



# Effect of Dry Needling on miR-939 and miR-25 Serum Levels in Myofascial Pain Syndrome With Shoulder Girdle Myofascial Trigger Points

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## Abstract

**Objectives:** Micro-RNAs (miRs) affect the gene expression of the pain and inflammation mediators. Myofascial trigger points (MTrPs) are one of the most common causes of skeletal and muscle pain. In this regard, dry needling (DN) was favored by physiotherapists as an effective method of treatment for mucopolysaccharidosis. The present study aimed to assess the effectiveness of DN on serum concentration of miR-939 and miR-25 before and after the treatment in patients with muscular pain caused by MTrPs in their shoulder girdle muscles.

**Methods:** Twenty patients with pain in their shoulders, upper limbs, along with heads and necks, who had 3-5 active MTrPs in their shoulder girdle muscles participated in the study. They were treated in 5 sessions with DN. One session was held per day every three days for 2 weeks. Before and after the treatment, the pain intensity of the patients was measured using the visual analogue scale (VAS), their peripheral blood samples were taken, and miR serum concentrations were estimated by real-time polymerase chain reaction.

**Results:** Comparing pain intensity in patients revealed a significant reduction after the treatment ( $P < 0.05$ ). In addition, statistical analysis indicated up-regulation of miR-939 and miR-25 with a significant difference ( $P < 0.05$ ) after the treatment compared to the time before the treatment.

**Conclusion:** According to the reduction in pain intensity and increased expression of miR-939 and miR-25 serum levels after treatment, it can be concluded that DN is an effective technique for treatment of mucopolysaccharidosis in shoulder girdle muscles.

**Keywords:** MicroRNA, miR-939, miR-25, Pain, MTrPs, Muscle pain, Dry needling

## Introduction

So far, 820 micro-RNAs (miRs) were identified in the human body. They can control one-third of the human mRNAs; in other words, each miR can identify up to 200 of its target mRNAs. Numerous recent studies reported miRs as biomarkers for early diagnosis of the patients (1, 2). All body fluids such as serum, plasma, saliva, and urine contain miRs which can be used as a supplementary diagnostic means in various health problems like heart disease, cancers, and chronic pains (3, 4). These molecules are effective in expressing genes and controlling vital factors which regulate different pathways involved in growth, cell differentiation, reproduction, and apoptosis. Previous studies revealed that different miRs are expressed in a cell and different tissues. Any deviation in the expression of miR scan causes different diseases (5). Currently, treatment with miRs is being investigated.

Pain due to myofascial trigger points (MTrPs) is one of the common causes of the patients' referral to the treatment centers. The patients have symptoms such as severe pain, referral pain, as well as sensory and motor disorders associated with the involvement of the autonomic system (6-9). The pain can become acute or chronic by various mechanisms. In this regard, the role of miRs in regulating the proteins affecting the mechanism of inflammation and pain is highlighted (10). Research on various pain models indicates that miRs affect GABA $\alpha_1$ , GABA and COX-2 molecules, along with sodium and calcium channels in order to regulate these mechanisms (11). Therefore, scientists confirm miR use as biomarkers in chronic pains.

In different research studies on experimental animal and human models of patients who suffer from pain, higher or lower expression of miRs in neural tissues,

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cerebrospinal fluid, and human blood is proved (12). Bjersing et al in their study on Swedish women with fibromyalgia, reported a decrease in 9 types of pain- and inflammation-related miR in their cerebrospinal fluid samples (13). In another study, changes in the expression level of 18 miRs were confirmed in patients suffering from complex regional pain syndrome (CRPS) (4). In addition, the results of the recent studies implicated that the effect of miR-25 on regulating Wnt signaling. In fact, the inhibitory role of miR-25 is confirmed in repressing the Wnt pathway and it is suggested that while miR-25 functions at the level of  $\alpha$ -catenin ( $\alpha$ -cat), it is a regulator of immune cell differentiation (12, 14). Further, previous studies predicted that Hsa-miR-939 can target several pro-inflammatory genes such as IL-6, VEGFA, TNF $\alpha$ , NF $\kappa$ B2, and nitric oxide synthase 2 (NOS2A). IL-6, NOS2A and NF $\kappa$ B2 mRNAs, IL-6, VEGFA, NOS2 proteins, and NF $\kappa$ B activation are reduced as a result of overexpressing miR-939 *in vitro*. Furthermore, decreased level of miR-939 can help in raising the levels of endogenous NOS2A and NO and may increase pain and inflammation (15,16). However, a number of studies investigated changes in miR before and after the treatment (17) whereas dry needling (DN) is used as an effective method for treating chronic muscle pain due to MTrPs (18, 19). Therefore, the present study aimed to study the effectiveness of DN method on the changes in serum levels of hsa-miR-939 and hsa-miR-25 before and after the treatment in patients with mucopolysaccharidosis associated with trigger points in the shoulder girdle muscles.

## Material and Methods

### Research Design

This study is a clinical trial with a quasi-experimental design. The samples were collected by simple random sampling technique. Considering the study design, each sample was his/her own control case who was compared with him/herself before and after the intervention.

### Target Population, Sample, and Sampling Method

The target population included individuals with shoulder or neck pain living in Tehran, Iran who referred to Behrad and/or other physiotherapy clinics of the University of Social Welfare and Rehabilitation Sciences. The sample size was calculated 17 people with a 95% confidence level ( $\alpha = 0.05$ ) and the test power of 80% ( $\beta = 0.2$ ) based on the following formula:

$$N = \frac{[(Z_1 - \alpha / 2) + (Z_1 - \beta)]^2 (S_1 - S_2)}{(X_1 - X_2)^2} \approx 17$$

The inclusion criteria were having pain in the shoulder, upper limbs, as well as head and neck with three important signs of trigger points (i.e., taut band, tender nodule in the taut band, and recognized pain under pressure). Exclusion criteria included having a history of severe head trauma, treating the trigger point in the past

month, using corticosteroids up to one month ago, as well as analgesics and non-corticosteroid anti-inflammatory drugs since 3 days before and during the study, and having contraindications for the DN such as local infection, pregnancy with the risk of abortion, menstruation, HIV, and the like (20,21). Finally, 20 patients were included. Their shoulder girdle muscles were examined and the trigger points were identified. Demographic data of the patients including their height, weight, and age were recorded. At least 3 and at most 5 trigger points were studied and marked in each participant's shoulder muscles.

### Intervention

In this study, DN was employed as the treatment technique. To do this, DongBang acupuncture needles of 25×30, 40×40, and 50×25 mm were used based on the body mass of the patients. Each patient received 5 doses of DN (one session per day) every three days for two weeks.

### Pain Intensity Assessment

To measure the intensity of pain, the visual analogue scale (VAS), a line graded from 0 to 10 cm, was employed. It was explained to the patients that 0 indicates "no pain" and 10 is considered the maximum pain, and then they were asked to determine their pain with respect to the numbers. The reliability and validity of this test were previously confirmed by the researchers. This measurement was performed before the initialization of the treatment and one day after the last session of treatment (22, 23).

### Blood Sampling and RNA Isolation From the Serum

A total of 10 mm blood samples were taken from the median cubital vein of all the patients before and one day after the treatment. After coagulation, the serum was isolated using a centrifuge of 12 000 rpm, and then poured in microtubes and kept at 70°C. Then, real-time polymerase chain reaction (PCR) test was performed. Additionally, QIAzol lysis solution and miRNeasy Serum/Plasma Kit (Qiagen) were employed to isolate RNA from the serum. In addition, after isolating the RNA, its quantity and quality were evaluated using a NanoDrop machine and agarose gel electrophoresis (24).

### Complementary DNA Synthesis

RevertAid™ First Strand cDNA Synthesis Kit was utilized to synthesize the complementary DNA (cDNA). In this method, cDNA was synthesized through the reverse transcriptase enzyme from isolated RNAs. Next, the PCR program was conducted to be ensured of the cDNA function. After the completion of the reaction, PCR products were analyzed by 1.5% agarose gel electrophoresis. In this study, primers of U6 were used as the reference gene.

### Real-Time PCR Reaction

Duplication was performed to measure the expression

of microRNA by relative real-time PCR method. The performance of the designed primers was evaluated and the standard curve was plotted for each primer. To this end, first, serial dilutions of 1, 1/5, 1/25, and 1/125 (0.6, 3, 15, and 75 ng/mL) were prepared from the synthesized cDNAs. Then, the real-time PCR was separately repeated twice for each dilution and each of the primers. Eventually, the standard curve for each primer was plotted based on the obtained Ct values versus the used dilutions. Using the obtained curve slope and the following Equation, the efficiency of the reaction was calculated for each primer.

$$E=10^{(-1/\text{slope})}$$

where, E denotes the reaction efficiency and slope refers to the slope of the curve. The relative quantity in real-time PCR was determined using the Exicycler™ 96 device by measuring the increase in fluorescence radiation resulted from binding by EvaGreen® Dye. The real-time PCR reaction was repeated twice.

### Data Analysis

The obtained data were statistically analyzed and the pain intensity diagrams were plotted using the SPSS software, version 19. In descriptive statistics, measures of central tendency and dispersion of studied variables were evaluated. Further, the Kolmogorov-Smirnov test was used to check the normality of data. Furthermore, the mean values and standard deviation were calculated to describe pain intensity. Paired *t* test was utilized to examine the significance of pain intensity before and after the treatment. Additionally, the raw Ct data were extracted from the device for statistical analysis of the microRNAs, and the expression was measured using the comparative CT ( $\Delta\Delta CT$ ) method. Finally, the gene expression was assessed by the REST software.

### Results

Twenty patients (16 women and 4 men) aged 20-60 years with a main body mass index of  $23.82 \pm 3.05$  were included in this study. Table 1 represents a summary of the demographic data of the participants.

The patients self-reported their pain intensity through a VAS before and after the treatment. Then, their results were compared using a paired *t* test to find if there were any significant differences in the pain intensities. The results of VAS and the comparison before and after the intervention are provided in Table 2.

**Table 1.** Demographic Data of the Subjects

N = 20	Minimum	Maximum	Mean ± SD
Weight	45	98	72.55±13.46
Height (m)	1.54	1.89	1.7405±.087
Age	26	50	41.95±6.73
BMI	15.15	29.34	23.82±3.05

Abbreviations: SD, standard deviation; N, case number; BMI, body mass index.

**Table 2.** Pre- and Post-measurement Scores for VAS in the Patients

Variables	Before Treatment Mean ±SD	After Treatment Mean ±SD	P Value
VAS	7.05	2.90	0.000

Abbreviations: VAS, visual analogue scale; SD, standard deviation.

The mean VAS value decreased from 7.05 before the treatment to 2.90 after the treatment (Figure 1).

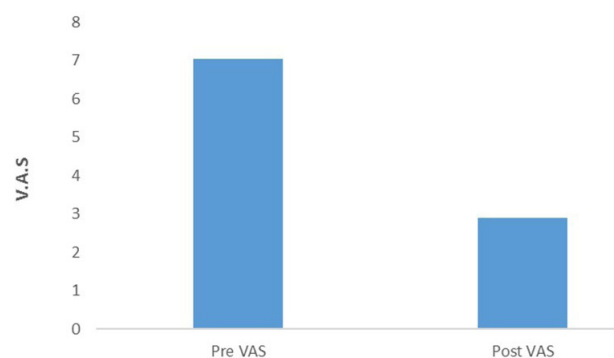
As previously explained, RNA was extracted. The results of the UV absorption of the extracted RNAs for cDNA synthesis were read by the NanoDrop instrument. At this step, samples with a wavelength of 260/280 nm and UV absorption of 1.7-2 were used to synthesize the cDNA. As noted, the qualities of the extracted RNAs were examined by 1.5% Agarose gel electrophoresis. The existence of two 18S and 28S ribosomal bands indicated the non-isolation of RNA.

As shown in Table 3, miR-939 and miR-25 are up-regulated after the treatment (compared to the time before treatment) by mean factors of 7.111 (S.E. range is 6.964-8.000) and 4.287 (S.E. range is 3.732-4.925), respectively. The results of the statistical analyses using the REST demonstrated a significant difference in serum levels of miR-939 and miR-25 in the studied variables before and after the intervention.

Finally, the results of miRs indicated an up-regulation of miR-939 and miR-25 after employing DN technique to treat the muscle pain resulted from MTrPs.

### Discussion

The results of the study represented the upregulation of miR-939 and miR-25 after the therapeutic intervention. Several studies were conducted to investigate the role of miRs in controlling various stages of chronic inflammation and pain while data on this topic have not yet been completed. Irina et al conducted a study on patients with CRPS and compared them with healthy individuals. They extracted 18 types of miRs from the patients' peripheral blood which demonstrated the down-regulation of miR-939 and miR-25. In addition, they found that VEGF



**Figure 1.** Pain intensity (VAS Score) Treatment After Compared to Before Treatment

Table 3. Relative Expression Report

Gene	Type	Reaction Efficiency	Expression	Standard Error	95% CI	P Value	Results
U6	Reference	1.0	1.000				
miR-939	Target	1.0	7.111	6.964 - 8.000	4.000 - 8.574	0.000	Up-regulation
miR-25	Target	1.0	4.287	3.732 - 4.925	3.482 - 5.278	0.000	Up-regulation

gene increased in the serum of patients with CRPS due to the decrease in miR-939 expression (4). Another study reported a decrease in miR-939 expression by examining the expression level of peripheral blood pain-related miRs in patients with CRPS. The miR-939 serum was found to inhibit the genes which express inflammatory mediators of VEGFA, TNF $\alpha$ , NF $\kappa$ B2, and NOS2A. Therefore, a decline in miR-939 level led to an increase in the expression of the above-mentioned molecules and exacerbated the inflammation and pain in this group of patients (16). These results, along with the results of the current study regarding pain reduction while the upregulation of miR-939 and miR-25 after the treatment indicate the effectiveness of DN method in reducing the pain and inflammation.

Yao et al examined the blood samples of 70 patients with sepsis, compared these samples with those of 30 healthy people, and noticed a decrease in the serum level of miR-25 in patients compared to healthy individuals. They attributed this reduction to the role of miR-25 in regulating the immune cells and considered it effective in the inflammation process. They further suggested that miR-25 can be used as a biomarker for sepsis compared to CRP and PCT (25). Several other studies investigated the role of miR-25 in inflammation process related to the acute aerobic exercise and ozone therapy. It was found that miR-25 can inhibit cell proliferation through the pathway of Wnt and the downstream gene expression of b-cat cellular proliferation and as a result, can affect VEGF receptors. Furthermore, it can target type B endothelial receptors and thus intervene in the regulation of VEGF serum levels in patients with chronic pain (4,14). The results of the current study suggested that upregulating miR-25 after the treatment can indicate the effectiveness of DN in reducing the pain and inflammation.

Testing the laboratory rats which suffered from sciatic nerve chronic constriction injury, Yan et al reported a decrease in the expression of miR-939 whereas an increase in the concentration of COX-2, IL-6, and TNF- $\alpha$  molecules in the spinal cord of the rats compared to the sham group (26). In a similar study, the increased expression of miR-150 while the decreased expression of COX-2, IL-6, and TNF- $\alpha$  molecules were reported in the laboratory rats with chronic constriction injury (27).

In a number of studies evaluating different models of pain, it was observed that miRs affect GABA $\alpha$ 1, GABA, and COX-2 molecules, as well as sodium and calcium channels in order to regulate the relevant mechanisms.

In the inflammatory process, several activated genes of signaling pathways such as Jack state and MAPK3 were activated, and they, in turn, activated phosphokinase and phosphoinositide 3-kinase enzymes. These molecules were the enzymes which were involved in the inflammation leading to the production of molecules such as TRPV1 and Nav1.8, and finally, an increase in the excitability of A ( $\delta$ ) and C neuronal fibers (12,28,29).

Additionally, some studies examined the effects of treatment methods on the expression of miRs. For example, Qureshi et al investigated the changes in miRNA in the blood samples of several rodent models with inflammatory pain caused by the complete Freund's adjuvant. In addition, they studied the effect of Celecoxib which is a selective non-steroidal anti-inflammatory drug (cyclooxygenase-2). Despite the differences in the identified miRNAs from the studied models, they detected common biological pathways in such miRNAs. Wnt signal pathway navigation was affected in all models and had an important role in pain pathogenesis. The results of their study indicated that miRNAs are useful as pain biomarkers in the blood and suggested that various clinical trials can determine the process of affecting the pain-related miRNAs at different stages of pain and inflammation (30). Further, Bai et al induced the inflammation by injecting complete Freund's adjuvant into the rat masseter muscle and reported a significant decrease in 7 different miRs in the ipsilateral mandibular division of the trigeminal ganglion. In this study, miR-124 was identified as an effective and key miR in the pain process (31). In another study, the researcher succeeded in reducing the pain while improving its behavior by intravenous injection of miR-124 in a laboratory animal model. However, in another group, the anti-miR-124 injection increased the level of pain (17).

Furthermore, Douglas et al used ketamine to treat patients with CRPS. Considering that the response to ketamine treatment may be strong or weak, blood samples were collected from the patients before and 5 days after ketamine intravenous injection, and then the changes in miRs in the blood samples of the patients were examined. Based on the results, strong and weak responders were 33 differed with regard to miR-33. The significance assessment of the mechanism of hsa-miR-548d-5p dilution in weak responders demonstrated that this miRNA may lead to a higher UDP-GT activity, higher levels of inactive glucuronide conjugates, whereas minimizing the therapeutic efficacy of ketamine in weak responders. The

results of this study revealed that assessing miRs before and after the treatment can be useful in providing effective types of therapies (32). Additionally, Asano et al. reported an increase in the level of miR-140-3p in peripheral blood of the patients with chronic myeloid leukemia suffering from musculoskeletal pain compared to painless patients with chronic myeloid leukemia and healthy individuals. Interestingly, a decrease in the blood level of miR-140-3p was observed in four patients with musculoskeletal pain after using analgesia (33). Therefore, the results represented that up-regulating this pain biomarker with DN treatment can optimistically be recommended as an applicable procedure in the treatment protocol of shoulder pain syndrome which enhances the procedure effectiveness.

### Limitations of the Study

In every human attempt, no doubt, there exist some limitations which need to be acknowledged. The financial constraints prevented the researchers from applying several complementary analyses such as gene expression and the real-time PCR follow-up. Therefore, future research is recommended to employ these developed analysis methods which may improve the findings of the present study. In addition, the researchers were compelled to collect data from more individuals than they expected, which wasted their time and costs due to the cultural barriers and thus prevented the eligible patients from participating in this research projects.

### Conclusions

In general, DN method is considered effective in reducing the muscle pain related to MTrP. Further, based on the results of the present study and those of other studies, there is a relationship between the pain, treatment, and the expression level of miRs (i.e., a change in one factor can cause a change in the two others). Therefore, pain intensity and recovery are effective in the expression (i.e., downregulation or upregulation) of miR-939 and miR-25 in the blood. Accordingly, further research is suggested to investigate this mechanism..

### Conflict of Interests

The authors declared that there is no conflict of interest.

### Ethical Issues

The present study was ethically approved by the Ethics Committee of Tabriz University of Medical Sciences (Reg No. 13950400). Before the study, the research conditions and procedure were explained to the patients and their written informed consents were obtained. They were allowed to discontinue the project whenever they wanted without any consequences.

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