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Investigation of the efficiency of pulsed electromagnetic field treatment and stretching exercise in experimental skeletal muscle injury model

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Abstract

Objective Pulsed electromagnetic fields (PEMF) and stretching exercises are safe and noninvasive methods that could have a therapeutic effect on tissue healing. This study aimed to assess the effectiveness of these methods in treatment of muscle injury (INJ).

Method Rats were divided into 5 groups (Control, INJ, INJ + Exercise, INJ + PEMF, INJ + Exercise + PEMF). At the end of the experiment, genetic, histopathological, and immunohistochemical evaluations were made in the muscle tissue.

Results Mononuclear cell infiltration, muscle degeneration, atrophy, and necrosis were found to be higher in the INJ group than in all groups ($p < 0.001$). On the 7th day, fibroblast growth factor (FGF) was found to be higher in the INJ group compared to both the control and the INJ + Exercise group ($p < 0.05$). On the 14th day, Vascular endothelial growth factor values were found to be higher in the injury group than the other groups except for the PEMF group ($p < 0.05$), and FGF values were higher in the injury group compared to all groups ($p < 0.001$). The expressions of transforming growth factor beta 1 (TGF-β1) and endothelial nitric oxide synthase (eNOS) on the 7th and 14th days showed a significant increase in the INJ group compared to the other groups ($p < 0.001$).

Conclusion In this study, it has been shown that PEMF and stretching exercise is effective in the treatment of muscle injuries as they balance the inflammatory process in the muscle, have a positive effect on muscle development, accelerate healing, prevent fibrosis development by reducing TGF-β1 signaling, and inhibit inflammatory-induced eNOS activity.

Keywords Muscle injury, Muscle stretching exercise, Pulsed electromagnetic therapy, Fibrosis

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Introduction

Skeletal muscles form about 40–45% of the total human body mass and are essential for sustaining life. Proper muscle function; allows mobility, joint stability, postural control, breathing, metabolic control, thermoregulation, and energy storage [1]. The incidence of traumatic skeletal muscle injuries is increasing dramatically throughout the world, and there may be many reasons for this. Sports-related injuries which are seen after surgical interventions such as tumor ablation, soft tissue reconstruction, or joint arthroplasty can be cited as examples [2–4]. Despite its high incidence, treating injured muscles and restoring their original structures and functions have not yet been fully achieved [5].

There are various conservative treatment strategies for acute and chronic skeletal muscle injuries, such as POLICE (Protection, Optimal Loading, Ice, Compression, and Elevation) [6], physiotherapy applications (exercise, stretching, mobilization, massage, ultrasound, laser, electrical stimulations, etc.), non-steroidal anti- antiinflammatory drugs, biological repair agents and surgical methods [7, 8]. Physiotherapy methods are among the first preferred treatment options for muscle injuries because of being affordable, noninvasive, safe, and effective and have been used for many years.

Stretching exercises can be used for treatment as they can show positive effects at the cellular level with the intermittent tension applied during the acute recovery period. Although the benefits of stretching exercises on injured fiber are still unveiled, it has been proven in a limited number of studies that it stimulates collagen synthesis, which has an important role in healing [6].

In addition to the physiotherapy modalities commonly used in rehabilitation clinics, alternative treatment modalities are also being tried. Pulsed electromagnetic field (PEMF) therapy, one of the physiotherapy methods, has been used for a long time for avascular necrosis, fracture, soft tissue injuries, osteoporosis, and musculoskeletal pain. However, despite numerous clinical studies, the effect mechanism has not been fully demonstrated. It is thought that magnetic field therapy improves the oxygen use of cells by increasing the blood supply of the tissues, and erythrocytes release more oxygen under the magnetic field's influence, thus positively affecting the circulatory system [9]. In addition, it has been reported that PEMF has angiogenesis, anti-inflammatory and proregenerative effects in animals and humans [10]. PEMF treatment is important because it has a wide application area, easy application, being a noninvasive, risk-free, economical, natural method without any side effects [11].

Reasons such as the slow and insufficient regeneration mechanism after skeletal muscle injury, the scarring of the region, and the fact that transforming growth factor-β1 (TGF-β1) increases collagen synthesis in the injured muscle tissue and heals with fibrosis lead scientists to study different regeneration models. There are studies in which various biological agents, such as antifibrotic agents, have been tried to inhibit this effect, stimulate regeneration and contribute to the average satellite cell migration [12]. Despite the invasive and expensive treatment modalities that cause positive effects in different steps in such myogenesis and muscle regeneration, the desired functional result has not been achieved sufficiently.

In order to eliminate this lack of knowledge, it is necessary to carry out studies in which current methods and new and easily applicable noninvasive treatment methods will be compared, and the pathogenesis of muscle injury and its repair mechanisms will be investigated in detail. Therefore, this study aims to examine the anti-inflammatory, injury-repairing, and antifibrotic effects of stretching exercises and PEMF applications on muscle tissue in an experimentally created injury model in the gastrocnemius muscles of rats.

Materials and methods

Animals and ethical approval

All experiments were conducted in accordance with ARRIVE (Animal Research: Reporting in Vivo Experiments) 2.0 guidelines. Animal experiments were approved by the Suleyman Demirel University Local Animal Ethics Committee with the ethical decision dated 10.06.2021 and numbered 04/08. The study was registered on ClinicalTrials.gov (NCT05988476).

According to the results of the power analysis (G Power 3.1) the minimum number of subjects to be included in the study was determined to be 48 in order to have a $p < 0.05$ with an effect size of 0.6, with 80% power. Considering the possibility of loss of animals, 2 rats were added to each of the experimental groups. In addition, since 5 rats were used in the preliminary study, the total number of rats was determined as 69.

Sixty-nine female, adult Wistar albino rats used in this study were obtained from Suleyman Demirel University Experimental Animal Production and Experimental Research Laboratory. The rats were kept in an environment between 22 and 24 degrees, 55–60% humidity, 12 h of light, and 12 h of darkness. Ad libutium feeding regimen was applied to the experimental animals. The PEMF-treated rats were housed in Euro type 2 cages during treatment, standard cages during rest hours, and all other rats were housed in standard cages.

Experimental design

Two experiments, a preliminary and a main study, were conducted on experimental animals within the scope of the project.

Preliminary study

This preliminary study aimed to find the ideal PEMF exposure duration. Thus, PEMF at a frequency of 27.12 MHz was applied to the rats at equal intervals throughout the day [13]. Muscle injury was generated in the right leg gastrocnemius muscle of the rats in all groups by using an 18 gauge (g) biopsy needle. Feed and water to be consumed daily were given, and an antiseptic dressing was applied to the wound site. A total of 5 rats were distributed into five groups (each containing 250–350 g 9 months old Wistar albino female rat) as;

1. *Muscle Injury (INJ) group*: This rat did not receive any additional treatment and was left to recover spontaneously.
2. *INJ + PEMF$_{1\,h}$ group*: In this group, PEMF was applied in two sessions per day, 30 min in the morning and 30 min in the evening.
3. *INJ + PEMF$_{2\,h}$ group*: In this group, PEMF was applied in two sessions per day, 60 min in the morning and 60 min in the evening.
4. *INJ + PEMF$_{4\,h}$ group*: In this group, PEMF was applied in two sessions per day, 120 min in the morning and 120 min in the evening.
5. *INJ + PEMF$_{6\,h}$ group*: In this group, PEMF was applied in two sessions per day, 180 min in the morning and 180 min in the evening.

At the end of the one-week experiment, histopathological analysis of gastrocnemius muscle tissues taken under 80–100 mg/kg ketamine (Ketalar, Pfizer, Turkey) and 10 mg/kg xylazine (Xylazin Bio %2, Bioveta, Czech Republic) anesthesia was performed. Following the abdominal incision, euthanasia with blood taken from the inferior vena cava, and surgical exsanguination method, muscle tissues were placed in 10% formaldehyde for histopathological examination and sent to the relevant unit.

As a result of the preliminary study, the ideal PEMF exposure duration was determined as 180 min (analysis results are given in the results section), and it was decided to use this duration in the main study.

Study design

Sixty four 9 months old Wistar albino female (250–350 g) rats were included in the study. These animals were divided into 5 groups, and the intact left feet of the INJ group were used for the control group. The muscle injury model was generated using an 18 g biopsy needle in all groups except the control group. The ideal PEMF duration was used for muscle healing at this stage, which was determined in the preliminary study. Feed and water were given to all animals, and an antiseptic dressing was applied to the wound site. Groups were;

1. *INJ group*: In this group, the injured muscle legs of these rats were allowed to heal spontaneously without any treatment.
2. *Control (CONT) group*: The left legs of the INJ group rats were used as the control group.
3. *INJ + Exercise group (EXE)*: In this group, a stretching exercise protocol was applied once a day.
4. *INJ + PEMF6h group*: In this group, PEMF was applied twice a day, for 180 min in the morning and 180 min in the evening.
5. *INJ + EXE + PEMF$_{6\,h}$ group*: In this group, PEMF was applied twice a day, for 180 min in the morning and 180 min in the evening, and a stretching exercise protocol was applied once a day, except for the hours when PEMF was applied.

Muscle injury procedure

Before the surgical procedure, the animals were anesthetized with 80–100 mg/kg ketamine (Ketalar, Pfizer, Turkey) + 8–10 mg/kg xylazine (Xylazin Bio %2, Bioveta, Czech Republic) anesthesia and placed in the prone position with fixation of the tail and extremities. Skeletal muscle injury was created by using an 18 g biopsy needle on the right leg gastrocnemius muscle, approximately 3 mm proximal to the muscle-tendon junction of the biopsy needle. The lesion was made to cover roughly 50% of the cross-sectional area of the medial gastrocnemius muscle [14]. After the biopsy, the needle was removed, and an antiseptic dressing was applied. The wound was covered with a bandage for 2–3 days, and the bandaging was terminated after epithelialization was achieved and the bleeding stopped.

Half of the animals in the groups were sacrificed on the 7th day and the other half on the 14th day under the same anesthesia protocol. Following the abdominal incision, euthanasia was performed with blood taken from the inferior vena cava as a surgical exsanguination method. Half of the muscle tissues were placed in 10% formaldehyde for histopathological examination and sent to the relevant unit. The remaining half of the muscle tissues were put into – 20° for genetic analysis.

Stretching exercise protocol

In order to comfortably position the rats during the stretching exercise, 2 restraints were designed and produced with a 3D printer suitable for the body structure of the rats. Animals were comfortably and calmly placed in the restrainer before exercise. At the beginning of the application, soft tissue massage and mobilization were applied to the gastrocnemius muscle of the rats before the stretching exercise to prepare the tissue for exercise, and then gentle passive stretching was applied to the feet of the rats in the direction of 30° dorsi flexion [7]. The knee joint is extended during the stretching exercise.

Stretching exercises were applied as 10 repetitions. Each repetition was performed as 1-minute of stretching and 30 s (s) rest. These processes took approximately 15 min for each rat [15, 16]. Stretching exercises were started the 3rd day after INJ in order to complete the inflammation process, strengthen the scar tissue and reduce the risk of recurrence [17]. Stretching practices were performed once a day, 5 days a week, and at the same times of the day. 5 sessions of stretching were applied to the rats sacrificed on the 7th day, and 10 sessions of stretching were applied to the rats sacrificed on the 14th day.Restrainers were cleaned daily with an antiseptic. All untrained rats were placed in the restrainer at the same time to be in the same conditions as the trained rats and to experience the same stress.

Setting up the PEMF system

As it is known from the scientific literature, if the exposure systems are based on electromagnetic principles, antennas can be designed for many frequencies, and these antennas can be driven with the desired frequency generators. Patch antennas are often used due to the space and size they cover. However, the geometries become very large below microwave frequencies (in the MHz area). Geometries using coils are very useful with path geometries suitable for the printed circuit surface to avoid the third dimension. 27.12 MHz radio frequency (RF) is reserved for medical use in the industrial, scientific, and medical (ISM) radio band. Diathermy devices approved by the FDA are also at this frequency and are frequently used in physiotherapy. The therapeutic effects of this frequency have been demonstrated in the extant literature; moreover, it is employed in the context of wound healing [18].

In order to see these beneficial effects, the applicator must be oriented so that it can see the wound surface. This study aimed to focus the radiation pattern of the antenna, which is the applicator of the oscillator circuit to the wound area.

Electrical measurements of the realized circuit and antenna parameter measurements were carried out by Department of Electrical-Electronics Engineering of Suleyman Demirel University. Circuit simulation results are shown in Fig. 1D. As understood from the simulations; two PCB antennas for two rats in the 20 cm x 12 cm living area of the Euro type 2-rat cage were sufficient for a smooth and homogeneous EMF at the targeted area.

In our tests, electromagnetic interference /electromagnetic compatibility measurements were based on the publication "Procedures for the Review of Telecommunication Systems for (Radio Frequency EMF Strength Meter, Extech 480836, USA). The shielding efficiency of EM insulated room value is known as 80 decibels (dB).

Simulation studies were carried out for 27.12 MHz frequency in a computer environment under the consultancy of a faculty member of the Electronic Communication Engineering department. Suitable antennas geometry was realized after the ideal PCB board dimensions were determined. In order to require simultaneous exposure of many animals to EMF in the same environment and to prevent one device from affecting the other, since the environment in the Experimental Animals Unit is metal-containing shelves, a buffet with 24 compartments was designed and made of wooden material (Fig. 1F). In order to ensure the homogeneous distribution of the electromagnetic field to each region, the cage covers, and drinker tips are also made with special 3-dimensional designs (Fig. 1A-B).

The constant power feeding the application input is limited to 1 W. Thus, an electric field of 15 V/meter could be obtained at every point in the cage, so it can be said that the target exposure strength value in the study was limited to 15 V/m at this frequency. The appropriate antenna for the oscillator/transmitter was made as a specific antenna. The wavelength is about eleven meters. So, the antenna could be too long to place in the circuit. In order to place the antenna, the geometry of the antenna was chosen as a hexegonal shaped PCB antenna. We can use a small place such size in the circuitry. We made some smilation by using CST to see the direction of the radiation at 27.12 MHz. The simulation shows that such size

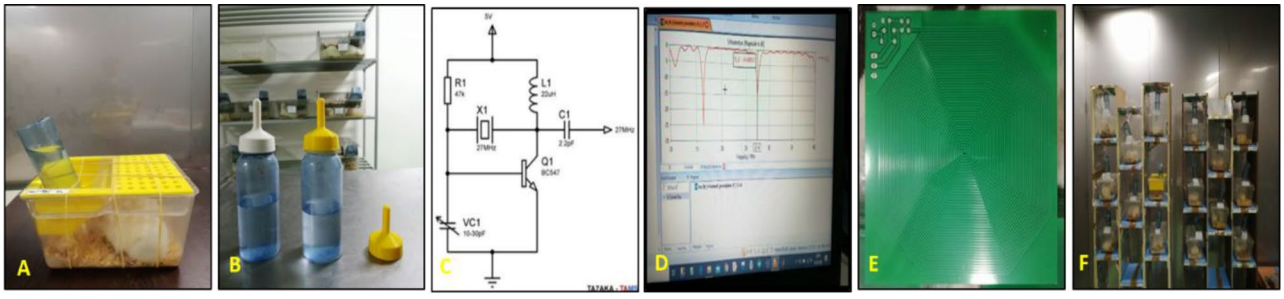


Fig. 1 Required Materials and Systems for PEMF Application. **A** Special design plastic cage cover, **B** Specially designed drinker tip, **C** Circuit Diagram of Oscillator, **D**-**E** Circuit and Applicator Finished with Printed Circuit Geometry. **F** PEMF device in Faraday room, which is planned to receive PEMF from the top of each cage and is not affected by the external environment

of the geometry is very directional. According to the simulations, this geometry can be said to be very directional. We were able to show that by using a Strength Meter (Extech 480836, USA). The size of the square antenna was 15 × 15 cm. We investigated the value of the electric field as 15 V/m below the antenna at a distance of 15 cm. The reading error was less than 10%. In this way, we can say that we have enough homegeneus electric fields. Namely, the operating electric field strength was highly homogeneous (13.5 V/m min., 16.5 V/m max.).

Histopathological analysis

Muscle tissue samples obtained at the end of the experimental period were fixed in 10% formalin for 24–48 h, and then embedded in paraffin blocks after routine light microscopic tissue monitoring. 5 micron (μ) sections taken from the prepared paraffin blocks using a Leica brand cylinder microtome, and a Hematoxylin + Eosin (H + E) stain was applied to these sections in order to reveal the general characteristics of the muscle tissue. Findings from the preparations were visualized using the Zeiss Axio A1 brand photo-microscope. In order to evaluate the histopathological changes that may occur in muscular damage under a light microscope, routine hematoxylin-eosin staining formed the basis of the analysis. Periodic histological follow-ups and staining were carried out in Alanya Alaaddin Keykubat University Faculty of Medicine Laboratories.

Immunohistochemistry test

Immunohistochemical examinations allowed us to see the expression level of the proteins we are investigating in cells semi-quantitatively, as well as show in which cell and in which region this expression was. We used the antibodies Vascular endothelial growth factor (VEGF) (Elabscience Cat: EAB-40004) and FGF (Santa Cruz Cat: sc-55520) for immunohischemical staining. Five μm thick sections were taken from the muscle tissue, and the sections were lowered into the water following the deparaffinization process. Sections lowered into the water were boiled for 5 min in a microwave oven in citrate buffer (pH = 6.0) to reveal the antigenic determinant regions. After cooling at room temperature for 10 min, the sections were washed with phosphate buffer PBS (PBS; pH 7.6). After this stage, it was treated with 3% H_2O_2 (hydrogen peroxide) prepared in methanol for 15 min to remove endogenous peroxidase activity. Sections passed through distilled water were washed with PBS. Blocking serum (Ultra V Block, LabVision) was applied to the sections to block non-specific antibody binding. Then, the sections were incubated for 1 h in a humid cabinet with the primary antibody to be diluted in the dilutions recommended by the manufacturer. Sections were kept in a secondary antibody solution (Biotinylated Link, Dako, K0609) for 30 min after washing 3 times with PBS. Sections washed 3 times in PBS will be treated with streptavidin peroxidase solution (Streptavidin HRP, Dako, K0609) for 10 min. After washing the sections with PBS 3 times, Diaminobenzidine (DAB) chromogen solution (Vector, SK-4100) will be applied at the specified times. After washing the sections with distilled water, counterstaining was performed by applying Mayer's hematoxylin for 30 s. Sections washed under running water for 5 min were covered with a coverslip, covered with a capping solution, and evaluated under a light microscope. Structural changes in muscle tissue sections of the experimental and control groups were evaluated according to the scoring of Abdel-Wahhab et al. ((-): No structural change, (+): Mild structural change, (++): Moderate. structural change, (+++): severe structural change) [19]. In addition, an analysis of the sections was made with the ImageJ program. Histopathological and IHC examinations and analysis were all performed by the same person.

Table 1 Primer sequence information designed for TGF-β1, eNOS, and GAPDH targets

Primers		Primer directories
TGF-β1	Forward	5′-CTTCAGCTCCACAGAGAAGAAC-3′
	Reverse	5′-CAGACAGAAGTTGGCATGGTAG-3′
eNOS	Forward	5′-GTGAAGGCGACTATCCTGTATG-3′
	Reverse	5′-CATGCTCTAGGGATACCACATC-3′
GAPDH	Forward	5′-CAAGGTCATCCCAGAGCTGAA-3′
	Reverse	5′-CATGTAGGCCATGAGGTCCAC-3′

GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase, eNOS: endothelial nitric oxide synthase, TGF-β1: Transforming growth factor-β1

Quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR)

Total Ribonucleic Acid (RNA) was extracted from rat tissues using TRIzol™ Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. RNA concentration and purity were determined in micrograms/microliter with a MySPEC microvolume spectrophotometer (VWR) instrument. For the synthesis of complementary Deoxyribo nucleic acid (DNA), 1 μg of isolated RNAs was used, and reverse transcription was performed with the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA) using oligo (deoxythymine) dT primers. For this purpose, complementary DNA was synthesized in a thermal cycler (Bio-Rad Laboratories, Hercules, CA) using a temperature program at 25 °C for 5 min, at 46 °C for 20 min, and at 95°C for 1 min. For real-time polymerase chain reaction, the fluorescence signal was detected by amplifying the iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) synthesis kit according to the manufacturer's instructions. The primer information used in the amplification process is shown in Table 1. The PCR conditions used

are, respectively; 10 min pre-denaturation at 95 °C, 10 s at 95 °C, and 30 s at 60 °C for 40 cycles. The total reaction volume was 25 µl (µl), and 100 ng cDNA was used. Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) housekeeping gene was used for normalization processes. Relative fluorescence changes were performed according to the $2^{-\Delta\Delta}$CT method. PCR products melting curve analysis was used to determine the specificity of amplification. All results are presented as fold changes in the graph.

Statistical analysis

Analysis of variance (ANOVA) post hoc LSD test was used to compare all histopathological, immunohistochemical and genetic scores. Statistical calculations were made using the SPSS 20.0 package program (SPSS Inc., Chicago, IL) and the values were considered significant for $p \leq 0.05$.

Results

Histopathological and immunohistochemical results

After the histopathological analysis, when the muscles were evaluated in terms of mononuclear cell infiltration, muscle degeneration, atrophy, and necrosis, all values in the INJ group were found to be significantly higher than the other groups at the 7th and 14th days ($p < 0.001$). In addition, it was determined that the values in the INJ + EXE + PEMF group were higher than the other groups except the INJ group ($p < 0.001$). There was no significant difference between the INJ + EXE and INJ + PEMF groups.

When the muscle tissues were examined in terms of pyknotic nuclei (PN), it was observed that the values in all groups were significantly higher than the CONT group on the 7th and 14th days ($p < 0.001$). It was determined that the values in the INJ + EXE + PEMF group were significantly lower than the other groups except for the CONT group ($p < 0.01$), and the values in the INJ + EXE group were significantly higher than all the groups except INJ + PEMF ($p < 0.05$) (Figs. 2, 3).

After the immunohistochemical examination, VEGF values on the 7th day did not differ significantly between the groups; FGF values were found to be statistically significantly higher in the INJ group compared to both the CONT group and the INJ + EXE group ($p < 0.05$). On the 14th day, VEGF values were found to be significantly higher in the INJ group compared to the groups other than the INJ + PEMF group, and higher in the INJ + PEMF group compared to the CONT group ($p < 0.05$). FGF values were found to be significantly higher in the INJ group than in all groups ($p < 0.001$), and the groups other than the INJ + EXE group were also significantly higher than the CONT group ($p < 0.05$) (Figs. 4, 5).

Genetic results

In PCR analysis made from genetically muscle tissue, a statistically significant increase was found on the 7th-day expressions of TGF-β1 in the INJ group compared to the other groups ($p \leq 0.001$). There was no statistically significant difference between the CONT and treatment groups. On the 14th day, expression of TGF-β1 was found to be significantly higher in the INJ group compared to the other groups ($p < 0.001$). In the treatment groups; TGF-β1 was found to be statistically significantly higher in the INJ + EXE group compared to the CONT group (p:0.041) (Fig. 6).

When the eNOS levels were examined, it was determined that the values in the INJ group were statistically significantly higher on the 7th and 14th day muscle tissues compared to all groups ($p < 0.001$). In the treatment groups; eNOS values of both the INJ + EXE group and the INJ + PEMF group were found to be significantly increased on the 7th and 14th days compared to the CONT group (INJ + EXE-CONT 7th and 14th days $p < 0.001$, INJ + PEMF-CONT 7th day p: 0.021, day 14 p: 0.003). It was observed that the 7th and 14th -day eNOS values of the INJ + EXE + PEMF group did not increase significantly compared to the CONT group ($p > 0.05$) (Fig. 6).

Discussion

The results of the study demonstrated that both stretching exercises and PEMF therapy resulted in a reduction in inflammation, degeneration, atrophy, and necrosis, while concurrently promoting accelerated muscle tissue recovery. In addition, by increasing the number of pycnotic nuclei, the muscle cell became more active, and the muscle mass increased. The application of PEMF and stretching exercise limited fibrosis formation by providing a controlled increase in VEGF and FGF, thus contributing to functional recovery. TGF-β1 level on both the 7th and 14th days; exercise, PEMF, and combined treatment groups were significantly reduced compared to the injury group, so single and combined treatment contributed to the reduction of fibrosis. It was observed that the single or combined use of stretching exercise and PEMF treatments significantly reduced eNOS levels compared to the injury group.

Inflammation is one of the first responses given at the tissue level when an injury occurs in any tissue due to various factors, such as trauma, as in skeletal muscle. In this inflammatory process, the body's defense cells, especially neutrophils, migrate to the injured area and try to repair that area in order to minimize the damage. Depending on the severity and duration of the injury, these cells, which are collected at the scene, can deepen the inflammation during repair and accelerate regeneration by increasing the synthesis of growth factors such

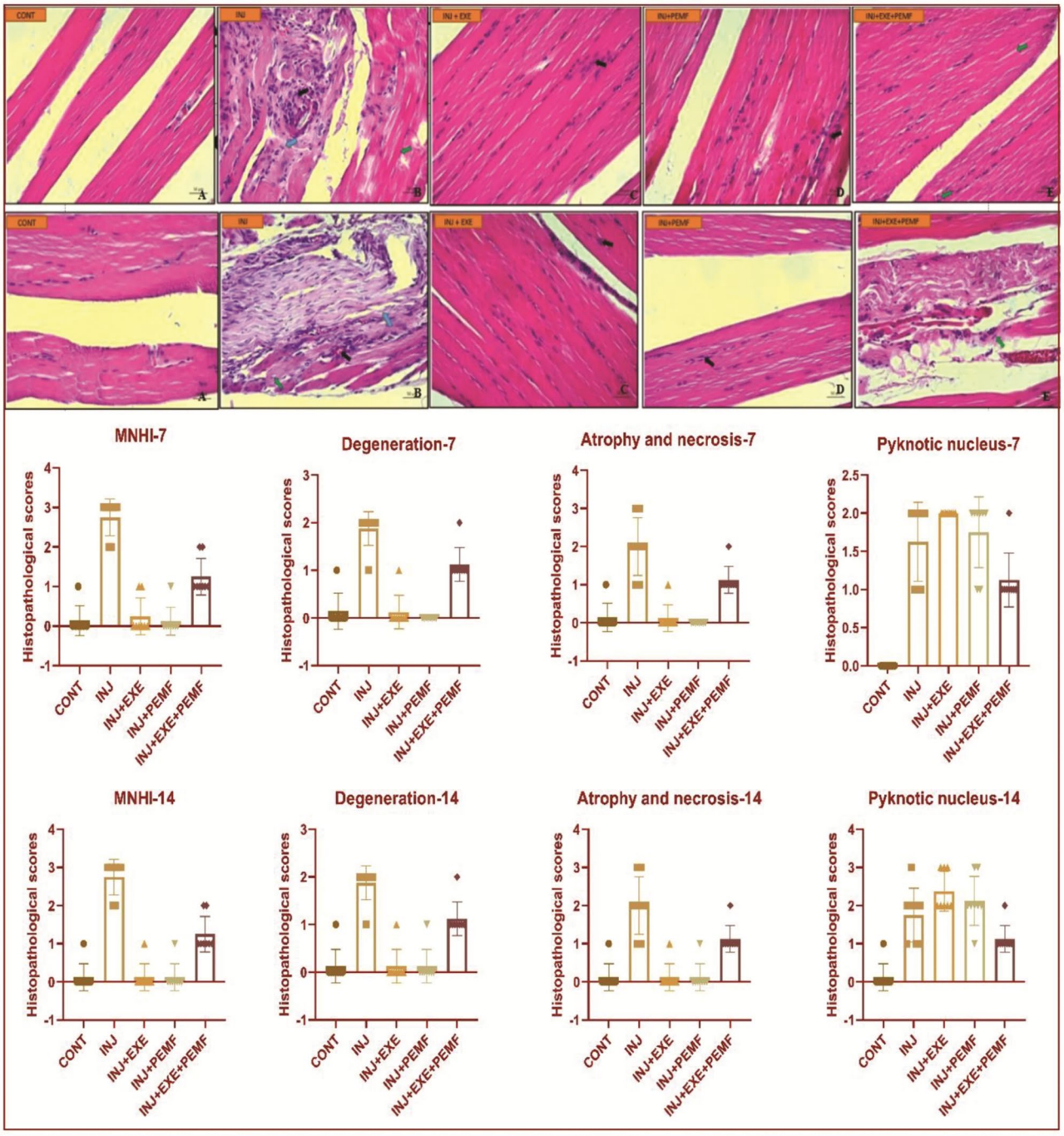


Fig. 2 Histopathological Results of Rats on The 7th (upper line) and 14th Days (line below) and Mean of The Data Obtained According to The Scoring of Histological Changes. There is no histopathological finding in the control group images. An increase in pyknotic nuclei, indicated by the black arrow, was observed in the images of the INJ + EXE and INJ + PEMF groups. In the images of the INJ group, mononuclear cell infiltration (black arrow), muscle tissue degeneration (blue arrow), and muscle tissue atrophy and necrosis (green arrow) are shown. In the INJ + EXE + PEMF group, muscle tissue atrophy and necrosis (green arrow) were indicated (CONT, INJ, INJ + EXE, INJ + PEMF, and INJ + EXE + PEMF scale bars: 50 µm, x20). **A** CONT, **B** INJ, **C** INJ + EXE, **D** INJ + PEMF, **E** INJ + EXE + PEMF, **MNHI-7 and MNHI-14**: 7th and 14th day mononuclear cell infiltration, **Degeneration-7 and Degeneration-14**: 7th and 14th day muscle degeneration, **Atrophy and necrosis-7 and Atrophy and necrosis-14**: 7th and 14th day atrophy and necrosis, **Pyknotic nucleus-7 and Pyknotic nucleus-14**: 7th and 14th day pyknotic nucleus

	MNHI-7		MNHI-14		Dejenere-7		Dejenere-14		Atr/Nec-7		Atr/Nec-14		Pyntotic-7		Pyntotic-14	
		P Value		P Value		P Value		P Value		P Value		P Value		P Value		P Value
CONT vs. INJ	***	<.001	***	<.001	***	<.001	***	<.001	***	<.001	***	<.001	***	<.001	***	<.001
CONT vs. INJ+EXE	ns	.632	ns	>.999	ns	.915	ns	>.999	ns	.938	ns	>.999	***	<.001	***	<.001
CONT vs. INJ+PEMF	ns	.936	ns	>.999	ns	.394	ns	>.999	ns	.535	ns	>.999	***	<.001	***	<.001
CONT vs. INJ+EXE+PEMF	***	<.001	***	<.001	***	<.001	***	<.001	***	<.001	***	<.001	***	<.001	**	.001
INJ vs. INJ+EXE	***	<.001	***	<.001	***	<.001	***	<.001	***	<.001	***	<.001	*	.041	*	.025
INJ vs. INJ+PEMF	***	<.001	***	<.001	***	<.001	***	<.001	***	<.001	***	<.001	ns	.484	ns	.169
INJ vs. INJ+EXE+PEMF	***	<.001	***	<.001	***	<.001	***	<.001	***	<.001	**	.001	**	.008	*	.025
INJ+EXE vs. INJ+PEMF	ns	.563	ns	>.999	ns	.440	ns	>.999	ns	.574	ns	>.999	ns	.166	ns	.356
INJ+EXE vs. INJ+EXE+PEMF	***	<.001	***	<.001	***	<.001	***	<.001	***	<.001	***	<.001	***	<.001	***	<.001
INJ+PEMF vs. INJ+EXE+PEMF	***	<.001	***	<.001	***	<.001	***	<.001	***	<.001	***	<.001	**	.001	**	.001

Fig. 3 Statistical Analysis of Histopathological Results. The relationships between the groups were evaluated with the One-Way ANOVA test (post hoc LSD test), Cont: Control, INJ: Muscle injury, EXE: exercise. PEMF: Pulsed electrromagnetic field, statistically significant at $p < 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, **ns**: non-significant, MNHI-7 and MNHI-14: 7th and 14th day mononuclear cell infiltration, Degeneration-7 and Degeneration-14: 7th and 14th day muscle degeneration, Atrophy and necrosis-7 and Atrophy and necrosis-14: 7th and 14th day atrophy and necrosis, Pyknotic nucleus-7 and Pyknotic nucleus-14: 7th and 14th day pyknotic nucleus

as VEGF and FGF. While this process may continue up to a certain level, if the irritation continues or due to the excessive severity of the injury, the intense inflammation may delay the repair. In order to ensure the restoration of the area, cytokines secreted from the defense cells coming to the scene can call other cells to the lesion area and increase the synthesis of growth factors [20]. In addition, in order to improve the migration to the lesion area during inflammation, NO is synthesized from the blood vessels via the eNOS enzyme, thereby expanding the vessel [21].

The presence of mononuclear cell infiltrations, atrophy, and necrosis detected in the INJ group induced by an 18 g biopsy needle in this study shows that the inflammatory muscle injury model required for stretching exercise and PEMF activities is formed. Both stretching exercise and PEMF applied in the treatment groups kept these inflammatory reactions at a lower level and accelerated muscle tissue recovery. In addition, by increasing the number of pyknotic nuclei, the muscle cell became more active, and the muscle mass increased. In order to increase the regeneration capacity of the injured muscle tissue, it is very important that these findings are parallel in both methods, and separate and intermittent applications of both methods can be recommended for the regression of inflammation. In addition, when combined treatment is applied, increasing the time between stretching exercise or applying stretching one day and PEMF the other day may facilitate tissue recovery and improve healing.

One of the main signs of cell death is nuclear changes that occur due to the fragmentation of chromatin and DNA. In pycnosis, which is also observed in the apoptotic process, the nucleus shrinks, and the chromatin density increases. The large pyknotic nuclei and high chromatin density in this study may also indicate that apoptosis, a negative effect for treatment, is reduced [22]. The fact that the number of pyknotic nuclei detected in this study increased in the single and combined treatment groups and was less in combined use than in single-use can be interpreted as suggesting single use for muscle development.

Being able to attenuate such an inflammatory response that can delay this repair time in the human body, which can repair itself, and reasonably reduce inflammatory cell infiltration is important to coordinate the inflammatory and regeneration process, which allows better functional recovery of the muscle after muscle injury [23]. In addition to routine stretching exercises, it is very important to support future studies to know that inflammation can be reduced more quickly and repair time can be accelerated with PEMF applications, which are easy to obtain and can be used repeatedly.

Berrueta et al. stated that stretching reduces inflammation, and active and passive stretching creates similar effects with a mechanical effect on tissues. In addition, it has been supported that stretching applied to the connective tissue reduces neutrophil migration within the tissue, which is a central aspect of the acute inflammatory response. Finally, stretching has been shown to have a direct mechanical effect on inflammatory regulatory molecules in connective tissue [24]. In other studies, both stretching exercises and exercises with a significant stretching component (e.g., yoga, Tai Chi) reduce circulating proinflammatory cytokine levels [25–27]. Achieving homeostasis in the face of acute inflammatory/immune challenges in the human body involves maintaining a balance of highly complex biochemical and cellular interactions, such as cytokine expression and signal transduction. The importance of maintaining healthy cytokine expression during this impactful time cannot be overstated. PEMF has the potential to regulate this very delicate balance [28]. In a rat rotator cuff injury and transosseous repair model to investigate the structural and functional effects of PEMF on tendon-to-bone healing, PEMF treatment was shown to improve recovery 3 months after surgery in patients with small or moderate

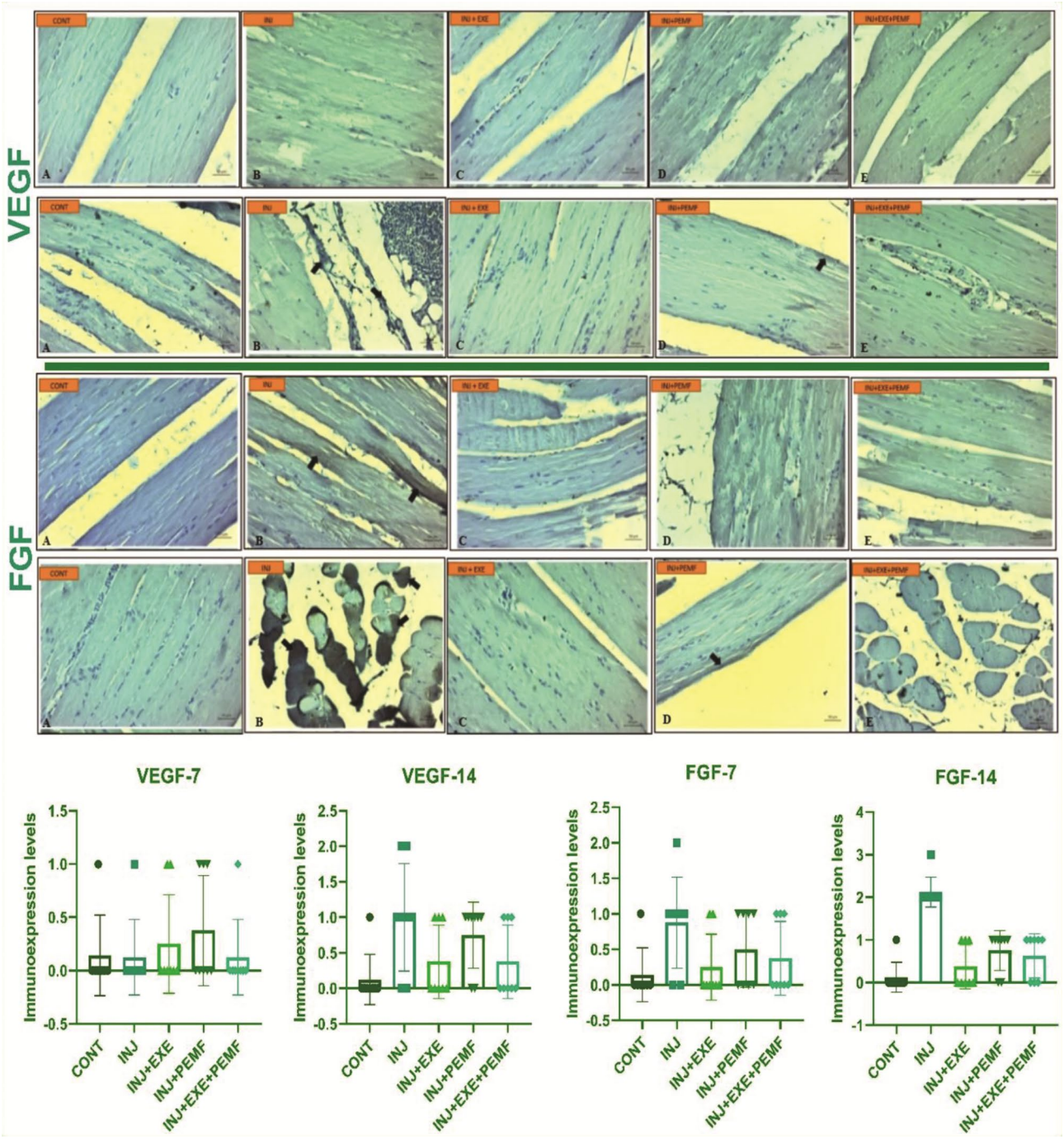
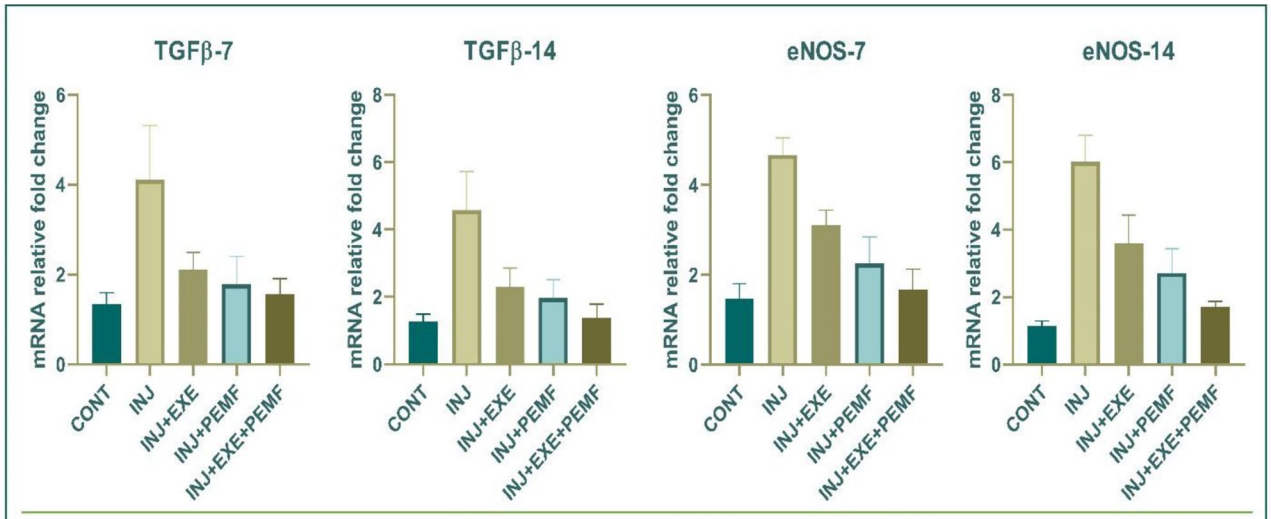


Fig. 4 VEGF and FGF Immunoexpressions of All Groups in The 7th (upper line) and 14th Days (line below) and Mean of The Data Obtained According to The Scoring of Immunohistochemical Changes. On 7th Day; No significant difference was observed between rat skeletal muscle tissues of all groups stained with VEGF. On 14th Day; VEGF staining was detected in INJ + PEMF and INJ groups. In particular, VEGF protein synthesis was more intense compared to the other groups (black arrow). It was observed that VEGF synthesis in the INJ group was more intense than the INJ + EXE + PEMF group. (CONT, INJ + EXE, INJ + PEMF, INJ and INJ + EXE + PEMF scale bars: 50 µm, x20), **A** CONT, **B** INJ, **C** INJ + EXE, **D** INJ + PEMF, **E** INJ + EXE + PEMF, VEGF-7 and VEGF-14: 7th and 14th day vascular endothelial growth factor. On the 7th Day, FGF (+) staining was detected only in the INJ group (black arrow), while no staining was observed in the other groups. On 14th Day; FGF protein synthesis was observed in the INJ + PEMF and INJ groups (black arrow). FGF synthesis and, accordingly staining in the INJ group was more intense, especially in the INJ + EXE + PEMF group (CONT, INJ + EXE, INJ + PEMF, INJ, and INJ + EXE + PEMF scale bars: 50 µm, x20), **A** CONT, **B** INJ, **C** INJ + EXE, **D** INJ + PEMF, **E** INJ + EXE + PEMF, FGF-7 and FGF-14: 7th and 14th day fibroblast growth factor

	VEGF-7		VEGF-14		FGF-7		FGF-14	
	P Value		P Value		P Value		P Value	
CONT vs. INJ	ns	.935	**	.003	**	.010	***	<.001
CONT vs. INJ+EXE	ns	.625	ns	.359	ns	.692	ns	.271
CONT vs. INJ+PEMF	ns	.292	*	.026	ns	.191	**	.008
CONT vs. INJ+EXE+PEMF	ns	.935	ns	.359	ns	.392	*	.032
INJ vs. INJ+EXE	ns	.555	*	.026	*	.021	***	<.001
INJ vs. INJ+PEMF	ns	.242	ns	.359	ns	.156	***	<.001
INJ vs. INJ+EXE+PEMF	ns	>.999	*	.026	ns	.062	***	<.001
INJ+EXE vs. INJ+PEMF	ns	.555	ns	.172	ns	.341	ns	.102
INJ+EXE vs. INJ+EXE+PEMF	ns	.555	ns	>.999	ns	.632	ns	.271
INJ+PEMF vs. INJ+EXE+PEMF	ns	.242	ns	.172	ns	.632	ns	.580

Fig. 5 Statistical Analysis of Immunohistochemical Results. The relationships between the groups were evaluated with the One-Way ANOVA test (post hoc LSD test), Cont: Control, INJ: Muscle injury, EXE: exercise. PEMF: Pulsed electromagnetic field, statistically significant at $p < 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, **ns**: non-significant, VEGF-7 and VEGF-14: 7th and 14th day vascular endothelial growth factor, FGF-7 and FGF-14: 7th and 14th day fibroblast growth factor



	TGFβ-7		TGFβ-14		eNOS-7		eNOS-14	
	P Value		P Value		P Value		P Value	
CONT vs. INJ	***	<.001	***	<.001	***	<.001	***	<.001
CONT vs. INJ+EXE	ns	.122	*	.041	***	<.001	***	<.001
CONT vs. INJ+PEMF	ns	.368	ns	.155	*	.021	**	.003
CONT vs. INJ+EXE+PEMF	ns	.661	ns	.820	ns	.523	ns	.212
INJ vs. INJ+EXE	**	.001	***	<.001	***	<.001	***	<.001
INJ vs. INJ+PEMF	***	<.001	***	<.001	***	<.001	***	<.001
INJ vs. INJ+EXE+PEMF	***	<.001	***	<.001	***	<.001	***	<.001
INJ+EXE vs. INJ+PEMF	ns	.489	ns	.475	*	.014	ns	.059
INJ+EXE vs. INJ+EXE+PEMF	ns	.253	ns	.064	***	<.001	**	.001
INJ+PEMF vs. INJ+EXE+PEMF	ns	.638	ns	.225	ns	.075	*	.036

Fig. 6 TGF-β1 and eNOS Expressions of All Groups on Days 7 and 14 and Statistical Analysis of Genetic Results. Statistical analysis of mRNA relative fold change Ct values was performed with one-way ANOVA posthoc LSD test. **TGF-β1**: Transforming growth factor-1 beta, **eNOS**: Endothelial Nitric Oxide Synthase, Cont: Control, INJ: Muscle injury, EXE: exercise. PEMF: Pulsed electromagnetic field, statistically significant at $p < 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: non-significant, **TGF-β1**–7 and **TGF-β1**–14: 7th and 14th day Transforming growth factor-1 beta, **eNOS**-7 and **eNOS**-14: 7th and 14th day Endothelial Nitric Oxide Synthase

rotator cuff tears [29]. In the study by Brown and Baker, calf muscle injury in rabbits was treated with shortwave diathermy (KDD) for 20 min twice a day for 8 and 16 days. After the treatment staining performed with the HE method and the results of the study indicated that 8-day treatment did not affect muscle recovery, the 16-day treatment program showed a trend towards increased recovery [30]. Contrary to the study of Brown and Baker [30], the decrease in the number of inflammatory cells detected on the 7th day in this study suggests that it may be related to the duration of PEMF use. The fact that different effects were observed at other times in the preliminary study also supports this result.

The increase in eNOS activity for repair at the site of inflammation in response to injury is a known physiological condition [31], and it was expected that PEMF application would deepen this increase [32–34]. However, the fact that the decrease in eNOS activities in PEMF groups was inversely related to the presence of inflammation within the scope of the study indicates that eNOS levels may decrease secondary to the anti-inflammatory activity of PEMF using other mechanisms. In many studies, PEMF, which increases eNOS increase-mediated repair upon healing, regressed inflammation in our study, resulting in a decrease in reactive endogenously synthesized eNOS levels. It is among the important outputs of the study that the stretching exercises act in parallel with the PEMF application and contribute positively to the repair process. In order to elucidate these situations, PEMF application times and different frequency values should be detailed with advanced molecular studies.

In the area of inflammation PEMF and stretching exercises limited the formation of fibrosis by providing a controlled increase in VEGF, which is an indicator of angiogenesis [35], and FGF, which performs fibroblast chemotaxis and is an indicator of fibrosis [20], thus contributing to functional recovery. The absence of an abnormal increase in the acute period and the fact that this increase becomes evident in the later period of the repair process can be interpreted as the regression of the inflammatory picture in the acute stage and the emphasis on the repair process in the later period.

In this study, VEGF levels in both INJ + EXE and INJ + PEMF groups and FGF levels in all treatment groups were higher than the control group, although not at a significant level, on the 7th day. On the 14th day, VEGF level increased in all groups except the control group while this increase was significantly higher in the injury group, except for the PEMF group, and FGF levels were significantly higher in the injury group than in all groups. As expected, the results support angiogenesis and wound healing, but the increase was more limited in combined treatments, contrary to expectations. We think that this may be due to the dosage of the treatment or the succession of applications without sufficient rest time. FGF level was also highest in the injury group on the 14th day. While the necessity of increasing growth factors has been shown in studies, it is not known exactly how much this increase should be and how long it should continue.

Several studies have shown that electrical stimulation of rat hind limb skeletal muscles can produce significant increases in the expression of the VEGF gene [36] and blood vessel density after stimulation [37, 38]. These results suggest that therapeutic modalities such as electrical stimulation of skeletal muscle may be promising to enhance angiogenesis following injury, potentially upregulating muscle regeneration events through stem cell activation and triggering of VEGF. In addition, mechanical stretching applied to muscle tissue can increase VEGF expression, while massage-like mechanical stretching can increase muscle regeneration through VEGF expression, stem cell, and inflammation modulation mechanism [39].

The amount of production of these factors may vary according to the type and severity of the injury, and it is known that intense VEGF and FGF increases also cause the development of fibrosis, which is an important problem in healing [20, 40]. Therefore, it is seen that the regular and certain level of expression of these growth factors rather than over or under production is more important. In this study, the application of both PEMF and stretching exercise provided a controlled increase in VEGF and FGF and limited the formation of fibrosis, thus giving the impression that it may have contributed to functional recovery.

Especially inadequacies in the repair process can cause the muscle tissue to heal with scarring and fibrosis. Different studies have been conducted to investigate the fibrosis formation process in muscle tissue and to determine the steps that cause it [41]. Current studies have focused on TGF-β1, which is an important mediator in the development of fibrosis, which is an undesirable condition in the healing of muscle injury. Many studies have shown that these protein's levels increase in injury groups and cause fibrosis. Although various growth factors such as VEGF and FGF released from neutrophils, macrophages, fibroblasts, and myogenic precursors increase fibrosis, the most profibrogenic growth factor identified in the literature is TGF-β1 [42, 43].

Depending on the inflammatory milieu, injury can result either in a tissue's complete regeneration or in its degeneration and fibrosis. TGF-β1 can modulate muscle repair not only by impacting at fibroblasts responsible for excessive extracellular matrix (ECM) deposition, but also by influencing the stem cells and inflammatory cells present and active within the injured area [44]. TGF-β1could prevent the apoptosis of Fibro-adipogenic progenitors (FAPs) and induce their differentiation into

matrix-producing cells, which results in a shift from transient matrix deposition to persistent fibrogenesis [45].

It has been determined that TGF-β1 increases collagen synthesis in injured muscle tissue and plays an important role in the process leading to fibrosis, and antifibrotic agents have been used to inhibit this effect. However, studies have been conducted on the use of various biological agents to stimulate regeneration and contribute to the average satellite cell migration [12, 41, 46]. Stretching exercises, a cheaper treatment method, can be used for treatment as they can show positive effects at the cellular level during the acute recovery period. Although the benefits of stretching exercises on injured fiber are not fully known, a limited number of studies suggest that they can have healing-stimulating effects. The reason for this thought is that when stretching exercises are performed in accordance with the muscle architecture, they create parallel intermittent tension and, as a result, stimulate collagen synthesis. In addition, with passive stretching in the muscle, capillary density, fibril cross-sectional area, and glycogen content increase, resulting in hypertrophy [6]. When the literature is examined, it is seen that studies generally focus on anti-fibrotic agents, but there are limited studies on the effect of physiotherapy methods such as exercise and electro-physical agents on fibrosis. Some animal models have shown that passive movements result in a reduction of fibrosis in healing tendons [47]. In other studies evaluating the effect of active stretching exercises, it has been shown that subcutaneous collagen formation is reduced after injury [48, 49]. In a study using an ex vivo model, tissue stretching was shown to decrease soluble TGF-β1 [50]. Although more studies are needed, controlled stretching programs may be a promising treatment option for preventing and treating fibrosis [51]. Hwang et al. assessed the effectiveness of stretching and decorin application on fibrosis after laceration injury and stated that whenever stretching exercise was started, it reduced the level of fibrosis as much as decorin [7]. Techniques such as massage and massage-like mechanical stretching may decrease the duration of the fibrotic phase by increasing the circulating clearance of TGF-β [39]. There are some studies showing that PEMF can also change the TGF-β level in wounds. Although TGF-β is well known to promote fibroblast to myofibroblast differentiation, there is a lack of evidence that PEMF alters TGF-β levels in wounds [52]. Looking at other soft tissues, Jasti et al. [53]. stated that PEMF had no significant effect on the systemic expression of TGF-β in a rat tendinitis model. In this study, it was observed that TGF-β1 level on both the 7th and 14th days, there was a significant decrease in exercise, PEMF, and combined treatment groups compared to the injury group, so single and combined treatment contributed to the reduction of fibrosis. Our applications are non-invasive, affordable and easy to apply, and do not have any known side effects, showing their superiority compared to antifibrotic treatments. It is obvious that stretching exercises and/or PEMF application can provide therapeutic effects with anti-fibrotic effects in any disease that is likely to develop fibrosis.

The limitations of our study include the following: evaluation could not be made in the early stages of the inflammatory process; different evaluations were not able to determine the effect of oxidative stress on NO level; healing with treatments may have occurred in a shorter time than expected due to the small damage in the wound model; since the study was conducted on female rats, the estral cycle may affect the study model. For this reason, the study can also be conducted on male rats; and it is difficult to make accurate comparisons because different methods have been applied in evaluation and treatment in the literature. More research is required to establish the method's effectiveness and examine various intracellular signaling pathways. The treatment protocols applied in this study were designed for animals. When the study will be carried out on humans, treatment protocols need to be rearranged.

Conclusions

This study showed that PEMF and stretching exercise may be effective in the treatment of muscle injuries, as they balance the inflammatory process in the muscle, have a positive effect on muscle development, accelerate healing, reduce TGF-β1 signaling, and prevent the development of fibrosis and inflammatory-induced eNOS activity. Both stretching exercises and PEMF can be used safely in the early period to accelerate the recovery and return to sports, which is the key point of rehabilitation, especially in professional athletes for whom even short periods of recovery are very important after muscle injuries. Further studies are needed to evaluate the effectiveness of these methods in more detail.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12891-025-08442-0.

Supplementary Material 1

Supplementary Material 2

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Author contributions

M.E.: Application of treatment methods to rats, conducted the data collection and dataanalysis, wrote the first draft of the manuscript, T.K.: Application of treatment methods to rats, conducted the data collection and data analysis, I.A.C.: Application of histopathological andimmunohistochemical tests, Y.E.: Application of genetic tests, H.A.: conducted the datacollection,

Methodology, Formal analysis, provided feedback on the first draft andcontributed to the final draft of the manuscript, S.C.: Supervision, Project administration,ınstallation and control of the PEMF device, contributed to the final draft of the manuscript, F.B.: Supervision, conceptualised the research project, led the review & editing process, contributed to the final draft of the manuscript.

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Data availability
The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
All experiments were conducted in accordance with ARRIVE (Animal Research: Reporting in Vivo Experiments) 2.0 guidelines. Animal experiments were approved by the Suleyman Demirel University Local Animal Ethics Committee with the ethical decision dated 10.06.2021 and numbered 04/08. The study was registered on ClinicalTrials.gov (NCT05988476).

Consent to publication
Not applicable.

Competing interests
The authors declare no competing interests.

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