure pain threshold,[7] muscle tension and the formation of the taut band[8] have been described. Due to the motor alterations associated with the presence of LTrPs, intervention could be relevant from a clinical point of view even if there were no spontaneous pain.

Some therapeutic treatments (e.g., ultrasound, manual therapy, or ischemic compression) have been proposed for the management of TrPs, but dry needling (DN) has gained in popularity.[4] DN is a therapeutic procedure in which a needle is inserted through the skin without the use of an anaesthetic.[9] One of the most commonly used procedures is to reach the muscle at the location of the TrP and manipulate the needle using a "fast in and out" technique.[2] DN is considered an effective treatment to reduce neck pain in the short term by treating TrPs.[10] Many studies have investigated the effect of DN treatment on pain [9] and pressure pain threshold [11, 12] (PPT) in TrPs, but few studies have investigated its effect on muscle activity with surface electromyography (sEMG). De Meulemeester, Calders [13] reported that a single DN session led to a significant decrease of the upper trapezius muscle sEMG activity after a typing task in office workers with trapezius myalgia. Aguilera, Martín [14] found a significant decrease in sEMG activity after treatment of LTrPs in healthy subjects with ischemic compression. Therefore, it could be that the direct mechanical action on LTrPs could cause the reduction of electromyographic activity.

Due to the clinical importance of evaluating DN treatment effects on hyperalgesia and electrical activity in upper trapezius muscle LTrPs, the aim of this

study was to evaluate the effect of DN intervention in PPT, root mean square (RMS), and mean frequency (MF) of sEMG at rest and during an isometric contraction in healthy subjects with LTrPs of the upper trapezius, and to compare the results with sham-DN treatment group. We hypothesised that patients who received DN on LTrPs in the upper trapezius muscle would achieve greater reductions in PPT and the EMG signal compared to those who received Sham-DN.

2. Material and methods

2.1 Study Design

A double-blind, randomised and placebo controlled clinical trial was designed. The study was approved by the General Hospital Complex in Toledo (number-507, dated:16/04/2020) and the clinical trial was registered with ClinicalTrials.gov (NCT046847XX). All participants gave written informed consent prior to their inclusion.

2.2 Participants

Forty-six participants were randomly allocated into two groups using a random number list generated by online software (http://www.randomization.com): the dry needling group (DN group) and the sham dry needling group (Sham-DN group). Sample size was calculated based on the work of De Meulemeester, Calders [13] who measured "sEMG activity". Considering the probability of loss during follow-up (20%) the minimal number of participants required to attain a power of 0.9 and a bilateral α level of 0.05 for analysis of variance (ANOVA) with repeated measures was calculated to be 17 per group.

Investigators and participants were blinded in allocation by using sealed opaque envelopes. Participants were excluded if they reported a history of: vertebral pathology, neck pain symptomatology, medical treatment, pharmacological treatment or physical therapies in the cervical region during the previous 6 months. They were also excluded if there were any red flags to DN and any history of head and upper extremity surgery or trauma. Participants aged between 18-35 years were screened for the presence of LTrP in the middle third of the upper trapezius muscle on the dominant side. A physiotherapist (JSI) with more than 5 years' experience in the identification of TrPs determined the presence of LTrP with the participants in a prone position with arms outside the stretcher. The criteria to determine TrPs presence were: 1) a a palpable taut band, 2) presence of a painful spot in the taut band and 3) presence of a local twitch response (LTR) of the taut band with palpation.[15] The LTrP was marked on the skin to identify it, perform the intervention and assess the outcomes.[11] During the palpation of LTrP, the participants were asked to report if they had referred pain pattern.

2.3 Intervention

The same physiotherapist (JSI) who identified the LTrP performed all the interventions. Before treatment, the skin was cleaned with chlorhexidine. A single DN session was performed in the middle third of the upper trapezius muscle on the dominant side with a solid filiform needle (0.30x50 mm). The needle was inserted through the skin and manipulated up and down using a "fast in and out" technique[16] within the LTrP 10 times, according to the DN protocol.[11] The local twitch responses of each intervention were recorded. For the Sham-DN group, non-penetrating needles (blunt needles) were applied on the LTrP with the same procedure as the DN group to provoke a pricking feeling without penetrating the skin.[17]

2.4 Outcome measures

The primary outcome of this research was to measure the changes in resting sEMG activity (amplitude, RMS). The secondary outcomes were to analyse the changes in resting sEMG activity (Median frequency (MF)), submaximal contractions sEMG activity of upper trapezius (RMS), submaximal contractions sEMG activity of upper trapezius (RMS), submaximal contractions sEMG activity of upper trapezius (MF) and PPT. The blinded examiners were positioned behind the participants who sat with their heads in a neutral position. PPT, RMS and MF of sEMG at rest and during an isometric contraction of the upper trapezius muscle were evaluated at the beginning of the study, 30 min after treatment, and after 24 hours and 72 hours of follow-up. Muscle thickness with ultrasound was measured at the beginning of the study in all subjects.

2.4.1 Pressure Pain Threshold

A manual mechanical algometer (FDK/FDN, Wagner Instruments, 1217 Greenwich, CT 06836 USA) was applied to assess PPT at the LTrP. With the subject seated with their head in a neutral position, the blinded examiner placed the tip of the algometer perpendicularly on the identified skin mark and applied an increasing pressure of 1 kg/cm² per second, controlled by a standard metronome. When the sensation of pressure changed to a feeling of pain, the participants had to say "now" and the applied pressure was recorded.[18] Participants carried out prior familiarisation, and a previous study had reported high intra-rater reliability of PPT.[19] The mean value of three repeated

assessments performed on the LTrP of the upper trapezius muscle in an interval of 30 seconds was recorded.[18]

2.4.2 Electromyographic Activity

Muscular activity of the upper trapezius muscle on the dominant side was recorded using a ME6000T8 device (Mega Electronics, Kuopio, Finland) and was analysed using MegaWin 3.1-b10 software (Mega Electronics, Kuopio, Finland). The signal was recorded at a sampling frequency of 1000 Hz with 14-bit analog-to-digital converter and an RMS was applied at intervals of 0.05 seconds. The signal was reamplified with a preamplifier located 6 cm from the electrodes (sensitivity 1 μ V, resolution 1 μ V, gain 1, CMRR 110dB, filter 8-500 Hz at 3dB). First, the skin was shaved and cleaned with alcohol to minimise skin impedance. The surface electrodes (Ambu BlueSensor N, Ballerup, Denmark) were placed on the muscle belly at the point marked with a distance between electrodes of 20 mm.[20] The reference electrode was placed on the C7 vertebra following sEMG for Non-Invasive Assessment of Muscles recommendations.[21]

A series of maximal voluntary contraction (MVC) values was recorded prior to the initial assessment and these values were used for normalisation of the RMS. For MVC, the examiner would give a verbal stimulus: "Try to raise your shoulders to the ceiling", with the subject positioned on a support specially designed for this activity,[22] which restricted the elevation of the shoulders (Figure 1). The participants were familiarised with the test prior to the measurements.

The evaluation of sEMG activity of the upper trapezius muscle was performed in two different tests: rest and isometric tests (Figure 1). During the resting sEMG exam,

the participants were instructed to remain in a sitting position with their backs fully supported, their feet parallel, with the third finger reaching the patella, and looking at a mark painted on the wall without speaking while relaxing neck and shoulders.[13] During isometric sEMG activity, the participants were instructed to remain in a sitting position with their backs fully supported, with their arms at 90° of abduction and the palms of the hands facing downward for 30 seconds. The RMS and the MF (Hz) were used for the analysis of muscle activity. The values of RMS were normalised and expressed as a percentage of the reference MVC to exclude variability due to interindividual differences (%refRMS).

2.4.3 Ultrasonography Scanning.

All the participants were examined with a Logiq S8 (GE Healthcare, Milwaukee, USA) with a 4 to 15-MHz linear probe (ML6-15-D; GE Healthcare system, Milwaukee, USA), using grey scale ultrasound. The centre of the linear transducer coincided with the skin mark over the dominant UT muscle. Thickness was defined as the vertical distance between echogenic fascial layers and was evaluated using ImageJ software, 1.47 version (National Institute of Health, Maryland, USA). Participants were instructed to sit relaxed with 0° of shoulder abduction while their forearms were on the armrest of the chair and in the pronation position. The head was in a neutral position. The UT muscle thickness was measured in the resting position at the end of the relaxed expiration.[11].

2.5 Statistical Analysis

Data were analysed using IBM SPSS Statistics v.24.0 software. The data was tested for normality with the Shapiro-Wilk test. All the variables presented a normal distribution (p>0.05). Mean and Standard deviation (SD) were calculated for all demographic data and all values. A 2-way repeated-measures ANOVA was performed for all outcome variables to analyse the interaction between groups (DN group and Sham-DN group) and the time of assessment (Baseline, 30 min, 24 hours, and 72 hours). Statistical significance was accepted when p<0.05. When differences were established, we applied a post-hoc Bonferroni multiple-comparisons test. The effect size (ES) was interpreted using Cohen's d: low (0.20), medium (0.50) and high (0.80).[23]

3. Results

Fifty patients volunteered to participate in the study (Figure 2). Two patients were excluded for not meeting inclusion criteria. One patient in the DN group and one patient in the Sham-DN group dropped out, as they did not attend on the follow-up days. There were no dropouts due to adverse effects of the therapies during follow-up. Therefore, the final sample was composed of 46 patients (23 in each group). No significant differences were found between the DN group and the Sham-DN group in demographic characteristics (Table 1). During needling in the DN group, subjects had 6.22 (2.11) local twitch responses.

In the PPT (Figure 3), there was significant intervention effect (F=18.7, p<0.01) and time effect (F=12.8, p< 0.01). The DN group showed lower values than the Sham-DN group at 24 hours (p<0.05) and higher values 72 hours (p<0.01). The mean change (SD) in PPT between baseline and 24 hours was -0.79 (0.57) kg/cm² for the DN group and 0.07 (0.60) kg/cm² for the Sham-DN group (mean difference -0.86 kg/cm², 95%)

confidence interval (CI) of the difference -1.22 to -0.51), and between baseline and 72 hours was 0.56 (0.98) kg/cm² for the DN group and -0.07 (0.81) kg/cm² for the Sham-DN group (mean difference 0.63 kg/cm², 95% CI of the difference 0.09 to 1.78).

In the sEMG at rest (Table 2), the DN group showed lower values in RMS than the Sham-DN group at 30 min (ES=0.6; p<0.05). The mean change (SD) in sEMG at rest between baseline and 30 min was -0.38 (0.38) %refRMS for the DN group and -0.05 (0.31) %refRMS for the Sham-DN group (mean difference -0.34 %refRMS, 95% confidence interval (CI) of the difference -0.54 to -0.13), and between baseline and 24 hours was -0.35 (0.35) %refRMS for the DN group and -0.06 (0.58) %refRMS for the Sham-DN group (mean difference -0.29 %refRMS, 95% CI of the difference -0.57 to -0.01). (Figure 4)

In the sEMG in isometric contraction (Table 2), the DN group showed lower values in RMS than the Sham-DN group at 72 hours (ES=0.6; p<0.05). The mean change (SD) in sEMG at isometric contraction between baseline and 30 min was -2.92 (3.51) %refRMS for the DN group and -0.50 (4.55) %refRMS for the Sham-DN group (mean difference -2.43 %refRMS, 95% CI of the difference -4.84 to -0.02), and between baseline and 72 hours was -4.18 (5.47) %refRMS for the DN group and -0.43 (6.12) %refRMS for the Sham-DN group (mean difference -3.75 %refRMS, 95% CI of the difference -7.20 to -0.30). On the other hand, the DN group showed higher MF values in isometric activity than the Sham-DN group at 72 hours (ES=0.7; p<0.05). (Figure 4)

4. Discussion

The main finding of our study was that one session of DN intervention in LTrPs in the upper trapezius muscle increased PPT and EMG muscle activity for the DN group

compared to the Sham-DN group. The DN group showed higher values of PPT after 72 hours follow-up compared to the baseline, indicating lower pain to pressure. Considering the values of the DN group, DN reduces the level of muscle activation at 30 min and 24 hours at rest, and up to 72 hours for the isometric contraction of the upper trapezius muscle. In addition, DN increases the MF (Hz) of the upper trapezius muscle when performing an isometric contraction in 90° shoulder abduction, indicating a reduction in muscle fatigue after the DN intervention.

Post-needling soreness directly influences PPT due to mechanical hyperalgesia caused by the DN intervention.[24] The PPT results of the present study are in consonance with previous articles using volunteers with LTrPs.[11, 12, 25] A decrease in PPT was described in the mechanical hyperalgesia observed at 30 min and 24 hours follow-up after needling in the DN group, although an increase in PPT was found 72 hours after treatment. This indicates that the mechanical hyperalgesia caused by the needle was present up to 24 hours, and that there was an increase in pressure pain tolerance at 72 hours after the intervention.

LTrPs can cause local motor dysfunctions such as muscle cramps and weakness, restricted joint range of motion, altered motor control strategy, an increase in electromyographic activity[6] and may reflect an additional recruitment of motor units to compensate for a decreased discharge rate of low-threshold motor units, associated with muscle pain and fatigue.[26] In our study we found a significant decrease in RMS from 30 min after intervention to 24 hours at rest and up to 72 hours in shoulder isometric contraction at 90° abduction, indicating a decrease in the electromyographic activity immediately after the intervention and its maintenance in the follow-up days. This has a potentially important clinical implication, as it means less motor unit

recruitment, which is associated with less muscle fatigue and prevention of motor dysfunction that may lead to future injury.[27, 28] Our results are in the line with other articles that found a decrease in the electromyographic activity of the upper trapezius muscle after application of a mechanical treatment of LTrP (ischemic compression[14] or DN[13, 29]), although Baraja-Vegas, Martín-Rodríguez [4] did not find changes in electromyographic activity of the gastrocnemius muscle after DN intervention. These discrepancies between the studies could be because the DN was performed on different muscles and with different population samples.

Several experiments have shown evidence that LTrPs may accelerate muscle fatigability with simultaneous overloading of the active motor units close to a LTrP.[5] The main evaluation of muscle fatigue is measured with sEMG that analyses the MF emitted by the active muscle, [30] and the decrease in MF is a recognised method of determining fatigue in an isometric muscle action.[31] Some studies have shown an increase of fatigue in patients with neck pain and presence of TrPs in the upper trapezius muscle during isometric contractions[22] and daily activities,[32] so their treatment is important. In our study, we found an increase in MF at 72 hours after DN when performing isometric exercise (90° degrees of shoulder abduction), which indicates that the DN intervention could reduce the muscular fatigue of the upper trapezius muscle with LTrPs in isometric activities. This finding is important, because it could be a useful technique for a person who performs isometric activity in their daily life or sports practice to present less muscle fatigue and a later onset of muscle soreness. In addition, this data are interesting because it could be an indicator of when it is effective to return to activities of daily life or sports training after needling. On the other hand, no effects were found on muscle fatigue at rest while the participant was sitting.

Some limitations are observed in our study. First, only the short-term (72 hours) effects of DN were collected, so the results cannot be interpreted in the medium and long term. Second, although the researchers indicated that physical activity should not be done on the days of the measurements, the upper trapezius is a muscle in which activity can be increased for different reasons (stress, body posture, how you have rested that night...) apart from physical activity. Third, the changes we observed are in healthy volunteers, so they cannot be extrapolated to the response in the clinical population. Future research is needed to determine the effects of the DN intervention in the medium and long term, compared to other techniques in patients with clinical pathology. Furthermore, it would be interesting to study in clinical population changes in functional movements, motor patterns and muscle activation with LTrPs and the effect of their treatment. Finally, the results of this study must be interpreted with caution, considering that the populations and clinical conditions.

5. Conclusion

In this study, we concluded that a single session of DN intervention was effective in reducing the electromyographic activity of the upper trapezius muscle in LTrP immediately after the intervention, up to 24 hours at rest and up to 72 hours at rest and during isometric contraction, as well as increasing PPT at 72 hours compared to the Sham-DN group. In addition, muscle fatigue decreased 72 hours after the intervention when performing the isometric contraction. Future research is needed to discover the extent of LTrP treatment in healthy people and the effect of LTrP treatment on motor patterns in different muscles and populations.

Declaration of conflicting interests

All authors report no conflicts of interest.

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Table 1: Demographic and clinical characteristics of patients

	DN Group (n=24)	Sham-DN Group (n=24)
Sex (male/female)	18/6	18/6
Age (years)	24 (3)	24 (3)
Weight (kg)	70 (12)	71 (9)
Height (cm)	174 (9)	174 (7)
Muscle thickness (cm)	1.14 (0.31)	1.10 (0.18)
Referred pain (positive/negative)	12/12	12/12
Dominant (right/left)	21/3	20/4

DN Group, Dry needling Group; Sham-DN, Sham Dry Needling Group; SD, standard deviation.

Values are reported as mean (SD).

	Baseline	At 30 min	Post 24 hours	Post 72 hours		F	р	n_p^2	Power
RMS _r (%refMV	/C)						K		
DN Group	1.05 (0.55)	0.67 (0.47) **#	0.70 (0.38) **	0.80 (0.68)	Group	1.70	0.20	0.04	0.25
Sham-DN	1.01 (0.56)	0.97 (0.49)	0.96 (0.52)	0.94 (0.51)	Time	6.64	<0.01	0.32	0.96
				30	Group x Time	3.74	0.02	0.21	0.77
RMS _i (%refMV	′C)		$\langle \langle \rangle$						
DN Group	15.64 (5.69)	12.71 (5.52) **	12.14 (4.56) *	11.46 (5.22) **#	Group	1.58	0.22	0.04	0.23
Sham-DN	15.35 (6.91)	14.86 (6.68)	14.37 (7.57)	14.92 (6.34)	Time	4.99	<0.01	0.26	0.89
					Group x Time	2.42	0.08	0.15	0.56
MF _r (Hz)									
DN Group	54.61 (20.65)	62.30 (25.72)	51.83 (19.16)	51.30 (17.37)	Group	0.68	0.41	0.02	0.13
Sham-DN	53.70 (23.77)	51.91 (19.69)	49.39 (21.37)	47.17 (27.39)	Time	2.03	0.13	0.13	0.48

Table 2: Outcome measurements of surface electromyography activity at rest and among patients at different time points.

					Group x Time	0.94	0.43	0.06	0.24
MF _i (Hz)									
DN Group	76.52 (11.68)	80.43 (12.64)	77.61 (12.32)	86.56 (15.00) *#	Group	3.49	0.07	0.07	0.45
Sham-DN	74.35 (13.21)	74.00 (13.98)	75.61 (13.56)	77.04 (10.66)	Time	3.62	0.02	0.21	0.76
					Group x Time	1.66	0.19	0.11	0.40

DN Group, Dry needling Group; Sham-DN, Sham Dry Needling Group; SD, standard deviation; RMS_r , Root Mean Square at rest; RMS_i , Root Mean Square in isometric contraction; MF_r , Median Frequency at rest; MF_i , Median Frequency in isometric contraction; MVC, Maximal voluntary contraction; Group, Main effect of group in the ANOVA results; Time, Main effect of time in the ANOVA results; Group x Time, Main effect of group x time in the ANOVA results; n_p^2 , partial eta squared.

Values are reported as mean (SD).

P < 0.05, P < 0.01, posttreatment, 24 hours follow-up, 72 hours follow-up compared with baseline.

#P < 0.05, #P < 0.01, comparisons between the DN and Sham-DN groups at corresponding time points.



Figure 1. A. Superficial electromyographic recording during Maximal voluntary contraction of the upper trapezius. **B.** Superficial electromyographic recording of the upper trapezius at isometric contraction. **C.** Superficial electromyographic recording of the upper trapezius at rest.

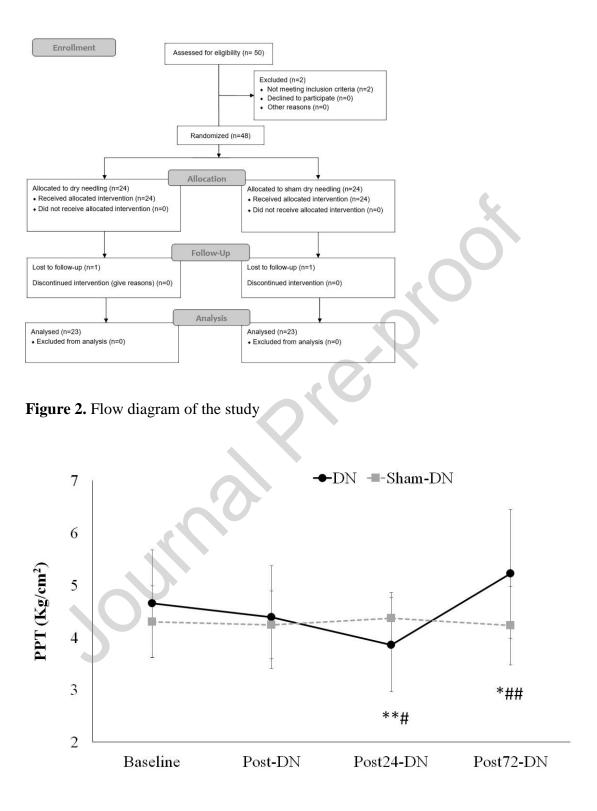


Figure 3. Mean changes in the pressure pain threshold (PPT) score in Dry Needling (DN) and Sham DN groups. Mean values and Standard deviation are shown. *P < 0.05,

**P < 0.01, posttreatment, 24 hours follow-up, 72 hours follow-up compared with baseline. #P < 0.05, ##P < 0.01, comparisons between the DN and Sham-DN groups at corresponding time points.

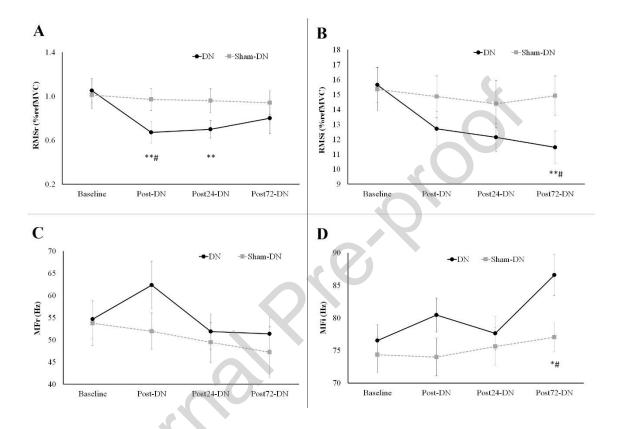


Figure 4. Mean changes: **A.** In the Root mean square of EMG at rest (RMSr). **B.** In the Root mean square of EMG at isometric contraction (RMSi). **C.** In the Median Frequency of EMG at rest (MFr). **D.** In the Median Frequency of EMG at isometric contraction (MFi). Mean values and standard error are shown. *P < 0.05, **P < 0.01, posttreatment, 24 hours follow-up, 72 hours follow-up compared with baseline. #P < 0.05, comparisons between the DN and Sham-DN groups at corresponding time points.