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ORIGINAL ARTICLE

Effects of low-level laser therapy and therapeutic ultrasound on Freund's complete adjuvant-induced knee arthritis model in rats

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ABSTRACT

Objectives: The aim of this study was to evaluate and monitor the effect of low-level laser therapy (LLLT) and therapeutic ultrasound (TU) alone, or combined with intra-articular prednisolone (P) in Freund's complete adjuvant (FCA)-induced knee arthritis model in rats.

Materials and methods: A total of 56 adult male Wistar rats were divided into seven groups: control (C), disease control (RA), P, TU, LLLT (L), P + TU (P+TU), P + LLLT (P+L) groups. The skin temperature, radiography, joint volume, serum rheumatoid factor (RF), interleukin (IL)-1β, serum tumor necrosis factor-alpha (TNF-α), and histopathological evaluation of joint were performed.

Results: Thermal imaging and radiographic examination provided results consistent with the severity of the disease. The mean joint temperature (°C) was the highest in the RA (36.2±1.6) group on Day 28. The P+TU and P+L groups significantly decreased radiological scores at the end of the study. The rat serum TNF-α, IL-1β, and RF levels in all groups were significantly higher compared to the C group ($p<0.05$). Compared to the RA group, serum TNF-α, IL-1β, and RF levels were significantly lower in the treatment groups ($p<0.05$). The P+TU and P+L group was showed minimal chondrocyte degeneration and cartilage erosion and mild cartilage fibrillation and mononuclear cell infiltration of synovial membrane compared to the P, TU, and L group.

Conclusion: The LLLT and TU effectively reduced inflammation. In addition, a more effective result was obtained from the use of LLLT and TU combined with intra-articular P. This result may be due to insufficient dose of LLLT and TU, thus further studies should be focus on at higher dose ranges on FCA arthritis model in rats.

Keywords: Low-level laser therapy, knee joint, prednisolone, rheumatoid arthritis, therapeutic ultrasound.

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation of joints and other organs, particularly in the synovial membranes and articular tissues. It is a destructive polyarthropathy associated with excessive synovial fluid accumulation in the joint, hyperplasia of synovial cells, and pannus formation, which can eventually cause articular cartilage degradation and joint deformities.[1,2] In the past few years, advances in imaging methods such as magnetic resonance imaging, radiography, and ultrasonography provide an advantage for diagnose RA and monitor disease activity.[3] These imaging methods, however, are limited to anatomical structures of interest. Infrared thermography is essential in terms of showing physiological changes and metabolic activities. Thermal imaging technology is a non-invasive technique employed to determine disease severity in both animal models and humans.[4] Thermography is a basic screening technology used to diagnose a variety of disorders.[5,6]

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Glucocorticoids are effective anti-inflammatory agents and have been used for many years in symptomatic treatment of RA. These agents inhibit proliferation, differentiation, function of macrophages and fibroblasts proliferation, as well as production and release of cytokines.[7] However, glucocorticoids have some disadvantages, such as weight gain, insulin resistance, and cardiovascular impairment.[8,9] Therefore, non-pharmacological treatments for RA management have been investigated.[10,11]

Low-level laser therapy (LLLT) has been used clinically to treat various inflammatory diseases since 1981.[12] Therapeutic advantages of LLLT for inflammatory pathologies have been suggested by several authors.[11,13,14] It is used to enhance tissue repair, decrease inflammation and edema, and pain relief. It can be used for the stimulation of cell function. Lasers photons are thought to be absorbed by chromophores within the cells, such as cytochrome c oxidase located in the mitochondria.[15] Alterations in cytochrome c oxidase activity result in increased production of adenosine triphosphate, a major source of cellular energy, which provides to normalization of cell function, pain relief, and healing process.[16] The anti-inflammatory efficacy of LLLT has been controversial, and some authors have not found any effect from LLLT on inflammation.[17] However, more recent studies have been able to find some dose-dependent effects on *in vitro*[18] and *in vivo*[13] studies. In addition, LLLT has been used for relief of chronic pain in RA, which causes by sensitization of neurons by inflammatory mediators.[19]

Therapeutic ultrasound (TU) is one of many physical therapy modalities. It consists of high-frequency vibrations, which can increase cell permeability by setting up cavitation, thus aiding cell communication across articular membranes and linings; this mechanical effect explains why ultrasound has anti-inflammatory qualities.[20] Stable cavitation also reduces the nerve conduction velocity of C fibers, thus decreasing pain. Ultrasound can elevate intra-tissue temperature when used in a continuous mode. The significant benefits of ultrasound thermal effects are reducing muscle spindle activity and, accordingly, reducing muscle spasms and pain.[21]

The objective of this study was to evaluate effects on either LLLT or TU and their combination with intra-articular prednisolone (P) in the Freund's complete adjuvant (FCA)-induced knee arthritis in Wistar rats and monitor inflammatory arthritis activity. We hypothesized that treatment with combinations of either LLLT or TU with intra-articular P might produce more effective results than their use alone in the FCA-induced knee arthritis model in rats.

MATERIALS AND METHODS

Animals

This experimental study included 56 adult male Wistar rats (approximately 90 days of age and average weight of 250 g) supplied from the Medical Experimental Application and Research Center of Atatürk University. The rats were housed four per cage prior to the initialization of experiments and were acclimated for one week. The humidity ranged from 40 to 60%. A uniform temperature of 22±2°C was maintained throughout with a 12:12h light: dark cycle.

Induction of arthritis

In this study, the rats were injected with 0.1 mL of FCA (Sigma Chemicals, St. Louis, MO, USA),

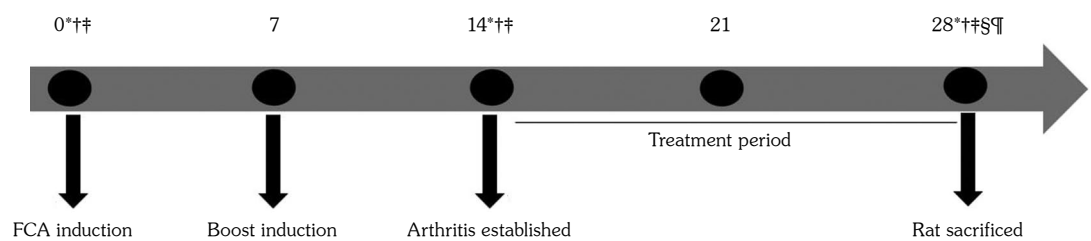

Figure 1. Timeline overview of the experimental study.

* Joint volume measurement; † Joint temperature measurement; ‡ Joint radiographic imagine; § Biochemistry measurement; ¶ Histological measurement.

containing 10 mg of heat-killed *Mycobacterium tuberculosis* per mL of paraffin oil, mixed with sterile saline in the ratio of 1:1 into the right knee joint. To increase the severity of arthritis, a booster injection with 0.1 mL of the emulsion was administered in the same manner on Day 7.[22,23] Figure 1 depicts the critical event linked with the FCA-induced arthritis model in further detail.

Study design

All animals were randomly divided into seven groups (n=8, Control (C), Disease Control (RA), P, TU, LLLT (L), P + TU (P+TU), and P + LLLT (P+L). Methylprednisolone sodium succinate (Prednol-L, Mustafa Nevzat, Istanbul, Türkiye) at a dose rate of 2 mg/kg/day was injected into the right knee joint (P, P+TU, and P+L groups). The same volume of sterile saline was injected into the right joint in the C and RA group.

Low-level laser therapy

The LLLT treatment was performed with a low-level laser (Eme Physio, Lasermed 2200; GaAs laser, 905 nm, 1 cm^2 beam area, 7,000 Hz, 17 mW, 1 J/cm^2, exposure time 60s; Pesaro, Italy) in the right knee joint once per day.

Therapeutic ultrasound

The TU treatment was employed with a TU device (Intelect Vet; 1 MHz, continuously mode, the intensity of SATA 0.5 W, exposure time: 300s; Chattanooga Group Inc., Chattanooga, TN, USA) in the right knee joint once per day. In C, RA, and P groups, LLLT and TU probes were placed, but no exposure was performed.

Measurement of knee joint volume

All joint volume measurements were performed immediately after the thermography examination. The inflamed joints of the rats were measured with a plethysmometer directly changes in knee volume by water displacement (mL) on Days 0 (baseline), 14, and 28.

Thermographic examination

The hair coat in the right knee joint area was shaved. A thermographic examination was performed in a room without sunlight and at a constant temperature of 22°C. The average temperature measurements of the knee joint were obtained from anterior, lateral, medial, and posterior views without administration of any sedative or anesthetic agents. All thermal camera measurements were recorded at the same time of day (10 to 12 AM). A thermal camera (IR Flexcam S, Infrared Solutions Inc., Plymouth, MN, USA) was used to measure on Days 0, 14, and 28.

The infrared camera was positioned at a distance of 1.0 m from the knee joint of rats and the thermal images were recorded on Days 0, 14, and 28. The SmartViewtm software (Fluke Corporation, Everett, WA, USA) was used for image visualization and analysis.

Radiological analysis

Before the begging of the study, the joints of rats were determined to be free of any radiological abnormality. The rats were anesthetized with sevoflurane and were positioned ventrodorsally on a table and X-ray image was obtained in the posterior-anterior view of the right knee joints with a stationary X-ray machine (Mex-100, Medical ECONET, Oberhausen, Germany) at 12 mA/s, 40 kV on Days 14 and 28. Two different blind observers performed radiological visual scoring, and visual scoring values were calculated based on the following conditions;[23] erosions: 0-3 (none, mild, moderate, severe); joint space narrowing: 0-3 (none, minimal, moderate, severe); joint space destruction: 0-3 (none, minimal, extensive, ankylosis).

Biochemical sampling and analysis

The rats were anesthetized with sevoflurane on Day 28, and approximately 3 mL blood samples were taken directly from the heart. The samples were transferred into sterile tubes containing ethylenediaminetetraacetic acid (EDTA) and centrifuged at 4,000 rpm, +4°C for 10 min and their plasma was separated and stored in a deep freeze at -70°C until biochemical analysis. Serum rheumatoid factor (RF), interleukin (IL)-1β, and serum tumor necrosis factor-alpha (TNF-α) were measured using a YLbiont enzyme-linked immunosorbent assay (ELISA) kit (Shanghai YL Biotech Co., Shanghai, China) according to the manufacturer's instructions.

Histopathological evaluation

The rats were sacrificed with a lethal dose of 200 mg/kg of sodium pentobarbital into intra-peritoneal injection on Day 28. The right

Table 1. Comparison of body weight, joint volume, joint temperature, radiological score, and biochemical analysis in all groups (n=56)

	C (n=8)	RA (n=8)	P (n=8)	TU (n=8)	L (n=8)	P+TU (n=8)	P+L (n=8)	
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	*p*
Body weight (g)†								
Baseline	244±36	255±24	283±40	252±19	258±36	250±51	266±29	0.834
At Day 14	242±18	246±44	276±32	248±33	252±22	252±18	258±41	0.575
At Day 28	254±41	241±48	277±21	244±20	250±13	248±44	260±11	0.678
Joint volume (mL)†								
Baseline	9.5±0.7	9.5±0.9	9.4±1.2	9.5±0.4	9.2±0.3	9.2±1.2	9.3±0.9	0.978
At Day 14	9.5±1^{a}	12.2±1.2^{b*}	12.3±1.8^{b*}	12.5±1.6^{b*}	12.4±1.7^{b*}	11.9±0.4^{b*}	12.2±2^{b*}	0.031**
At Day 28	9.6±1^{a}	13.3±1.3^{b*}	11.1±2.5^{c*}	11.7±1.3^{c*}	12.1±1.6^{bc*}	11.3±0.2^{c*}	10.8±1.6^{ac*}	0.022**
Joint temperature (°C)†								
Baseline	30.3±0.9	30.3±0.7	31±0.8	31±0.6	30.1±0.6	30.4±0.5	30.6±0.5	0.742
At Day 14	30.6±1.2^{a}	34.3±1.3^{b*}	34.2±0.8^{b*}	34.8±0.7^{b*}	34.7±1.2^{b*}	34.3±0.9^{b*}	34.9±0.7^{b*}	0.001**
At Day 28	30.5±1.4^{a}	36.2±1.6^{b*}	33.9±1.9^{c*}	35.5±0.8^{bd*}	35.1±1.5^{bd*}	34.2±1.3^{cd*}	33.1±1.3^{cd*}	0.001**
Radiological score‡								
At Day 14	0.00^{a}	1.61±0.61^{b}	1.58±0.44^{b}	1.62±0.51^{b}	1.55±0.32^{b}	1.61±0.42^{b}	1.49±0.28^{b}	0.017**
At Day 28	0.00^{a}	1.98±0.63^{b*}	1.45±0.53^{c}	1.55±0.49^{c}	1.45±0.11^{c}	1.11±0.53^{dc*}	1.05±0.32^{dc*}	0.021**
Biochemical analysis†								
TNF-α (pg/mL)	35.4±15.2^{a}	122±19.2^{b}	84.3±17.3^{c}	100±21.3^{bc}	99.6±29.4^{bc}	72.6±19.4^{c}	71.6±19.7^{c}	0.001**
IL-1β (pg/mL)	325±22.8^{a}	522±31.2^{b}	380±33.6^{c}	439±42.2^{bc}	478±45.1^{bc}	376±39.5^{c}	330±38.2^{c}	0.001**
RF (u/L)	1.5±0.2^{a}	5.7±0.5^{b}	3.9±0.2^{c}	4.5±0.3^{d}	3.9±0.3^{c}	4.1±0.6^{dc}	4.0±0.9^{dc}	0.032**

SD: Standard deviation; C: Control group; RA; Disease control group; P: Prednisolone group; TU: Therapeutic ultrasound group; L: Low-level laser therapy group; P+TU: Prednisolone + therapeutic ultrasound group; P+L: Prednisolone + low-level laser therapy group; TNF-α: Tumor necrosis factor-alpha; IL-1β: Interleukin beta; RF: Rheumatoid factor; † Kruskal-Wallis test; ‡ Two-way analysis of variance; * Within-group comparison statistically significant (p<0.05); ** Between-group comparison statistically significant (Different letters denote difference between-groups, p<0.05).

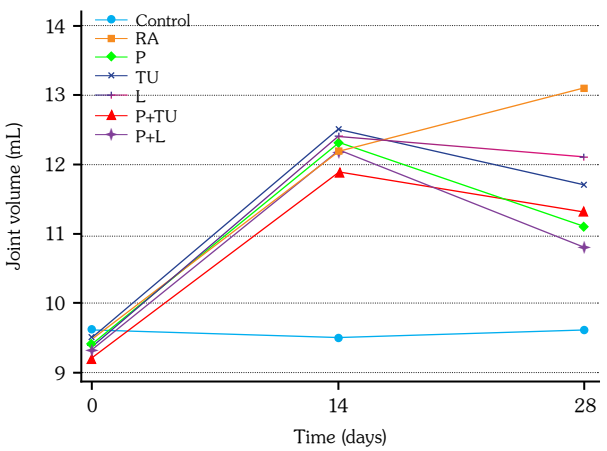

Figure 2. The change of rats' joint volume over the time.

Control (C) group, Disease Control (RA) group, Prednisolone (P) group, Therapeutic Ultrasound (TU) group, Low-level Laser Therapy (L) group, Prednisolone + Therapeutic Ultrasound (P+TU) group, and Prednisolone + Low-level Laser Therapy (P+L).

knee joint samples of the rats were removed and fixed in 10% neutral phosphate-buffered formalin for 48 h. After this process, they were decalcified with the Osteosoft decalcification solution (Merc KGaA, Darmstadt, Germany) for 48 h, and the samples were dehydrated in 70 to 99.9% ethanol series, cleared in xylene and embedded in paraffin blocks and submitted to 4 µm thickness transversal section. They were stained with hematoxylin-eosin and Masson's trichrome, examined and photographed using a light microscope (Leica DM 1000; Leica Microsystems Wetzlar GmbH, Wetzlar, Germany). The knee joint was histologically scored as chondrocyte degeneration, cartilage erosion/ulceration, cartilage fibrillation, mononuclear cell infiltration of synovial membrane

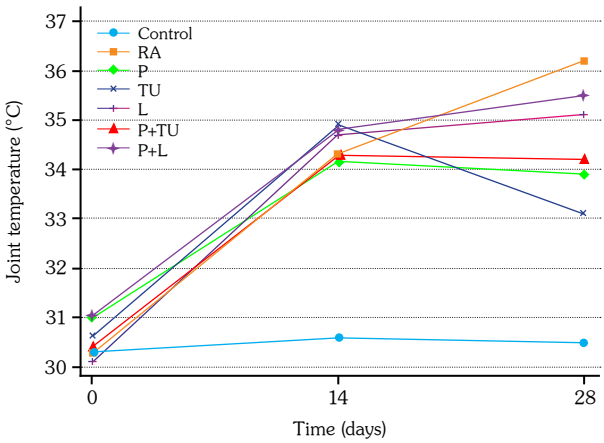

Figure 3. The change of rats' joint temperature (average of anterior, lateral, medial, and posterior view of joint temperature) over the time.

Control (C) group, Disease Control (RA) group, Prednisolone (P) group, Therapeutic Ultrasound (TU) group, Low-level Laser Therapy (L) group, Prednisolone + Therapeutic Ultrasound (P+TU) group, and Prednisolone + Low-level Laser Therapy (P+L).

(- normal, + minimal, ++ mild, +++ moderate, ++++ severe, Table 2).

Statistical analysis

The sample size was performed assuming 80% power to detect a 20% improvement in temperature with a standard deviation of 0.5°C and a significant level of 5%. The needed sample would be eight animals per group. This calculation was based on data from previously an experimental study evaluating FCA-induced arthritis in a Wistar rat model.[22]

Statistical analysis was performed using the SPSS version 22.0 software (IBM Corp., Armonk, NY, USA). The normality of data distribution was evaluated using the Shapiro-Wilk test. Descriptive data were expressed in mean ± standard deviation (SD). The normally distributed data was the two-way analysis of variance (ANOVA) with Bonferroni post-hoc test. For non-normally distributed data, the Kruskal-Wallis test was used to compare each group, followed by Dunn's multiple comparison test. A p value of <0.05 was considered statistically significant.

RESULTS

Evaluation of knee joint volume

The joint volume was not significantly different among groups at the baseline measurements ($p>0.05$). Compared to the C group, all groups were significantly increased joint volume on Days 14 and 28 ($p<0.05$, Figure 2). No significant

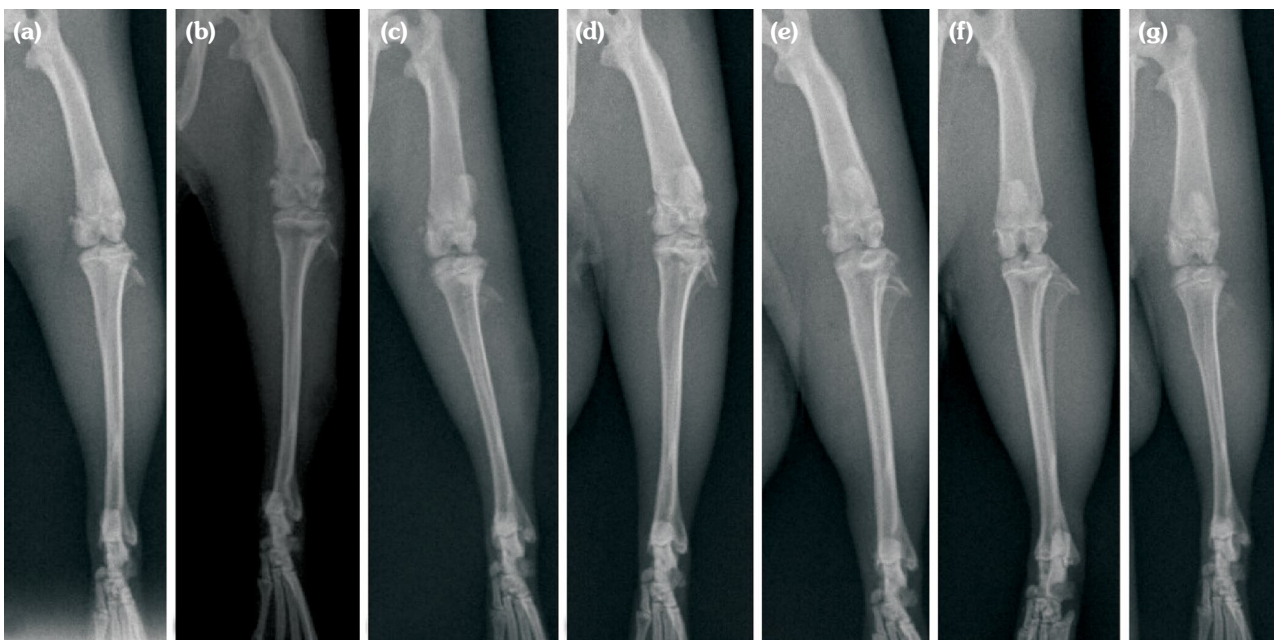

Figure 4. The X- ray images on Day 28 after FCA-induced in rats. **(a)** Control (C) group, **(b)** Disease control (RA) group, **(c)** Prednisolone (P) group, **(d)** Therapeutic ultrasound (TU) group, **(e)** Low-level laser therapy (L) group, **(f)** Prednisolone + therapeutic ultrasound (P+TU) group, and **(g)** Prednisolone + low-level laser therapy (P+L).

difference was found among the FCA-induced groups on Day 14 ($p>0.05$). The mean joint volume was significantly highest in the RA group (13.3±1.3) compared to L (12.1±1.6), TU (11.7±1.3), P+TU (11.3±2.2), P (11.1±2.5), P+L (10.8±1.6), and C group (9.6±0.9) on Day 28. The P+TU and P+L were significantly reduced joint volume on Day 28 compared to Day 14 (Table 1).

Evaluation of joint temperature

The baseline temperature of the knee joint was ranged from 30.2 to 31.0°C with had no significant difference among groups. No significant difference was observed among FCA-induced groups on Day 14 ($p>0.05$). Compared to the C group, the joint temperature was significantly higher in all groups on Days 14 and 28 ($p<0.05$, Figure 3). The mean joint temperature (°C) was the highest in the RA group (36.2±1.6) followed by TU (35.5±0.8), L (35.1±1.5), P+TU (34.2±1.3), P (33.9±1.9), P+L (33.1±1.3), and C group (30.5±1.4) on Day 28. The P and P+L groups were significantly decreased joint temperature compared to the RA, TU, and L groups at the end of study ($p<0.05$, Figure 3, Table 1).

Evaluation of radiological score

The radiological abnormality was not observed any time points in C groups (on Days 14 and 28 radiological score= 0, Table 1). The radiological score was not significantly different among groups on Day 14 ($p>0.05$, Figure 4). Compared to the RA groups (1.98±0.6), the mean radiological score was lower TU (1.55±0.5), P (1.45±0.5), L (1.45±0.1), P+TU (1.11±0.5), and P+L (1.05±0.3) group on Day 28 (Figure 4). The P+TU and P+L groups significantly decreased radiological scores at the end of the study ($p<0.05$, Figure 5). The P, TU, and L groups also decreased radiological scores, but were not statistically significant ($p>0.05$, Table 1).

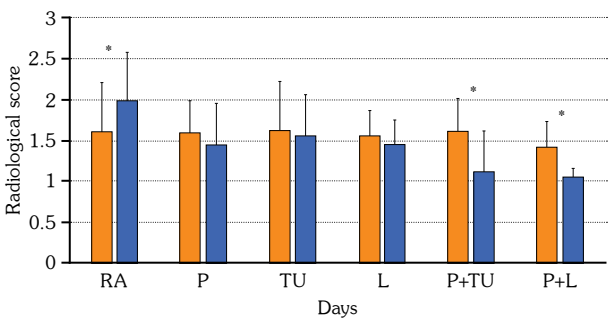

Figure 5. The change of radiological score on Days 14 and 28.

Control (C) group, Disease Control (RA) group, Prednisolone (P) group, Therapeutic Ultrasound (TU) group, Low-level Laser Therapy (L) group, Prednisolone + Therapeutic Ultrasound (P+TU) group, and Prednisolone + Low-level Laser Therapy (P+L). * $p<0.05$ significantly difference between 14 and 28 days.

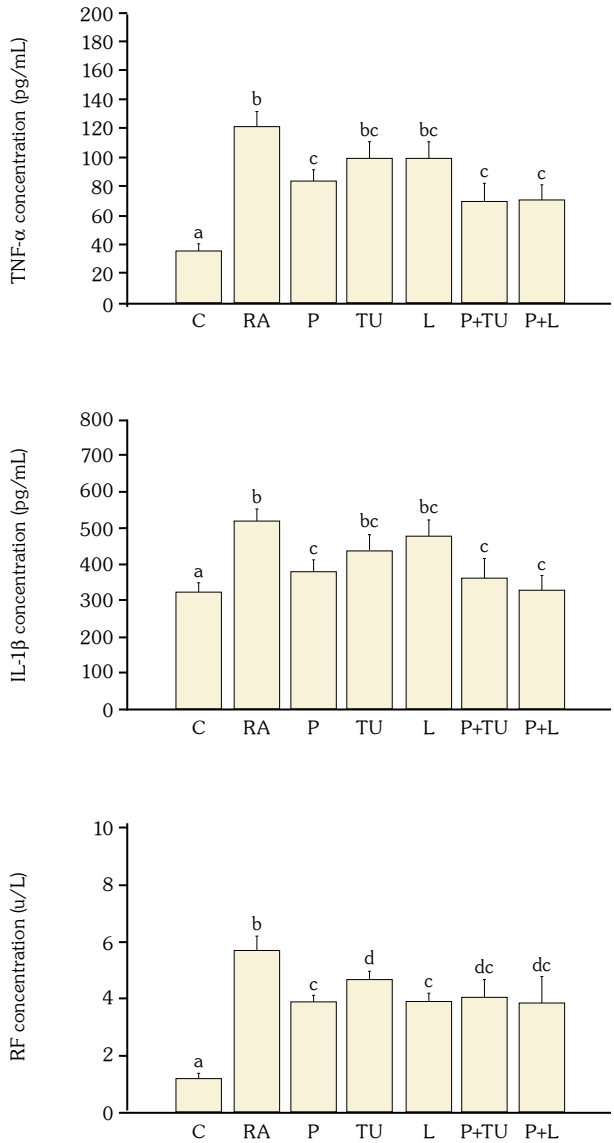

Figure 6. The change of serum concentration of TNF-α, IL-1β, and RF.

TNF-α: Tumor necrosis factor alpha; IL-1β: interleukin-1 beta; RF: Rheumatoid factor; C: Control group; RA: Disease Control group; P: Prednisolone group; TU: Therapeutic Ultrasound group; L: Low-level Laser Therapy group; P+TU: Prednisolone + Therapeutic Ultrasound group; P+L: Prednisolone + Low-level Laser Therapy. The different letters in the same sampling day indicate significant differences ($p<0.05$).

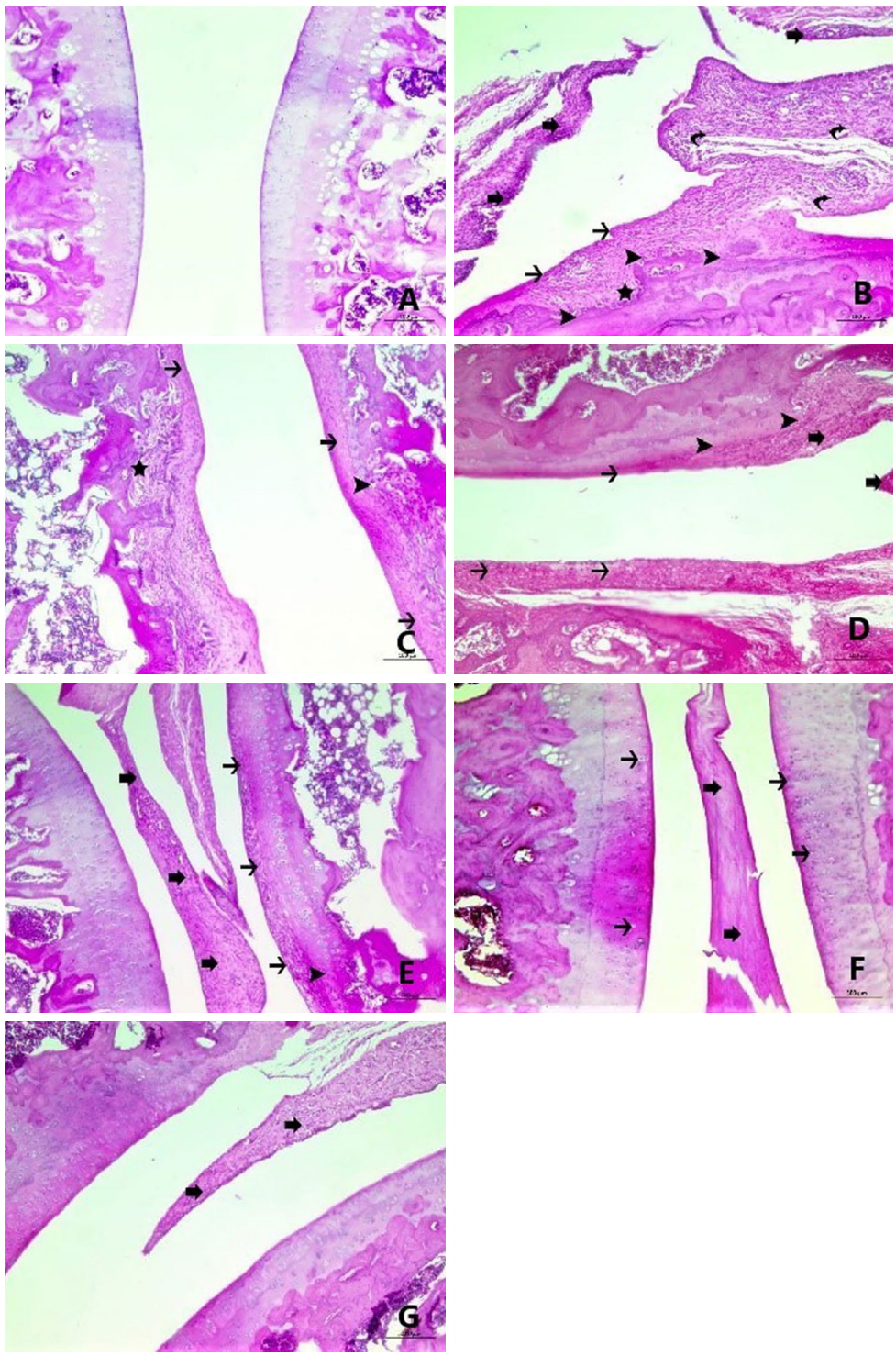

Figure 7. The histopathological evaluation of Freund's complete adjuvant-induced arthritis in Wistar rats (H-E, ×100). **(a)** Control (C) group, **(b)** Disease control (RA) group, **(c)** Prednisolone (P) group, **(d)** Therapeutic ultrasound (TU) group, **(e)** Low-level laser therapy (L) group, **(f)** Prednisolone + therapeutic ultrasound (P+TU) group, and **(g)** Prednisolone + Low-level laser therapy (P+L). The black thin arrow, arrowhead and asterisk indicate cartilage fibrillation, erosion and ulceration, respectively. The thick arrow indicates mononuclear cell infiltration of synovial membrane. The curved arrow indicates Pannus formation.

Table 2. Histopathologic score of the groups

Groups	Chondrocyte degeneration	Cartilage erosion/ulceration	Cartilage fibrillation	Mononuclear cell infiltration of synovial membrane
C	-	-	-	-
RA	++++	++++	++++	++++
P	+	++	++	+++
TU	++	++	++	+++
L	++	++	++	+++
P+TU	+	+	++	++
P+L	+	+	++	++

- None; + Minimal; ++ Mild; +++ Moderate; ++++ Severe; C: Control group; RA; Disease control group; P: Prednisolone group; TU: Therapeutic ultrasound group; L: Low-level laser therapy group; P+TU: Prednisolone + therapeutic ultrasound group; P+L: Prednisolone + low-level laser therapy group.

Evaluation of serum levels RF, TNF-α, and IL-1β

The rat serum TNF-α, IL-1β, and RF levels in all groups were significantly higher compared to the C group, ($p<0.05$, Figure 6). Compared to the RA group, the serum TNF-α, IL-1β, and RF levels were significantly lower in the treatment groups ($p<0.05$). The TNF-α and IL-1β levels in the P, P+L, and P+TU group were lower compared to TU and L group ($p<0.05$, Figure 6). Additionally, there was no significant difference between the TU and L group in terms of the TNF-α and IL-1β ($p>0.05$, Table 1).

Evaluation of joint histopathology

The joint sections of the rats in the C group were examined histopathologically. The joint capsule, synovium, cartilage, and bone tissues were in normal histological structure (Figure 7). The RA group showed severe chondrocyte degeneration, cartilage erosion/ulceration, cartilage fibrillation, and mononuclear cell infiltration of synovial membrane compared to P, TU, L, P+TU, and P+L group ($p<0.05$, Table 2). The P+TU and P+L group was showed minimal chondrocyte degeneration and cartilage erosion and mild cartilage fibrillation and mononuclear cell infiltration of synovial membrane compared to the P, TU, and L group ($p<0.05$, Figure 7).

DISCUSSION

The objective of this study was to investigate the effectiveness of either LLLT or TU and their combination with intra-articular P, on an experimentally established FCA-induced arthritis model in Wistar rats. The FCA-induced arthritis model of chronic inflammation is considered the best available experimental model of RA.[24,25] Also, it is a model of chronic polyarthritis with features that resembles RA.[26] Therefore, the C group was formed instead of using collateral knee joints in rats.

Rheumatoid arthritis is a long-term form of autoimmune disorder that primarily affects the joints identified by inflammation and swelling of the synovium of the joint.[27] The current study showed that, following intra-articular injection of FCA, knee edema of the effected joint increased as a result of inflammation. Our results showed that all treatment groups had significantly reduced joint volume compared to the RA group. The activity of glucocorticoids is mediated via glucocorticoid receptor, which is significantly decreased in synovial inflammation.[28]

Previous studies reported that LLLT[11,16,29] and TU[30,31] treatments showed positive effects in several types of joint inflammation. Similar to our result, Tomasek et al.[32] reported that LLLT reduced edema and inflammatory cell infiltration and increased the collagen and elastic fibers at the injury site. Also, another study reported that LLLT (785 nm) produced a decrease in leukocytes in anterior tibialis muscle of rats.[33] These results showed that the LLLT had anti-inflammatory effects and decreased leukocytes infiltration at the injury site.

Change of tissue temperature is one of the essential physical features of the inflammatory

process.[34] Increasing joint temperatures indicate the appearance of inflammatory reactions in RA.[35] Administration of FCA into the knee joint has been shown to cause localized inflammatory response characterized by increased vascular leakage, infiltration of inflammatory cells, and the following swelling of the effected joint. In the current study, the temperature of the inflamed joint in all groups was significantly higher than in the C group. The maximal mean temperature of the effected knee joint was noted RA group on Day 28. The P and P+L groups were more effective and significantly decreased the temperature of the inflamed joint. The L group decreased the joint temperature compared to the RA group, but was not effective by the P+L group. The LLLT significantly reduced the level of inflammatory cytokine prostaglandin E2 (PGE2) in the synovial membrane of RA.[36] This anti-inflammatory effect may provide to decrease the temperature of inflamed joint. The TU is capable of producing thermal therapeutic effects.[37] Therefore, it was noticed that, in the treatment groups with TU, even if the tissue temperature decreased due to the severity of arthritis, the thermal effect of TU might prevent us from detecting severity of arthritis. Thus, the thermal camera was ineffective for the follow-up of the disease in thermal applications.

During the process of adjuvant induced RA, joint inflammation is caused by the infiltration of immune cells, synovial hyperproliferation, and the over production of proinflammatory cytokines, such as TNF-α and IL-1β, following causing cartilage and bone damage.[38] Moreover, Uddin et al.[39] reported that the TU stimulation prevented cartilage damage by inhibiting the IL-1β and stimulating chondrocyte migration, proliferation, and differentiation. The proinflammatory cytokines may cause bone erosion indirectly by promoting differentiation of osteoclast precursors and afterward by activating osteoclasts.[40] Therefore, the radiological examination is a useful measure of disease severity, as well as progression in RA.[41] Our study showed that the RA groups' radiological score was higher than all treatment groups. On Day 28, the P+TU and P+L groups radiologically reduced the severity of arthritis compared to Day 14, possibly by inhibiting the infiltration of proinflammatory cytokines. This result is consistent with our histopathological findings.

Serum IL-1β and TNF-α, which are two mainly mediatory of RA in experimental models,[42] are produced in chondrocytes in pathological conditions.[43] In addition, diagnostic studies proposed previously that circulating pro-inflammatory cytokines such as TNF-α and IL-1β could be dependable biomarkers of RA.[44] Also, serum RF is considered a specific rheumatoid biomarker.[45] In this study, the rat serum TNF-α, IL-1β, and RF levels in FCA-induced groups were significantly higher compared to the C group. Treatment groups (P, TU, L, P+TU, and P+L) of serum TNF-α, IL-1β, and RF levels were lower compared with the RA group. This result indicated that either LLLT or TU and their combination with intra-articular P were produced in anti-inflammatory effects on FCA-induced knee arthritis. Similarly, previous studies reported that LLLT reduced the levels of TNF-α and IL-1β.[12,46] The LLLT emerges to reduce the TNF-α and IL-1β by affecting processes downstream of nuclear factor kappa B (NF-kB) pathway activation.[47] In addition, a previous study indicated that TU stimulation was inhibited the IL-1β.[40] Although this study was also showed that LLLT and TU reduced serum TNF-α and IL-1β level, their combination with intra-articular P more effectively result was obtained. These combinations may produce a synergistic effect on reducing inflammation of the inflamed joint.

Histopathological analyses of inflamed joints obtained from rats with the RA group have shown the severity of chondrocyte degeneration, cartilage erosion, cartilage fibrillation, and mononuclear cell infiltration of synovial membrane. This study observed that all treatment groups decreased mononuclear cell infiltration of synovial membrane compared to the RA group. However, differences were found between these groups, showing that mononuclear cell infiltration in P+TU and P+L was lower than in the P, TU, and L groups. This result may show that the anti-inflammatory effect increases, when L and TU are used together with glucocorticoids. Also, previous studies reported that LLLT[13,48] and TU[49] produced anti-inflammatory effects in various inflammation diseases.

Nonetheless, this study has some limitations. We examined only short periods after the arthritis. It would be remarkably fascinating to investigate the short and long tissue response to the LLLT and TU application in different periods after inflammation. Another limitation of this study is that functional evaluation (such as gait analysis) and synovial fluid analysis were unable to be performed.

In conclusion, thermal imaging and radiographic examination can give results consistent with the severity of the disease. However, thermal imaging can be ineffective for follow-up thermal application. Although either LLLT or TU have positive effects on RA, a more effective result obtained from the use of LLLT and TU combination with intra-articular P. Also, LLLT and TU may become a better therapeutic opportunity for the treatment of RA. However, further studies are needed to investigate the effects of using various doses of LLLT and TU in rat models of RA.

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