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Research Article

Tarantula Cubensis Extract and Low-Level Laser Therapy: A Histopathological, Radiological and Serological Analysis of Bone Repair on an Experimental Bone Defect Model

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Abstract: In this study, it was aimed to investigate the effects of *tarantula cubensis* extract (TCE) and low-level laser therapy (LLLT) on cancellous and cortical bone repair. A circular unicortical bone defect was created on both the cancellous and cortical regions of the tibia of each subject. The subjects, totaling 54 mature New Zealand rabbits, were randomly allocated into three groups (n:18 each): control, LLLT (Galium-aliminum-arsenide laser at a wavelength of 780 nm and 4 J/cm^2, 5 min/day) and TCE (1 µg/kg) groups. Relative optic density (ROD) level was higher in the TCE group than the control group on day 28 as radiological (P<0.05). In both cancellous and cortical bone, inflammatory cell densities were less on the 14 and 21st day in the TCE and LLLT groups, bone tissue formation and qualities were higher 7 and 14th days in TCE and LLLT and collagen maturation were higher 28th day in the TCE group as histopathological (P<0.05). In this study, TCE accelerates bone repair as much as LLLT and more than the control group. In conclusion, that TCE is an effective, easier to apply and more economical supportive treatment in bone defects like LLLT.

Keywords: *Bone repair, Homeopathy, Low-level laser therapy, Tarantula cubencis extract, Rabbit*

Tarantula Cubensis Ekstraktı ve Düşük Seviye Lazer Tedavisi: Deneysel Kemik Defekti Modelinde Kemik Onarımının Histopatolojik, Radyolojik ve Serolojik Analizi

Öz: Bu çalışmada *tarantula cubensis* ekstraktı (TCE) ve düşük seviyeli lazer tedavisinin (LLLT) süngerimsi ve kortikal kemik onarımı üzerindeki etkilerinin araştırılması amaçlandı. Her deneğin tibiasının hem süngerimsi hem de kortikal bölgelerinde sirküler unikortikal kemik defekti oluşturuldu. Toplam 54 olgun Yeni Zelanda tavşanından oluşan denekler rastgele üç gruba ayrıldı (her biri n:18) kontrol, LLLT (780 nm dalga boyunda ve 4 J/cm^2, 5 dk/gün'de Galium-alüminyum-arsenid lazer) ve TCE (1µg/kg) grupları. Rölatif optik yoğunluk (ROD) düzeyi radyolojik olarak 28. günde TCE ve LLLT gruplarında kontrol grubuna göre daha yüksekti (P<0.05). Histopatolojik olarak hem süngerimsi hem de kortikal kemikte, inflamatuvar hücre yoğunlukları 14. ve 21. günde TCE ve LLLT gruplarında daha azdı kemik dokusu oluşumu ve kalitesi 7. ve 14. günlerde TCE ve LLLT gruplarında daha yüksekti ve kollajen olgunlaşması 28. günde TCE grubunda daha yüksekti (P<0.05). Bu çalışmada TCE, kemik onarımını LLLT kadar ve kontrol grubuna göre daha fazla hızlandırmaktadır. Sonuç olarak, LLLT gibi kemik defektlerinde TÇE'nin etkili, uygulaması kolay ve ekonomik bir destekleyici tedavi olduğu sonucuna varıldı.

Anahtar sözcükler: *Kemik onarımı, Homeopati, Düşük seviye lazer tedavi, Tarantula cubencis ekstraktı, Tavşan*

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Introduction

Today, both bone metabolism the disorders and the impairment of bone integrity due to traffic and work accidents have made bone healing an important health problem in both humans and animals. The main targets in the treatment of these bone defects in orthopedic clinics are to make the damaged area painless by performing the necessary conservative or surgical intervention as soon as possible and to help the patient return to normal life by helping the bone repair as quickly as possible [1,3].

To accelerate bone repair, biomaterials, bone morphogenetic proteins (BMP), physical stimulation such as ultrasound, electromagnetic fields, and laser applications and chemical stimulants such as drugs and homeopathic remedies are used [4,5]. Tarantula cubensis extract (TCE), a venom of spider, as an injectable agent that is potentized (6x) according to the German Homeopathic Pharmacopeia after maceration in alcohol, has been used as homeopathic in many animal species in recent years. TCE, which has a peptide structure, removes necrotic tissues and thus accelerates epithelialization and heals the wound [6,7]. It has been used in the treatment of breast tumors, pododermatitis, painful abscesses involving gangrene, septicemia, and toxemia by creating a demarcation line around the lesion [8,10]. In addition, it causes a decrease in inflammation in rats, accelerates uterine involution, treats genital microbial diseases, oral ulcers, cutaneous and oral papillomatosis [6,8-10]. It has also been reported to be effective for tendon, nerve, and wound healing [10-12]. However, studies on bone tissue are very scarce [13].

Low-level laser therapy (LLLT) is a supportive treatment application that aids biological tissue regeneration processes such as bone repair, skin lesions and muscle tissue repair [14,15]. With LLLT, photobiomodulation (nonthermal phototherapy) occurs in the tissues by creating reactions such as increased cell proliferation, osteoblast activity, vascularization, bone formation and collagen deposition in bones and various tissues [15-18]. LLLT also accelerates the healing process with its analgesic and anti-inflammatory effects [19,20]. Most studies using LLLT have been planned for the repair of defects formed in the cortical bone [15,19,21]. Although the repair of cancellous bone is known to be faster than that of cortical bone [22], it is not evident whether there is a difference between the repair of LLLT and TCE on both cortical and cancellous bone defects at the same time.

Briefly, since bone healing is basically connective tissue healing [1], it is thought that TCE may positively affect healing by accelerating collagen synthesis from fibrin networks and fibroblasts that fill the defect area in the early stages of bone repair. In addition, due to the vascularization-enhancing effect of TCE in the repair area [12], it may cause an increase in VEGF (Vascular endothelial growth factor), which stimulates new vessel formation, and increases bone stabilization in the reparative phase of the bone healing process, leading to the formation of fibrocartilagenous callus. Because of the above-mentioned features, TCE is thought to be able to provide a faster repair of the bone by accelerating angiogenesis, collagen synthesis and anti-inflammatory effect, which have a primary role in bone repair like LLLT. Moreover, LLLT and TCE are thought to be important in terms of revealing the expression and localization changes of BMPs with osteoinductive properties in the repair of bone and their roles in regeneration. In light of these hypotheses, it was aimed to find answers to the following questions. First, does TCE increase the expression of VEGF receptors (flt1/fms, flk1/KDR and flt4, respectively) and BMPs in damaged bone tissue? Second, is there a difference in the efficacy of TCE and LLLT on cancellous and cortical bone regions? Third, since homeopathic agents are known to significantly reduce cost compared to conventional agents [23], does TCE as effective in bone repair as LLLT, which requires specialized equipment and cost?

Material And Methods

Ethical Statement

This study was conducted with the principles of animal welfare in mind. All experimental protocols were thoroughly revised and approved by the Animal Experiments Local Ethics Committee, Dicle University, Diyarbakır (Approval No: 2016/8, 13.04.2016)

Animals

Healthy 54 male mature New Zealand white rabbits, which have a tibial diameter of 0.5-0.7 cm, were included in this study. Rabbits were purchased from the experimental animal production unit for the University of Dicle. The rabbits were properly housed in individual cages in the rabbit room with the same humidity and temperature in the same unit before and after the surgical procedures. Standard feed and water were provided *ad libitum*. The acclimation period before surgery for all the rabbits was one week.

Anesthesia and Surgical Procedure

Before the surgery, rabbits were anesthetized with ketamine HCl (50 mg/kg, IM) (Ketasol®, Richter-Pharma AG, Wels, Austria) following premedication with xylazine HCl (10 mg/kg, IM) (Rompun® 2%, Bayer, Turkey) and butorphanol (0.3 mg/kg, IV) (Butomidor®, Richter-Pharma AG, Wels, Austria). After providing asepsis-antisepsis of the operation area of all rabbits under general anesthesia, the right tibias of the rabbits were opened surgically with an appropriate anatomical surgical approach, then

a unicortical bone defect was created on the cortical and cancellous bones of the right tibia with a 3.5 mm diameter drill. Drilling was performed slowly to avoid possible bone necrosis, accompanied by saline irrigation [19]. After the defected area was irrigated with saline to remove bone debris, the muscles and skin were covered with 3-0 suture material. Rabbits were administered 400.000 IU of procaine penicillin (Procaine Pen® 400.000, Tümekip İlaç, İstanbul) for 5 days for postoperative antibiotherapy and carprofen (1.5 mg/kg/day, SC) (Rimadyl®, Zoetis, İstanbul) for 2 days for postoperative analgesia. Since unicortical defects were performed, there was no need to apply any support bandage on the respective legs of the rabbits since no problems with walking were expected. All rabbits received daily wound care for 10 days. If spontaneous fracture in the defect areas and development of osteomyelitis were accepted as exclusion criteria in the experimental process.

Study Design and Treatment Protocols

After the experimental bone defect model was created, the rabbits have randomly allocated into 3 main groups as follows:

Group 1: LLLT (n = 18): Low-level laser therapy was applied with a [Galium-aliminum-arsenide-GaAlAs-Chattanooga Intellect-USA] laser at a wavelength of 780 nm and 4 J/cm^2, 5 min/day] at the same time for 4 weeks starting on the 4th postoperative day [21].

Group 2: TCE (n = 18): *Tarantula cubensis* alcoholic extract (Homeopathic group) (Theranekron® D6, 1 mg/mL, Richter-Pharma AG, Wels, Austria) was administered to rabbits subcutaneously (SC) on the 3rd, 7th, 10th, and 15th days at 1 µg/kg on the skin of the defect-created tibia [11].

Group 3: Control (n = 18): Rabbits were administered SC saline only once after the surgical procedure, and these rabbits were used as the control group.

The three main groups then were allocated into 3 subgroups according to the duration of treatment (7, 21 and 28 days), with six animals in each subgroup.

Laboratory (Serological) Evaluations

Serum samples were obtained from the rabbits by centrifugation of blood from the ear vein on preop (0) and postoperative 7th, 14th, 21th and 28th days. Bone Alkaline phosphatase (B-ALP), Osteocalcin (OC), Prokollagen I N-terminal peptide (PINP) bone formation factors, and Interleukin-1β (IL-1β), Interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) proinflammatory cytokines were analyzed in the serum samples using rabbit-specific commercial ELISA kits (Elabscience Biotechnology Co., Ltd, Wuhan, China).

Radiological Evaluation

Each animal underwent radiological evaluation just postoperatively and once a week for 4 weeks. The radiological technique was performed according to Matos et al.[24]. In contrast to conventional radiological evaluation to confirm the assessment and follow-up of fracture repair, the Fracture Healing Monitor Module of the Relative Optical Density Image Analysis (RODIA) a system developed by Polish researcher Glinkowski [25] was utilized for image evaluation. For this purpose, the web-based Fx-Expert 2014 program was used, which is called Orthopedic Intelligence System v.1.0. [26] *(Fig. 1-A).* This program works on the principle of determining the optical density (OD) of a fracture or defect area on the

Fig 1. The Fx-Expert program was used to determine the relative optical density (ROD) value and the sequence of images showing the ROD analysis with the program. A: Accessing the program via the internet, B: Opening the X-ray image in the program; C: Displaying the solid and defect regions on the x-ray image; D: Measuring the OD values in the solid and defect regions, and the program calculating the ROD value automatically *(green arrow)*

radiological image transferred to the computer. Briefly, digital DICOM X-ray images obtained with a CR system were transferred to a personal computer. The images were then opened with the Fx-Expert program running on the web *(Fig. 1-B)*. The optical density of the intact region adjacent to the defect area was first determined on the images *(Fig. 1-C)*. The optical density of the defect was then determined. After these two OD values were determined, the program automatically determined the relative optical density (ROD) value with the ratio of the OD value of the defective region to the intact region *(Fig. 1-D)*. These analyzes were performed separately for the defect site in both the cancellous and cortical bone. All analyzes were performed by a blinded researcher.

Histopathological Analysis

The rabbits were euthanized end of the experiment in days postoperative 7th, 14th, 21th, and 28th days after administration of a high-dose anesthetic (50 mg/kg) (Pentothal Sodium®, Tümekip İlaç, İstanbul) and pancuronium (1 mg/kg) (Pavulon®, Organon, İstanbul). Histopathologic sections were prepared according to Pretel et al.[27]. For the histopathological and immunohistochemical examinations, serial sections of 5 µm thickness were taken from the prepared blocks. Histopathological sections were stained with Crosmann's triple and Solochrome Cyanine. In the areas where new bone formation occurred in Crosmann triple stained preparations, collagen fibrils, distribution and morphology of trabecular structure, determination of the presence of osteoblasts and osteocytes, inflammatory cell density, blood vessels, etc. were evaluated. In the preparations stained with Solochrome Cyanine, osteids, mineralized and calcified bone areas of newly formed bone tissue were evaluated.

Immunohistochemistry Staining

The streptavidin peroxidase method was used to determine the localization and expression of VEGF and its receptors, osteopontin and BMPs in the bone tissue of the rabbits. Immunohistochemical staining procedures were performed according to the staining principles in our previous study [28]. All sections were treated according to the same protocol.

After staining, the preparations were photographed on a Nikon-Eclipse 400 DSRI Nikon digital camera (NIS Elements Imaging Software-version 3.10) attachment research microscope and evaluated.

Semi-Quantitative Evaluation

The scoring method belonging to Pretel et al.[27] was used to evaluate the histopathologic differences in the formation stages of new bone tissue after sections were stained with Crosmann's triple and Solochrome Cyanine staining. Histopathologic analysis was performed under a light microscope. Each prepared sample was evaluated blindly by two independent observers. In cases of disagreement, the samples were re-evaluated and a consensus was reached among independent observers.

Statistical Analysis

The data were analyzed with the SPSS 24.0 program and are presented as mean ± SD. P-value less than 0.05 was considered statistically significant. The Kruskal-Wallis test was used to determine the difference between treatment days and groups and Mann-Whitney-U test was used to determine the group that showed differences in histopathologic data. Bone-specific markers and radiological ROD evaluations were performed using One-Way ANOVA, and the Post-hoc Duncan test for multiple comparisons was used to detect differences. Two independent samples test (student t-test) was used to evaluate the difference between cortical and cancellous bone defects.

Results

There were no postoperative complications in any of the rabbits during the experimental period.

Clinical and Radiological Findings

Clinically, the rabbits had no problems walking during their daily controls, and there were no signs of discharge, swelling, etc. in the surgical wounds. In radiological evaluations, conventional radiographs were presented *Fig. 2* and statistical data of the ROD analysis obtained by

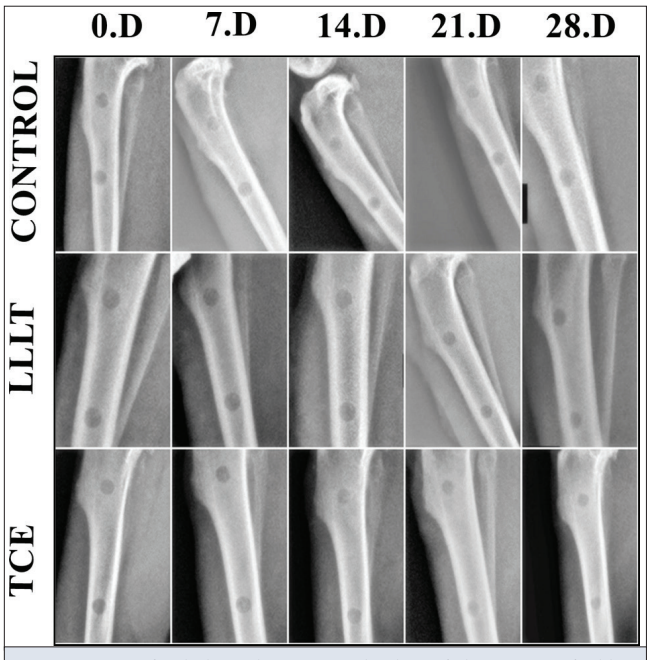

Fig 2. A series of radiological images on the days of observation of rabbits from each group

rabbits immediately after the surgery and on the 7th, 14th, 21st, and 28th days are presented in *Table 1*.

Histopathological Findings

The histopathological results obtained by scoring techniques are summarized in *Table 2*. Histopathological images of tissues are given on *Fig. 3* and *Fig. 4*.

Immunohistochemical Findings

Intensity scores of osteopontin, BMPs, VEGF and VEGF receptors (flt1/fms and flk1/KDR) expression in the cortical and cancellous bone defect during the applications are presented in *Table 3* and immunohistochemical staining of VEGF are shown in *Fig. 5*.

Laboratory (Serological) Findings

The results of the ELISA test carried out on the serum samples are presented in *Table 4*.

Discussion

In clinical and experimental studies, it has been revealed that LLLT increases vascularization in damaged tissue with its photobiological and photochemical effects, accelerates reepithelization, and accelerates tissue healing by increasing fibroblast activity and collagen synthesis [14,15,17,19]. Clinically, it is known that LLLT have beneficial effects on pain management and bone formation [14,29]. In this study, it was tried to determine whether there was a difference in the effects of LLLT on cancellous and cortical bone. By direct histopathological examination of the bone tissue, cells can be determined quantitatively and functionally, structural changes in the cortical and cancellous bone can be analyzed, and changes in the fracture healing cycle, bone diseases, and bone development cycle can be determined most accurately [30,31]. Since the research was an experimental study, changes in the bone healing process, such as the inflammatory phase, cellular proliferation

Table 1. *Statistical data (Mean±SD) of radiological relative optical density (ROD) analysis*

Bone Tissue	Groups	0. Day	7. Day	14. Day	21. Day	28. Day
Cancellous	C	0.80 ± 0.01^{D}	0.84 ± 0.02^{C}	0.87 ± 0.03^{B}	0.89 ± 0.03^{AB}	0.91 ± 0.03^{Abc}
	TCE	0.81 ± 0.01^{D}	0.85 ± 0.03^{C}	0.90 ± 0.03^{B}	0.92 ± 0.03^{AB}	0.95 ± 0.03^{Aa}
	LLLT	0.80 ± 0.02^{D}	0.85 ± 0.01^{C}	0.88 ± 0.02^{B}	0.90 ± 0.02^{B}	0.94 ± 0.02^{Aab}
Cortical	C	0.77 ± 0.01^{C}	0.79 ± 0.03^{C}	0.83 ± 0.02^{B}	0.86 ± 0.02^{A}	0.88 ± 0.02^{A}
	TCE	0.78 ± 0.01^{D}	0.82 ± 0.02^{C}	0.86 ± 0.03^{B}	0.88 ± 0.03^{B}	0.91 ± 0.02^{A}
	LLLT	0.77 ± 0.02^{C}	0.79 ± 0.02^{C}	0.83 ± 0.03^{B}	0.87 ± 0.02^{A}	0.89 ± 0.03^{A}

Superscripts in the same line (A, B, C, D) and in the same column (a, b, c) are statistically significant (P<0.05).
C: *Control,* ***TCE:*** *Tarantula cubensis extract,* ***LLLT:*** *Low-level laser therapy*

Table 2. *Scores of histopathological findings in the defect area in cortical and cancellous bone according to the follow-up periods (Means ± SD)*

Histopathological Scores	Follow-up Periods	Cortical Bone			Cancellous Bone		
		Control	LLLT	TCE	Control	LLLT	TCE
Inflammation	7	2.80 ± 0.447^{a}	2.40 ± 0.547^{a}	2.60 ± 0.547^{a}	2.90 ± 0.457^{a}	2.50 ± 0.547^{a}	2.60 ± 0.547^{a}
	14	1.60 ± 0.547^{bA}	0.60 ± 0547^{cB}	0.40 ± 0.547^{cB}	1.50 ± 0.547^{bA}	0.40 ± 0547^{bB}	0.20 ± 0.547^{bB}
	21	0.20 ± 0.447^{cA}	0.0 ± 0.0^{dB}	0.0 ± 0.0^{dB}	0.20 ± 0.447^{cA}	0.0 ± 0.0^{cB}	0.0 ± 0.0^{cB}
	28	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
Formation and quality of bone tissue	7	0.50 ± 0.20^{aA}	1.60 ± 0.547^{aB}	1.80 ± 0.836^{aB}	0.70 ± 0.30^{aA}	1.70 ± 0.447^{aB}	1.90 ± 0.836^{aB}
	14	1.40 ± 0547^{bA}	2.80 ± 0.447^{bB}	2.60 ± 0.547^{aB}	1.60 ± 0547^{aA}	2.90 ± 0.447^{bB}	2.70 ± 0.447^{aB}
	21	3.20 ± 0.837^{c}	3.80 ± 0.447^{c}	3.60 ± 0.547^{b}	3.50 ± 0.547^{b}	3.90 ± 0.447^{c}	3.70 ± 0.447^{b}
	28	3.80 ± 0.447^{cd}	4.0 ± 0.0^{cd}	4.0 ± 0.0^{bc}	3.80 ± 0.447^{b}	4.0 ± 0.0^{c}	4.0 ± 0.0^{b}
Collagen maturation	7	1.40 ± 0.547^{a}	1.40 ± 0.547^{a}	1.80 ± 0836^{a}	1.40 ± 0.547^{a}	1.60 ± 0.547^{a}	1.80 ± 0836^{a}
	14	1.80 ± 0.447^{a}	2.40 ± 0.547^{b}	2.40 ± 0547^{a}	2.40 ± 0.547^{b}	2.60 ± 0.547^{b}	2.50 ± 0547^{a}
	21	2.80 ± 0.447^{bA}	3.60 ± 0.547^{cAB}	3.80 ± 0.447^{bBC}	2.80 ± 0.447^{bA}	3.70 ± 0.447^{cAB}	3.80 ± 0.447^{bBC}
	28	3.80 ± 0.447^{c}	4.0 ± 0.0^{cd}	4.0 ± 0.0^{bc}	3.60 ± 0.447^{b}	4.0 ± 0.0^{c}	4.0 ± 0.0^{b}

Different superscripts (a, b, c, d) in the same column indicate significant differences among treatment days (P<0.05). Different superscripts (A, B, C) in the same line indicate significant differences among treatment groups in cortical and cancellous bone (P<0.05)

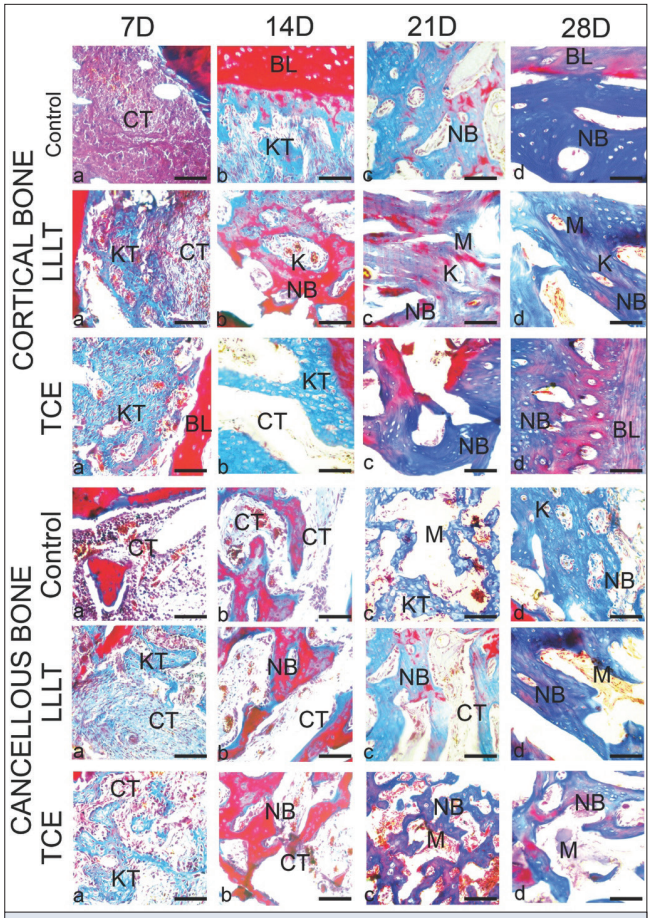

Fig 3. Histological sections of cortical and cancellous bone according to applications days after the bone defect is created**.** Cortical and Cancellous bone: repair process in bone tissue at 7 (a), 14 (b), 21 (c) and 28 (d) days in the control, LLLT and TCE groups. TCE groups; new bone tissue (NB) formed at the defect region, and observed cancellous bone matrix formation (M) and blood vessels (K) in the cortical bone. In the cancellous bone, on days 21 and 28, the defect is completely filled with new bone tissue (NB), the presence of bone marrow cells (M), and numerous capillaries in the TCE groups. CT; connective tissues, KT; cartilage tissue, BL; intact bone line, NB; new bone tissue, FT; fibrotic tissue, M; bone matrix formation and bone marrow cells, K; blood vessels. Crossman's triple stain, scale bars: 25 µm

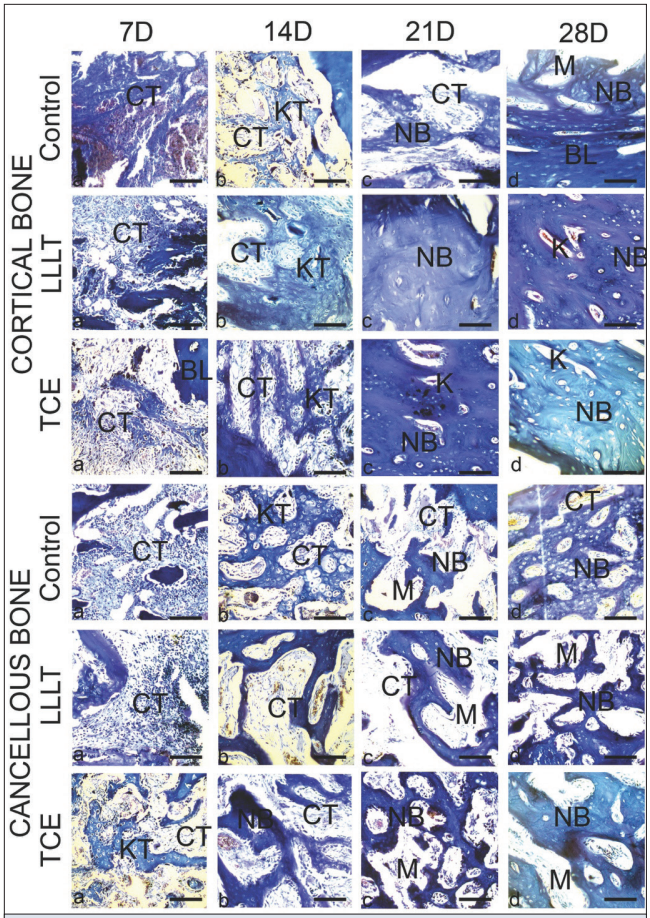

Fig 4. Histological sections of cortical and cancellous bone according to applications days after the bone defect is created. In both cortical and cancellous bones, the appearance of mineralized bone (M-light blue), calcification front (dark blue), and osteoids (K-light red) in the bone defect region during the repair on days 7, 14, 21, and 28 in the control, LLLT, and TCE groups. CT; connective tissue, KT; cartilage tissue, BL; bone line, K; osteoids, M: bone matrix, NB; new bone-mineralized bone. Solochrome cyanine R staining, Scale bars: 25 µm

or fibroplasia phase and bone remodeling phase, could be determined more easily by histopathological examination. It was noticed that compared to the control group, the LLLT group was found to be lower in terms of inflammatory cell density and to be higher bone tissue formation and collagen density as expected. In the study, it was also investigated whether TCE, a homeopathic agent whose effectiveness has been reported in many tissues other than bone, is effective and whether it can be an alternative to LLLT, which has equipment costs and difficulties in application. In the histopathological examination, bone tissue formation and quality 7th day and collagen maturation were significantly better on the 21st day in the TCE group compared to the control group. In the LLLT group, both cortical bone and cancellous bone showed histopathologically similar collagen density and bone tissue formation. These similarities observed in the study showed that TCE is at least as effective in bone tissue repair as LLLT.

Histopathologically, the increase in cell proliferation, osteoblastic activity, vascularization, new bone formation, and collagen deposition in bone tissue could accept as an indicator that TCE enables the amelioration of necrotic structures, a decrease of inflammatory cells and thus the healing of bone by accelerating osteogenesis [7-13]. This is also an indicator that LLLT has a photobiomodulatory effect on bone tissue [15,19,21]. In this study, the fact that bone tissue formation and quality were more prominent in the TCE and LLLT group on the 7th and 14th day than in the control group, and that collagen maturation was higher than in the control group on the 21st day. However, the great similarities between the TCE group and LLLT at all observation times indicate that the homeopathic regenerative effect of TCE is significant on bone tissue as

Table 3. *Intensity scores of osteopontin, bone morphogenetic protein-1, -2, -3, vascular endothelial growth factors, and its receptors (flt1/fms and flk1/KDR) expression in the cortical and cancellous bone defect regions during the applications*

Groups	Days	Cortical Bone							Cancellous Bone						
		OP	BMP-1	BMP-2	BMP-3	VEGF	Flt1/fms	Flk1/KDR	OP	BMP-1	BMP-2	BMP-3	VEGF	Flt1/fms	Flk1/KDR
Control	7	++	+	+	+	+++	+++	+++	+	+	+	+	+++	+++	+++
	14	++	+	++	+	++	++	++	++	+	++	+	++	++	++
	21	+++	++	+	++	+	+	+	+++	++	+	++	+	+	+
	28	+++	++	++	++	+	+	+	+++	++	++	++	+	+	+
LLLT	7	+	+	+	+	+++	+++	+++	+	+	+	+	+++	+++	+++
	14	++	+	+	+	++	++	++	++	+	+	+	++	++	++
	21	+++	++	+	++	+	+	+	+++	++	+	++	+	+	+
	28	+++	++	++	++	+	+	+	+++	++	++	++	+	+	+
TCE	7	+	+	+	+	+++	+++	+++	+	+	+	+	+++	+++	+++
	14	++	+	+	+	++	++	++	++	+	+	+	++	++	++
	21	+++	++	++	++	+	+	+	+++	++	++	++	+	+	+
	28	+++	++	++	++	+	+	+	+++	++	++	++	+	+	+

OP: *osteopontin,* ***BMP-1:*** *bone morphogenetic protein-1,* ***BMP-2:*** *bone morphogenetic protein-2,* ***BMP-3:*** *bone morphogenetic protein-3,* ***VEGF:*** *vascular endothelial growth factors,* ***flt1/fms:*** *fms related receptor tyrosine kinase 1,* ***flk1/KDR:*** *Tyrosine Kinase Growth Factor Receptor*

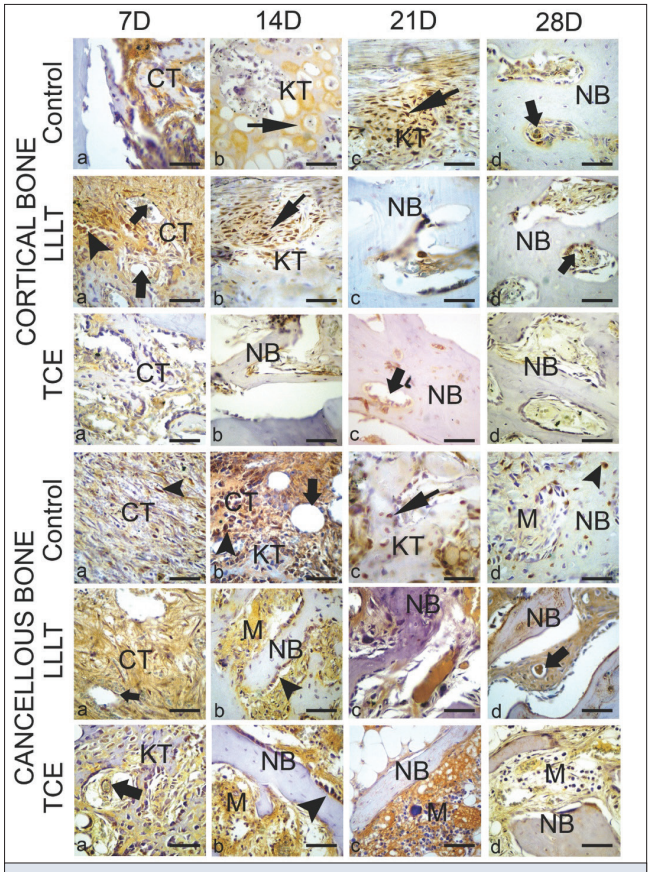

Fig 5. Immunohistochemical localization of vascular endothelial growth factor (VEGF) in the defect area and newly formed bone tissue according to the control, LLLT, and TCE groups in the cortical and cancellous bones. Expression of VEGF in connective tissue cells in the defect area and cartilage cells, osteoblasts, and osteocytes in newly formed bone tissue at 7, 14, 21, and 28 days of application. CT; connective tissue, KT; cartilage tissue, BL; bone line, M: bone marrow, NB; new bone-mineralized bone, K; blood vessel. Arrowhead; VEGF positive connective tissue cell, thin arrow; VEGF positive cartilage cell (chondrocytes or Chondroblast), thick arrow; VEGF positive osteocytes or osteoblast. Scale bars: 25 μm

well as in other tissues (tendons, skin, etc.). In addition, the fact that the inflammatory phase was less in the TCE and LLLT groups compared to the control group and that no inflammatory cells were observed on the 21st day may be evidence that the healing process starts more quickly with the anti-inflammatory effects of these applications.

Histopathological examination of bone tissue is important in terms of quantitative and functional determination of cells, analysis of structural changes in cortical and cancellous bone, and determining the borders of the bone healing cycle [11]. In clinical practice, histopathological examination by taking a biopsy sample from the fracture site or applying expensive high-resolution imaging techniques is not a very simple and convenient option in routine. Therefore, the clinician should be able to make this assessment with easier and more economical measures to determine the healing status. For this purpose, one of the most preferred methods to evaluate the fracture healing process is conventional radiography. Despite its advantages these radiographic features are not sufficient in the evaluation of clinical improvement (esp. mechanical strength), and quantitative evaluations are needed. On the other hand, it is known that there is a strong correlation between optical densitometric analysis and clinical evaluation in bone healing assessments performed on digital radiography [25,26]. For this reason, optical density analyzes have encouraged researchers to develop web-based methods, especially since they offer a quantitative evaluation opportunity and are non-invasive. Web-based assessment tools are a method that can generally be worked asynchronously, based on user-user interaction, offering various solutions for clinicians with different specialties, including orthopedic surgery. In the field of orthopedics,

Table 4. *Statistical data of proinflammatory cytokines and bone-specific markers measured by ELISA carried out on the serum samples*

Parameters	Groups	0. Day	7. Day	14. Day	21. Day	28. Day
TNF-α (pg/mL)	C	0.19 ± 0.06^{c}	0.43 ± 0.13^{Aa}	0.38 ± 0.17^{ab}	0.26 ± 0.12^{Bbc}	0.43 ± 0.12^{ABa}
	TCE	0.19 ± 0.06	0.23 ± 0.14^{B}	0.26 ± 0.13	0.22 ± 0.06^{B}	0.30 ± 0.08^{B}
	LLLT	0.19 ± 0.06^{b}	0.28 ± 0.14^{ABb}	0.34 ± 0.15^{ab}	0.52 ± 0.19^{Aa}	0.53 ± 0.15^{Aa}
IL-1β (pg/mL)	C	0.10 ± 0.04	0.23 ± 0.27	0.10 ± 0.02	0.16 ± 0.07	0.24 ± 0.10^{A}
	TCE	0.10 ± 0.04	0.07 ± 0.02	0.10 ± 0.05	0.11 ± 0.04	0.12 ± 0.04^{B}
	LLLT	0.10 ± 0.04^{b}	0.19 ± 0.07^{a}	0.12 ± 0.06^{b}	0.10 ± 0.03^{b}	0.09 ± 0.04^{Bb}
IL-6 (pg/mL)	C	3.08 ± 0.69^{b}	4.02 ± 1.0^{Aab}	5.23 ± 1.90^{a}	5.23 ± 2.10^{Aa}	5.82 ± 1.29^{a}
	TCE	3.08 ± 0.69^{b}	2.65 ± 0.5^{Bb}	3.75 ± 1.17^{b}	3.22 ± 0.67^{Bb}	5.87 ± 2.31^{a}
	LLLT	3.08 ± 0.69^{b}	4.10 ± 1.57^{Ab}	4.40 ± 1.20^{b}	4.12 ± 0.92^{ABb}	6.12 ± 1.46^{a}
OC (pg/mL)	C	0.38 ± 0.12	0.39 ± 0.16	0.52 ± 0.18	0.47 ± 0.13^{A}	0.38 ± 0.22
	TCE	0.38 ± 0.12	0.35 ± 0.11	0.30 ± 0.11	0.34 ± 0.06^{AB}	0.27 ± 0.05
	LLLT	0.38 ± 0.12	0.37 ± 0.15	0.33 ± 0.21	0.27 ± 0.08^{B}	0.28 ± 0.14
B-ALP (ng/mL)	C	3.21 ± 0.08^{a}	2.32 ± 0.72^{b}	2.31 ± 0.37^{Bb}	2.24 ± 0.35^{Cb}	2.37 ± 0.40^{b}
	TCE	3.21 ± 0.08^{a}	2.23 ± 0.36^{b}	2.49 ± 0.55^{ABb}	2.71 ± 0.31^{Bab}	2.60 ± 0.26^{b}
	LLLT	3.21 ± 0.08^{ab}	2.83 ± 0.48^{bc}	3.12 ± 0.36^{Aab}	3.40 ± 0.08^{Aa}	2.66 ± 0.34^{c}
PINP (ng/mL)	C	0.68 ± 0.80	0.68 ± 0.12	0.67 ± 0.07	0.69 ± 0.10	0.69 ± 0.14^{B}
	TCE	0.68 ± 0.80	0.76 ± 0.11	0.81 ± 0.28	0.91 ± 0.27	0.91 ± 0.23^{A}
	LLLT	0.68 ± 0.80^{ab}	0.68 ± 0.12^{ab}	0.77 ± 0.13^{a}	0.78 ± 0.20^{a}	0.55 ± 0.04^{Bb}

Tumor necrosis factor-alfa (TNF-α), Osteocalcin (OC), Interleukin 1-Beta (IL-1β), Interleukin-6 (IL-6), Procollagen I N-terminal peptide (PINP). The superscripts in the same line (a, b, c) and same column (A, B, C) are statistically significant (P<0.05)

telemedicine-style techniques are mostly performed on radiological images [25,26,31]. In contrast, Werkman et al.[5] performed optical density analysis with a free computer program called Image Tool (UTHSCSA Image Tool version 3.0) to evaluate the repair in the bone defect model they created in rats with osteoporosis, and they argued that the optical density analysis they performed did not take into account the differences between trabecular and lamellar structures in newly formed bone and did not fully explain the morphological course of the bone callus, since it added fibrous tissue to the analyzes, and therefore it was not significant in the evaluation of bone repair. Through a specially developed web-based program, it was determined the optical density in this study. In the study, the best group according to the results of ROD was the group in which TCE was applied, followed by the group in which LLLT was applied, and the group with the lowest ROD value was the control group. It was also observed that the ROD value in the cancellous bone was higher than in the cortical bone in all groups and on all follow-up periods. This elevation in optical density is generally thought to be related to the degree of mineralization of the callus after the reparative phase. Because bones hold 30-40 times more radiation than soft tissue. Since the mineralization level of a callus is determined by densitometry of x-ray images [32], it shows that an osseous callus is formed due to better mineralization in the TCE group. In addition, since these results show similarity with histopathological evaluations, contrary to Werkman et al.[5], optical density was thought to be useful in the evaluation of bone repair.

Clinically, fractures of the cancellous bone repair much more rapidly and have a different healing process compared to diaphyseal fractures in the cortical bone structure. While the periosteum is very important for the healing process in cortical bones, the trabeculae structures are important in cancellous bones. Compared to cortical bone fractures, cancellous ones have a larger internal contact surface area and greater blood supply capacity, allowing adequate fixation. In addition, the cancellous bone tissue is largely in contact with the bone marrow cells. Therefore, cancellous bone fractures have a tighter affinity with multiple sources of mesenchymal stem cells that can differentiate into the osteoclast-osteoblast cell line to promote cancellous bone healing [33,34]. Han et al.[33] reported in their study that less bone tissue necrosis, less hematoma formation, a more limited inflammation event, and no external callus were observed in the cancellous bone healing phase compared to cortical fractures. Moreover, unlike cortical bone healing, the healing cycle consists of five phases: hematoma or inflammatory phase, cell proliferation phase, secondary

bone formation phase, lamellar bone formation phase, and bone remodeling phase [33]. In the present study, it was not possible to make this histopathological distinction with precise limits since the evaluation was performed on only 4 different observation days (7, 14, 21, and 28 day). However, according to the histopathological evaluation data, the healing of the cancellous bone was better than the cortical bone, although it was not statistically significant. On the other hand, in terms of ROD values performed on radiological images, the mineralization level in the cancellous bone was found to be statistically better than in the cortical bone.

During the inflammatory phase of the fracture healing process, cellular debris and the fracture hematoma are bounded by a fibrous capsule consisting of irregular granulation tissue that is dense with type 3 collagen. At this early stage of bone healing, a small amount of type I collagen may be found in the callus in isolated areas close to the cortical bone. Collagen tissues are also found in high amounts in the primary callus and have very important functions, especially in the reparation phase of healing. Collagen types I, III, and V are more intense in the reparation phase, and collagen type I in the remodeling phase. Therefore, the presence of collagen tissue in tissue damage is very important for wound healing and healing quality [31,35,36]. In the present study, collagen tissue densities were evaluated both histopathologically and serologically in terms of PINP. Histopathologically, only their density in the repair phase was evaluated without making any distinction of collagen type. Accordingly, the collagen tissue density in both the cancellous and cortical bone defect areas in the TCE-treated group was found to be higher, although not significant, on the 7th day, but significantly better on the 21st day compared to the control group. Considering the PINP level, the TCE group showed an increase insignificant level of PINP on the 7, 14 and 21st days compared to the other two groups, but showed an increase significant level of PINP on the 28th day, which was consistent with the histopathological evaluation. Similar to its effects on tissues [11,12], it can be said that TCE accelerates repair in bone tissue. Moreover, the fact that collagen type I is more intense in the remodeling phase of the bone tissue [10,35] may be an indication of the completion of the healing phase in the TCE group.

Many inflammatory mediators, such as interleukins and TNF-α, are significantly elevated after fracture for several days. These pro-inflammatory mediators have a chemotactic effect on other inflammatory cells. After this process, angiogenesis occurs for fibrin deposition and a greater supply of nutrients. Vascular proliferation within the developing callus tissue is regulated by angiogenic factors such as FGF, VEGF, and angiopoietins. In the present study, the expression levels of VEGF and its receptors (Flt1/fms, Flk1/KDR), which are responsible for vascular proliferation, were examined immunohistochemically. Because the expression of VEGF is very important for endochondral bone healing [3,37,38]. As expected, the expression of VEGF and its receptors was observed at a higher level on the 7th day in both the cancellous and cortical bone defect areas. In the later phases, since these cells need to be expressed to provide the blood reserve required for repair activities [3,38], the expression continued, albeit at a low level. However, expression levels of VEGF and its receptors were similar between the groups at all follow-up periods (7, 14, 21, and 28 days), and no difference was found in terms of scoring. In the present study, VEGF and its receptors showed similar localizations and expressions in the defect areas shaped in new bone tissue, and the lack of relative differences in expression densities suggested that VEGF and its receptors together perform similar functions in the bone defect region in rabbits as mentioned above. However, further experimental studies are needed to reveal the precise physiological roles of VEGF and its receptors in bone tissue remodeling in rabbits.

Although activation of the fracture repair process appears to depend on an adequate pro-inflammatory response, the resolution of this inflammatory condition is critical for continued healing [39]. Pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, which are molecular components that initiate the fracture healing process, first increase significantly for a few days after the fracture, and their levels decrease rapidly within 3 days. The fact that these cytokines are significantly high in the inflammatory phase is very important in terms of activating other inflammatory cells for the initiation of angiogenesis with fibrin deposition at the fracture line. In addition, they accelerate the healing and callus consolidation by being expressed again in the later stages of fracture healing, namely in the remodeling phase [3,39,40]. A situation that can be said to be a deficiency in this study is the lack of early postoperative period data. However, since the treatment started on the 3rd day at the earliest in our study, it is thought that the analysis of pro-inflammatory cytokines in this process would not make a difference between the groups. Therefore, it is not possible to comment on the approach of these cytokines to normal levels in the first 3 days. However, on the 7th day, TNF- α, one of these three pro-inflammatory cytokines, was found to be significantly higher in the control group compared to normal values (pre-op), whereas the other two cytokines were not. On the other hand, although the inflammatory cell density in the control group was not statistically significant on the 7th day, histopathologically, the fact that it was higher than in the other groups and the serologically higher TNF-α level could be evidence of the control group's continued

inflammation. During the remodeling phase of fracture healing, late cytokine expression accelerates healing and callus consolidation [39,40]. Therefore, it can be interpreted that repair of the bone is better in the LLLT group.

Since serum bone markers are less invasive and more economical than biopsies, they can be used to evaluate bone metabolism and diseases. These markers reveal bone formation, matrix degradation, and enzymatic activity of bone cells. Commercial serum markers are currently available for determining bone formation activity such as bone-specific alkaline phosphatase (B-ALP), procollagen type-I N-terminal propeptide (PINP), and osteocalcin (OC) [31,41]. Bone alkaline phosphatase (B-ALP), which are thought to play a role in bone formation and/or mineralization, is an osteoblastic ectoenzyme. Change in these enzyme levels, may be an indicator of collagen formation and bone formation in bones and osteoblast cells [15,41,42]. It was stated that B-ALP levels were higher in groups with better ossification with LLLT in bone injuries. In addition, it has been revealed that LLLT application modulates the inflammatory process and changes the level of osteoblasts and osteoclasts with alkaline phosphatase activity [15,39]. In contrast, Nissan et al.[29] suggested that LLLT on the bone defect created in the rat mandible was not different from the control group in terms of serum B-ALP levels at 1, 2, and 4 weeks. In the present study, serum B-ALP levels were observed to be significantly lower on days 7, 14, 21, and 28 compared to normal values (pre-op), except for days 14 and 21 in the LLLT group. It has been suggested that the observed early decrease in serum B-ALP activity may be related to the suppression of bone formation and/or mineralization during the bone remodeling phase in the defect area [41]. However, this situation does not show compatibility with the histopathological and radiological findings determined in the study. Therefore, it can be said that there was no significant change in terms of bone repair in this study, contrary to what is known, except for the group in which LLLT was applied serologically at the B-ALP level.

In conclusion, TCE may be a good alternative to LLLT in terms of accelerating bone repair. Thus, it could be an effective, easily applicable and relatively economical supportive treatment option in bone defects. In addition, since TCE accelerates the cancellous bone a little more than cortical bones, it could be said that the rehabilitation period may be shorter for cancellous bone defects. However, further studies are needed on the specific efficacy of TCE on VEGF, its receptors and BMPs.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author (S. Altan). The data are not publicly available due to privacy or ethical restrictions.

Conflict Interests Statement

The authors declare that they have no conflicting interests.

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None

Author Contribution

SA and HS conceived and supervised this study. SA, BEK, FA and RÇ completed the main experimental content. SA, HS, EA, BEK, and FA collected and analyzed data. SA wrote the first draft of the manuscript. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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