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The Immune Base Therapy of Pain with Magnesium Sulfate on the Trigger Axis of the TNF-α-TRAF6-NF-κB and Its Inhibitor (miR-146a-5p) in Rats

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ABSTRACT

Neuropathic pain can arise from injury or illness affecting the somatosensory system. It can also be triggered by cancer or chemotherapy drugs like paclitaxel. Researchers have indicated that magnesium sulfate may help in preventing neuropathy. This study aimed to investigate the effect of magnesium sulfate on paclitaxel-induced neuropathic pain by inhibiting the Tumor Necrosis Factor (TNF) Alpha - receptor-associated factor 6 - Nuclear factor kappa-light-chain-enhancer of activated B cells (TNF-α-TRAF6-NF-ϰB) axis.

Twenty-four male rats were divided into four groups: experiment group (E)-1, E2, E3, and the control group (Co). The experimental groups and the control group received paclitaxel at a dosage of 8 mg/kg every other day, totaling four injections over seven days. In addition, magnesium sulfate was administered daily in three doses of 300, 150, and 75 mg/kg, amounting to seven injections over the course of seven days. On the seventh day, peripheral blood samples were collected from the rats, and sera were used for the analysis of TNF-α serum levels and MicroRNA-146a-5p expression using ELISA and qRT-PCR methods, respectively.

The serum levels of TNF-α increased in the E1, E2, and E3 groups compared to the control group. However, there was a gradual decrease in the E1, E2 and E3 groups. The miR-146a-5p expression declined in the E1 group and increased in the E2 and E3 groups compared to the control group.

This study demonstrated that administering 300 and 150 mg of magnesium sulfate decreased TNF-α synthesis and reduced the function of the TNF-α-TRAF6-NF-ϰB axis during the initiation step.

Keywords: Immunotherapy; Magnesium sulfate; MIRN146a microRNA, Paclitaxel; Pain; Rat; Tumor necrosis factor-alph

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INTRODUCTION

Neuropathic pain is caused by injury or disease to the somatosensory system. Aging and illnesses such as cancer, as well as chemotherapy treatment, significantly contribute to the development of pain.[1] The intensity of pain is a major complication for cancer patients, and it depends on factors such as sensitivity (hyperalgesia), the type of cancer, and the location of cancerous tissue.[2] Additionally, treatments like radiotherapy, chemotherapy, and surgery can lead to painful conditions. These treatments have been reported to cause pain complaints in over 30% of adults and 60% of children with cancer.[3,4] The chemotherapeutic drug paclitaxel is commonly used to treat breast, lung, neck, and ovarian cancers as well as melanoma. However, it often causes painful neuropathy.[5] Paclitaxel, which is insoluble in water, is a derivative of the Vinca alkaloids family. It works by binding to microtubules, which prevents polymerization, inhibits mitotic division, and disrupts cell structure formation.[6,7] Neuropathy is ultimately caused by the toxic properties of paclitaxel neurons.[8,9] In addition, patients may experience muscle pain (myalgia) and joint pain (arthralgia).[10,11] Paclitaxel is distributed to both peripheral and central nervous tissues, where it accumulates in the dorsal root ganglia, brain (in low concentrations), sciatic nerve, and spinal cord (in moderate concentrations).[9,12] It has been well-documented that paclitaxel-induced peripheral neuropathy and pain are related with the expression of Tumor Necrosis Factor Alpha (TNF-α) and Interleukin 6 (IL-6) genes.[13] The use of anti-inflammatory drugs to reduce their expression may help alleviate peripheral neuropathy.[14] Transcription factors known as signal transducer and activator of transcription (STAT) also regulate the production of cytokines. Specifically, STAT3 controls the expression of TNF-α, indicating a relationship between the expression of the target gene (TNF-α)[15] and its regulators, such as STAT3. Furthermore, small non-coding nucleic acids known as miRNAs are responsible for regulating the expression of STAT transcription factors.[16]

Research has shown that magnesium sulfate functions in the opposite way to paclitaxel.[17] It is believed that the analgesic properties of magnesium sulfate may work by inhibiting N-methyl-D-aspartic acid (NMDA) receptors, which in turn prevents the release of acetylcholine from the neuromuscular terminals. Additionally, magnesium sulfate inhibits the activation of phosphoinositide 3-kinase/ Protein kinase B (Akt) pathway,[18,19] which is closely related to the TNF-α-TRAF6 pathway, ultimately activating the NF-κB transcription factor. Regulating them has has emerged as a novel therapeutic strategy.[20]

MiRNAs are[21] small nucleic acids, each approximately twenty-two nucleotides long, are responsible for recognizing the 3' tail (The 3′ untranslated region) of mRNA before translation is completed. This recognition results in a decrease in mRNA lifespan, ultimately impacting protein levels, including TNF-α.[22] One well-known example is miR-146a-5p, which regulates the serum level of TNF-α. This capability positions miR-146a-5p as a potential regulator of the TNF-α-TRAF6-NF-κB axis.[22] This study aimed to measure TNF-α serum levels and miR-146a-5p serum expression in response to a dose-dependent intraperitoneal injection of magnesium sulfate in an experimental pain model.

MATERIALS AND METHODS

All research procedures were approved by the Research Ethics Committee of Laboratory Animals at Zabol University of Medical Sciences on May 2, 2023, and received the ethics code No: IR.ZBMU. AEC.1401.001.

Prediction of miR-146a-5p Target Sites

To predict the m-RNA targeting of the gene TNF-α by rno-miR-146a-5p, we utilized the online miRBase database (https://mirbase.org/mature/MIMAT0017132), RNAhybrid, and the website https://www.ncbi.nlm.nih.gov. The specific targeting of the 3' UTR-mRNA was identified as five bands with a minimum free energy (MFE: -23.3 kcal/mol). see Figure 1.

Animal and Study Design

Twenty-four male Wistar rats (mean±SD, 200±40 g) were obtained from the animal house of Zabol University of Medical Sciences. The animals were kept in a controlled environment (22±2°C, 12-hour light/dark cycle) with access to adequate food and tap water.

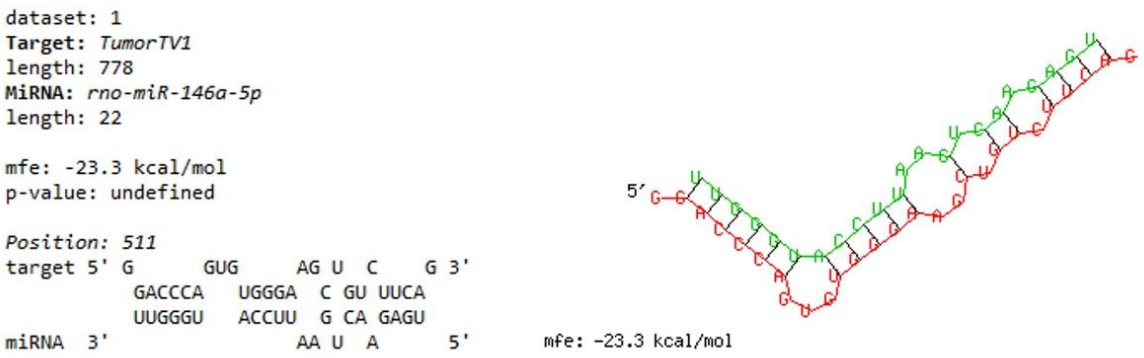

Figure 1. This is an example of a predicted target for rno-miR-146a-5p within the promoter overlapping transcript (TNF-α) sequence.

Randomization

This study used a randomized block approach, with blocks prepared and numbered from 1 to 4. Block number 4 was designated as the control group. The ratswere kept under identical conditions and were randomly divided into four groups of six: Experiment 1 (E1), Experiment 2 (E2), Experiment 3 (E3), and Control (Co).

Induction of Pain and Protection

To induce neuropathy, all groups were given intraperitoneal paclitaxel at a dosage of 8 mg/kg, administered every other day intervals, for a total of four times.[23] Additionally, the E1, E2, and E3 groups received intraperitoneal magnesium sulfate in doses of 75, 150, and 300 mg/kg daily, respectively. This was done over seven days, excluding the control group.[24,25] One hour after the paclitaxel injection on the first day, the rats received doses of magnesium sulfate. The control group was given seven doses of saline (0.9%). Peripheral blood samples were collected from the mice in heparinized tubes on the seventh day, and the serum was separated using a centrifuge. The serum was then analyzed for TNF-α levels using ELISA and miR-146a-5P expression using RT-PCR.

Hot-plate Test

To evaluate the neuropathic pain induced by paclitaxel, we performed the hot-plate test on the 7th day, we placed the animals on a heated hot-plate surface (Ugo Basile 35100, United Kingdom). We considered the time taken for the animals to lick, lift paws, or jump from the hot-plate surface (with a cut-off time set at 45 seconds) as the end-point for assessing the response to heat latency. We measured latencies at 30, 60, 90, and 120 minutes after the magnesium sulfate injection.[26] (Data not shown)

ELISA Assay

The TNF-α ELISA kit, which follows the sandwich protocol for rats, was procured from Karmania Pars Gene Company in Iran (www.armanbiotech.com) with Lot number RTNF0821001.

The sensitivity of the kit was 2 pg/mL. A direct serum sample (without dilution) was used, and the experimental process was carried out following the instructions provided with the kit. Thelight absorption at 450 nm was measured using an ELISA microplate reader.

Design of Primer

First, the forward and reverse primers for miR-146a-5-p were obtained from the miRbase system (https://mirbase.org) and synthesized by Arian Gene Gostar (https://www.ariangene.com) Table 1.

Table 1. The primer sequences are shown below.

MIR-146A-5P PRIMERS	
R	GTCGTATGCAGTGCAGGGTCCGAGGTATTCGCACTGCATACGACAACCCAT
F	ACGCTGAGAACTGAATTCC

MiRNA Extraction

We used a micro RNA extraction kit (miRNA Isolation Kit) from Favorgen Co in Taiwan (Cat. No: FAMIK002, Lot. No: BH516117724) to extract miRNAs from rat serum. This kit utilizes a mini spin column and is designed based on a silica-fiber matrix. In this method, RNA molecules of various sizes are selectively attached to the silica-fiber matrix. We measured the RNA concentration using the NanoDrop (Nano Mabna Iranian, Iran).

Real-Time PCR

A cDNA synthesis kit was purchased from Karmania Pars Gene Company in Iran, utilizing the stem-loop method for the cDNA synthesis process. Subsequently, the Rotor-Gene 6000 machine from Qiagen was utilized for real-time PCR. The temperature program for the Real-Time PCR employed the SYBR green method. U6 RNA served as an internal control for miR-146a-5p expression, and the fold change (2^-ΔΔct) method was used to measure the relative change in miR-146a-5p expression.

Statistical Analysis

A one-way ANOVA test was performed for data analysis, with a p-value of less than 0.05 considered statistically significant.

RESULTS

ELISA Analysis

The levels of TNF-α in serum increased in all experimental groups compared to the control group (Figure 2). However, a sequential reduction was observed across the groups, with the E1 group showing a mean of 3.33±0.92, followed by the E2 group at 2.31±0.27, and the E3 group at 1.55±0.35. The mean±SD for the control group was 3.97±1.50. Notably, only the E3 group exhibited a significant difference compared to the control group (p=0.046) (Figure 2).

RT-PCR Analysis

The analysis of miR-146a-5p expression showed a decrease in the E1 group (Fold Change=0.29±0.08) compared to the control group. In contrast, the E2 group exhibited an increase in expression (3.14±1.2), while the E3 group showed a substantial increase (11.49±3.7) relative to the control group (Figure 3).

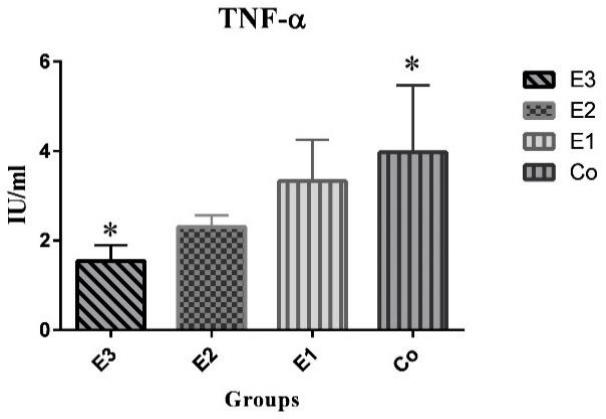

Figure 2. This figure illustrates the mean±SD serum levels of TNF-α in experimental groups E1, E2, and E3. All experimental groups (E1, E2, and E3) exhibited significantly different levels compared to the control (Co) group (*p=0.046).

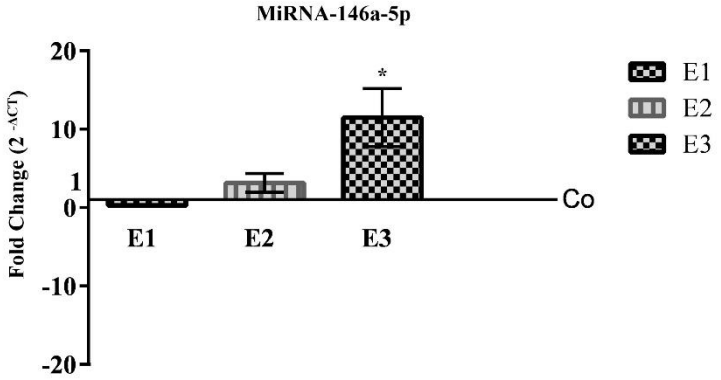

Figure 3. The data in this figure illustrate differences in miR-146a-5p expression levels (fold change) between groups. Both experimental (E) groups -3 (*p*=0.025) and E2 exibit higher expression levels compared to the control (Co) group. In contrast, the expression level in the E1 group is lower than that in the Co group.

DISCUSSION

Understanding the pain mechanisms associated with cancerous diseases has been challenging due to scientific limitations. Additionally, the lack of animal models has hindered the study of the molecular, biochemical, and neurobiological pathways of cancer-induced pain. To address this issue, various models have been developed to enhance our understanding of the pain mechanism.

This study investigated the inhibitory effect of magnesium sulfate on the pain mechanism triggered by paclitaxel, known as "TNF-α-TRAF6-NF-κB" pathway. Several studies have established a connection between paclitaxel-induced neuropathy and the production of inflammatory cytokines IL-6 and TNF-α by resident macrophages in brain and spinal tissue.[14] Duan et al (2022) also supported the immunologic basis of pain and neuropathy, demonstrating that TNF-α, the most crucial inflammatory cytokine with hormone-like properties and cascading activation, leads to irreparable damage to nerve tissues.[27] Leung et al (2010) also found that increased serum levels of inflammatory cytokines, including TNF-α, can contribute to pain. Taking non-steroidal anti-inflammatory drugs (NSAIDs) can alleviate the pain by reducing TNF-α levels in the bloodstream.[13]

In recent studies, a dose of 8 mg/kg of paclitaxel was administrated intraperitoneally four times to rats weighing 200±40 g. Similarly, Cuozzo et al (2021) also utilized paclitaxel (8 mg/kg, four times, intraperitoneally) to induce neuropathy in mice weighing approximately 25-30 g.[23]

In addition, Scialli et al (1997) administered paclitaxel (10 mg/kg, intravenously) to induce embryotoxicity in rats.[28] Our study assessed the protective effects of varying doses of magnesium sulfate (75 mg, 150 mg, and 300 mg) in a mouse model of paclitaxel-induced neuropathy. Aisenbrey et al (1992) had previously recognized the protective and analgesic qualities of magnesium sulfate in the context of paclitaxel-induced neuropathy following surgery and in cases of preeclampsia.[17]

Our findings indicated that the serum level of TNF-α in the E1 group (75 mg magnesium sulfate) was higher compared to the E2 (150 mg) and E3 (300 mg) groups suggesting successful administration of magnesium sulfate in the E2 and E3 groups. The decrease in TNF-α levels in these groups may suggest a lower activity of the TNF-α-TRAF6-NF-κB axis, potentially leading to reduced inflammation and neuropathy. Huehnchen et al (2020) showed that paclitaxel can induce neuropathy and inflammation in the dorsal root ganglia. This effect was exacerbated by the presence of macrophages and the release of inflammatory cytokines, such as IL-6 and TNF-α.[14]

Further supporting our findings, a clinical trial conducted by Aryana et al, demonstrated that administering magnesium sulfate before and after surgery in patients undergoing coronary artery transplants resulted in decreased serum levels of IL-6, TNF-α, and MMP1.[29] In our study, we evaluated microRNAs using RT-PCR.

The expression level of miR-146a-5p in the serum of E3 group increased compared to groups E1 and E2. This

finding aligns with the ELISA results, which showed a decrease in TNF-α serum levels in E3 group.

Our research also revealed a decrease miR-146a-5p expression in the E1 group compared to the control group. This suggests that the effective dose of magnesium sulfate needs to exceed 75 mg. This observation is supported by a study conducted by Stickel et al who demonstrated that miR-146a inhibits the function of the TNF-α-TRAF-6 axis and the expression of NF-κB. Their research indicated that decreased miR-146a expression leads to the dysregulaed TNF-α synthesis, exacerbating the inflammatory condition. They proposed that miRNAs could serve as alternatives to drugs targeting the TNF-α cytokine in inflammatory diseases.[30]

To summarize, we found that injecting paclitaxel into the peritoneum of rats or mice (please clarify) caused neuropathic pain by increasing TNF-α serum levels. However, administering varying amounts of magnesium sulfate reduced the risk factor for neuropathic damage, particularly with doses of 150 mg and 300 mg in the E2 and E3 groups, respectively. These doses led to increased miR-146a-5p expression, decreased TNF-α production, and inhibition of the TNF-α-TRAF6-NF-κB pathway. We suggest further investigation of TRAF6 and NF-κB levels within the TNF-α-TRAF6-NF-κB inflammatory pathway, as well as exploration of other inflammatory pathways associated with miR-146a.

STATEMENT OF ETHICS

This research was approved by the Research Ethics Committee of the Zabol University of Medical Sciences. (Reference number: IR.ZBMU.AEC.1401.001).

FUNDING

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CONFLICT OF INTEREST

The authors declare that they do not have any known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Data Availability

Data are available upon reasonable request (by the correspoding author's Email).

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