Experimental Repair of Rabbit Segmental Bone Defects by Using Autologous Bone Marrow and Electrical Stimulation

Otolog Kemik İliği ve Elektriksel Uyarı ile Tavşan Segmental Kemik Defektlerinin Deneysel Onarımı

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ABSTRACT Objective: In this study, we have aimed to investigate the potential role of autologous bone marrow cell injection and muscular electrical stimulation as a separate and concomitant application on bone healing in experimental rabbit ulnar segmental bone defect model. **Material and Methods:** Forty New-Zealand rabbits, all over three months of age and weighing between 2500 and 3500 grams were divided into four groups. Four groups of rabbits were the control group (I), electrical stimulation group (II), bone marrow cells injection group (III) and bone marrow cells injection with electrical stimulation group (IV). Bone defect healing was evaluated radiologically according to the modified Lane and Sandhu scoring system and at the end of the sixth week, rabbits were sacrificed and their forearms were sampled for histopathological investigation. **Results:** When one-to-one comparison between all groups was performed, defect healing was found to be better in Groups II, III, and IV compared to Group I based on the radiological and histopathological parameters evaluated. This evaluation revealed that the healing was better in groups treated with bone marrow cell injection with or without electrical stimulation as well as in group treated with electrical stimulation compared to the control group with no treatment. **Conclusion:** Autologous bone marrow cells with or without electrical stimulation, would be used in healing of segmental bone defect with an adequate efficacy. Future stem cell studies combined with electrical current are required to demonstrate and to confirm that electrical current enhances in vivo cellular differentiation.

Key Words: Fractures, bone; bone marrow cells; electric stimulation

ÖZET Amaç: Bu çalışmada otolog kemik iliği hücrelerinin enjeksiyonu ve kas elektriksel uyarımının ayrı ayrı ve birlikte uygulanmasının tavşan modeli üzerinde segmental ulnar defektin iyileşmesine olan potansiyel rolünü araştırmayı amaçladık. **Gereç ve Yöntemler:** Üç aylıktan büyük ve 2500-3500 gram ağırlığında olan 40 Yeni Zellanda tavşanı dört gruba ayrıldı. Bu dört grup (I) kontrol, (II) elektriksel uyarımın yapıldığı grup, (III) kemik iliği hücreleri enjekte edilen grup ve (IV) elektriksel uyarı ve kemik iliği birlikte uygulanan gruptan oluşturuldu. Kırık iyileşimi modifiye Lane ve Sandhu skorlama sistemi kullanılarak radyolojik açıdan ve altı hafta sonunda sakrifiye edilen tavşanların ön kol örneklerinin histopatolojik incelemesi sonucu değerlendirildi. **Bulgular:** Bütün gruplar radyolojik ve histolojik parametrelere göre karşılaştırıldığında, defekt iyileşimi Grup II, III ve IV de grup I'e oranla daha iyi bulundu. Bu değerlendirme, kemik iliği enjeksiyonu yapılan grubun elektriksel uyarım yapılan veya yapılmayan grup ile tedavi verilmeyen gruba oranla daha iyi sonuç verdiğini gösterdi. **Sonuç:** Otolog kemik iliği, elektriksel uyarım olsun ya da olmasın kemik defektlerinin iyileşiminde etkin şekilde kullanılabilir. Elektriksel uyarının kemik iliği hücreleriyle etkinliğinin gösterilmesi ve onaylanması için yeni kök hücre çalışmaları yol gösterici olacaktır.

Anahtar Kelimeler: Kemik kırıkları; kemik iliği hücreleri; elektriksel uyarı

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Bone defects generally emerge from oncological surgery, following revision arthroplasty, or trauma and are orthopedic problems that are difficult to treat. When it has been proven that bone marrow shows a

capacitance characteristic of bone and used as a percutaneous graft, it is considered that it may also be used for healing of defects. The osteogenic potential of bone marrow is also demonstrated in *in-vitro* conditions which increase the interest in studies on this subject.[1,2] Since autologous bone marrow involves a structure with primitive osteogenic cells rich for cytokines, some investigators support the use of bone marrow with autologous and allogeneic grafts. Connolly et al. showed that bone marrow alone may recover, and they provided recovery in 18 of 20 tibial non-unions with bone marrow injection following intra-medullary stabilization.[3] Recently, autologous bone marrow injection was shown to accelerate healing in long bone non-unions.[3-5]

There are many reports regarding the effect of different types of electrical current on acceleration of fractures and defect recovery; however, the positive effects of neuromuscular electrical stimulation on fractures and defect healing, preferred in the present study and used mostly in physiotherapy clinics.[6] Burr et al. showed that neuromuscular electrical stimulation in extremities immobilized using plaster casts increased bone turnover after 17 days.[7] Bloomfield et al. showed that functional electrical current therapies caused an increase in bone mass.[8] Although it is not known how neuromuscular stimulation by electrical current affects fracture healing, it is thought that it provides healing by increasing the peripheral blood circulation, bone turnover, and exerting an effect on the mesenchymal stem cells by creating a magnetic field.[6,9-12]

An agent that can be considered to increase defect and fracture healing should be inert, dissolve and dissociate in the body and therefore, it should be osteo-inductive and osteo-conductive. In the present study, it has been investigated whether the weak osteo-conductive effect of bio-inert bone marrow may be increased or not by neuromuscular electrical current in bone defects experimentally created in rabbits' ulnas, and their clinical effects have been observed.

MATERIAL AND METHODS

Forty New-Zealand rabbits, all over three months of age and weighing between 2500 and 3500 grams were divided into four groups: Group I: only the defect-induced group, Group II: Group received electrical current after defect induction, Group III: Group injected bone marrow after defect induction, and Group IV: Group received electrical current and bone marrow after defect induction. All experiments were performed in compliance with the relevant scientific local laws and institutional guidelines after obtaining permission from local animal ethics committee, and all procedures were performed according to guide for the Care and Use of Laboratory Animals principles.

Following local sterilization with povidone iodine solution on left upper extremities of rabbits, a 0.8-1 cm defect area from the diaphysis of one third middle ulna was extracted with its periosteum. The surgical field was washed with isotonic saline solution (%0.9 NaCl) and bone debris was washed away. The skins of all four groups were sutured without any other interventions.

The first group of rabbits were left to normal activities without application of any procedures. Rabbits in the second group were connected to electrodes beginning on the fourth day of surgery, leaving the extremity surgery-free, one electrode 1 cm distal to the elbow joint on the anterior part, and the other one 1 cm proximal to the waist joint posteriorly. An Electroform Medical 12 instrument with 25 mA amplitude, 50 μm wave length and 40Hz frequency was used to deliver biphasic electrical current for an hour per day. All rabbits in these groups received sedatives during exposure to the electrical current. Rabbits were left to normal daily cage activities on the other days.

On the fourth day after surgery, bone marrows of the rabbits in the third group were aspirated with heparin-washed injectors 1.5-2 ml from the right distal femur. One drop a time was taken on the slide and was confirmed to contain bone marrow macroscopically and was looking for under the microscope before a percutaneous injection of 1 ml aspirate.

The fourth group received bone marrow and received electrical current as described previously. The rabbits were observed for six weeks continuing

their daily cage activities. At the end of the sixth week, rabbits, seven in each group, were sacrificed and their forearms were sampled for histopathological investigation.

RADIOGRAPHIC EVALUATION

Radiographic evaluation of ulnar defect was performed according to the *modified Lane and Sandhu scorring system* (Table 1).[13] According to the established scoring system, third and sixth week radiographs were evaluated based on 10 points. All the X-rays were taken by using the same visualization system by the same technician. Radiographic analyses were performed by an independent radiologist blinded, without any information regarding the procedures and groups.

HISTOLOGICAL EVALUATION

Tissue Processing, Histology, and Histomorphometry

The ulnar bones of rabbits were cut 5 mm distal to defect area. Following the fixation and decalcification procedure, bone samples were dehydrated in high degree alcohol, washed with xylene and embedded in paraffin before being cut with a microtome RM 2145 (Leica GmbH, Wetzlar). Longitudinal sections of 3 μm thickness from each ulna were obtained and spread on glass slides covered with poly-L-Lysin and the sections were dyed with hematoxylene eosin. The cancellous bone of ulna was examined under the light microscope for bone histomorphometry (Olympus BX-51 light microscope and Olympus C-5050 digital camera, Olympus Europa GmbH, Germany). The results were compared with the Image-Pro Express analysis software (Media-Cybernetics, 2002, USA). Nomenclature and symbols used in conventional bone histomorphometry were the same as those used by Parfitt et al.[14] Histomorphological parameters were evaluated as follows;

- Total tissue area (Tt. Ar, μm^2),
- Total trabecular area (Tb. Ar, μm^2),
- Trabecular perimeter (Tb.Pm, lμm) was measured,
- Cancellous bone surface (BS= Tb.Pm, μm),
- Cancellous bone volume (BV/TV= Tb.Ar/Tt.Ar, %),
- Trabecular thickness [Tb.Th= (BV/TV)/0.5 BS, μm)],
- Trabecule number [Tb.N= (BV/TV)/Tb.Th, mm^{-1}],
- Trabecular space [Tb.Sp= (1/TbN)-Tb. Th. μm] was calculated.

STATISTICAL ANALYSES

Statistical analysis of radiological parameters was performed using SPSS Software release 13.0 for

TABLE 1: Modified Lane ve Sandhu scorring system.

Bone formation	
No evidence of bone formation	0
Bone formation occupying 25% of defect	1
Bone formation occupying 50% of defect	2
Bone formation occupying 75% of defect	3
Full gap bone formation	4
Union	
Nonunion	0
Possible union	1
Radiographic union	2
Remodelling	
No evidence of remodelling	0
Remodelling of medullary canal	1
Full remodelling of cortex	2

Total point possible per category	
Bone formation	4
Proximal union	2
Distal union	2
Remodelling	2
Maximum score	**10**

Windows (Apache Sofware Foundation, Chicago, USA). Wilcoxon Test, Kruskal–Wallis Test,Mann–Whitney U Test, One-way ANOVA Bonferroni's Multiple Comparison Tests were performed in the evaluation for radiographical analysis. For histopathological evaluation, the collected data was used for intra- and inter-group comparisons by using the One-way ANOVA with Bonferroni multiple comparison test and the statistical analysis was performed using the GraphPad Instat 3.05 software (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

When one-to-one comparison between Groups I, II, III, and IV was made, defect healing was found to be better in Groups II, III, and IV compared to Group I based on the evaluated radiological and histopathological parameters ($p< 0.05$).

EVALUATION OF RADIOLOGICAL CHANGES AND THE COMPARISON OF THE GROUPS ACCORDING TO RADIOLOGICAL EVALUATION

Radiological statistical analysis was performed with the SPSS Software release 13.0 for Windows (Apache Sofware Foundation, Chicago, USA) Firstly, in-group analysis of bone graphs were performed and evaluated. Radiological analysis of Group I without any treatment revealed that there was no improvement in the bone healing based on either each case or the sum of the whole group (Table 2a, Figure 1a). Groups II, III and IV were also evaluated with respect to radiological status and significant improvement in bone healing was documented for either each cases in groups or for whole groups in in-group analyses (Table 2b,c,d; Figure 1b,c,d). No significant difference was observed in Group I between the third and sixth week values for defect healing ($p> 0.05$). There was a significant difference in Groups II, III, and IV between the third and sixth week values for defect healing ($p< 0.05$) (Group II $p= 0.024$, Group III $p= 0.026$, Group IV $p= 0.026$). When all groups were evaluated together, there was no difference between groups according to third week defect healing ($H= 5.065$, $p= 0.167$), on the other hand there was a significant difference between groups according to sixth week defect healing ($H= 12.675$, $p= 0.005$).

Paired comparison of the groups with respect to radiology was summarized as follows;

There were a significant difference between Group I and II ($p= 0.026$, $p< 0.05$), Group I and III ($p= 0.017$, $p< 0.05$), Group I and IV ($p= 0.001$, $p< 0.05$). However there were no differences between Group II , III, and IV ($p> 0.05$).

EVALUATION OF HISTOLOGICAL CHANGES AND THE COMPARISON OF THE GROUPS ACCORDING TO HISTOPATHOLOGICAL EVALUATION

The collected data was used for intra- and inter-group comparisons using the One-way ANOVA with Bonferroni multiple comparison test and the statistical analysis was performed using the GraphPad Instat 3.05 software (GraphPad Software Inc., San Diego, CA, USA).

Histopathological analysis of defect areas were performed at the 6th week of the experiment after sacrificing the rabbits. Samples were obtained and treated as described in material methods section. All samples from each group were analyzed and the means of groups was used for comparison. Cancellous bone volume ratios were found as 21.40%, 50.80%, 50.40% and 50; 20% in control group, electrical stimulation group, bone marrow injection group and electrical stimulation plus bone marrow injection group, respectively. Trabecular thicknesses were found as 84.25 mm, 128.80 mm, 127.3 mm and 130.8 mm in control group, electrical stimulation group, bone marrow injection group and electrical stimulation plus bone marrow injection group, respectively. Trabecule numbers in healing areas were found as 2.75, 5.25, 5.0, and

TABLE 2a: Group I: Radiological evaluation of the group I with only defect induction.

	Control 1	Control 2	Control 3	Control 4	Control 5	Control 6	Control 7
Third week	4	1	1	1	5	3	3
Sixth week	5	1	1	7	6	4	3

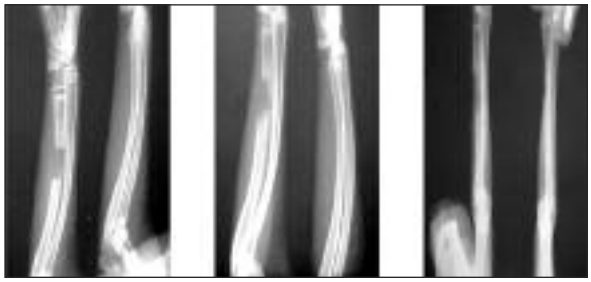

FIGURE 1a: Radiological controls of Group I on the surgery day, third and sixth week.

5.0 in control group, electrical stimulation group, bone marrow injection group and electrical stimulation plus bone marrow injection group, respectively. Trabecular spaces were measured as 71.50 mm, 122.0 mm, 122.5 mm and 125.3 mm in control group, electrical stimulation group, bone marrow injection group and electrical stimulation plus bone marrow injection group, respectively (Table 3).

When cancellous bone volume (BV/TV= Tb.Ar/Tt.Ar, %), trabecular thickness [Tb.Th= (BV/TV)/0.5 BS, μm)], trabecule number [Tb.N= (BV/TV)/Tb.Th, mm^{-1}] and trabecular space values were compared in all groups , the results were summarized as follows and illustrated in Figures 2a-d. There were significant differences between Groups I and II, I and III, I and IV ($p< 0.001$). On the other hand, there were no differences between Groups II, III and IV ($p> 0.05$).

DISCUSSION

Segmental bone defect is one of the orthopedic problems that constrain the clinician. Autografting or allografting, as the most commonly utilized treatment method recently, have created some problems. Therefore, there have been tremendous efforts to develop new methods. Allogeneic or autologous stem cells injection, muscular electrical stimulations are innovative examples of these interventions. Friedenstein et al. demonstrated that although hematopoietic cells die in in-vitro conditions in bone marrow aspirates, fibroblast precursors survive and continue to generate bone tissue which reflected the osteogenic potential of bone marrow.[1] Bone marrow aspirates may be easily inoculated to the defect area by using a 14-gauge Jam-Shidi needle. In this easily harvested and practically applied method, osteogenic activity of bone marrow could be maintained without destruction unlike bone grafts.[15] It has been shown that, in 1 ml of each 4 ml bone aspirates, there were adequate precursor cells with high osteogenic potential.[16] Their use in clinical treatment of non-unions began with Connolly et al. who treated 10 tibial fractures with non-union by bone marrow injections.[3]

TABLE 2b: Group II: Radiological evaluation of the group with only electrical current (EC) application after defect induction.

	EC1	EC2	EC3	EC4	EC5	EC6	EC7
Third week	3	5	3	4	6	5	7
Sixth week	5	7	5	7	7	7	7

TABLE 2c: Group III: Radiological evaluation of the group with only bone marrow (BM) injection after defect induction.

	BM 1	BM 2	BM 3	BM 4	BM 5	BM 6	BM 7
Third week	7	7	6	4	1	2	3
Sixth week	8	8	8	6	3	8	7

TABLE 2d: Group IV: Radiological evaluation of the group with both electrical current and bone marrow application after defect induction.

	EC+BM1	EC+BM2	EC+BM3	EC+BM4	EC+BM5	EC+BM6	EC+BM7
Third week	4	3	5	5	3	4	4
Sixth week	7	8	9	8	7	7	7

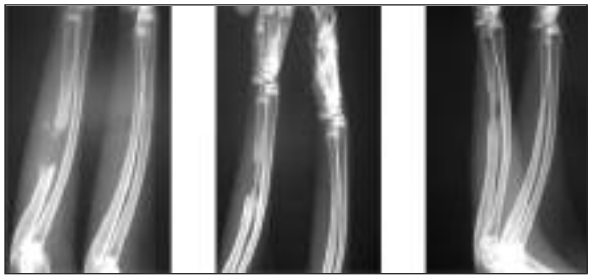

FIGURE 1b: Radiological controls of Group II on the surgery day, third and sixth week.

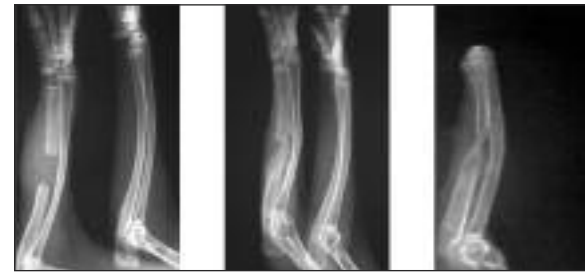

FIGURE 1c: Radiological controls of Group III on the surgery day, third and sixth week.

There are many studies demonstrating that bone marrow may also be used successfully in accelerating fracture healing besides non-union treatment. It is also believed that immediate application of bone marrow accelerates bone healing with osteogenic precursor cells and the growth factors it includes.[17-20] Besides, as it is easy to apply and has no complications, it is a successful alternative treatment method in non-union of long bones.

There is not much information about the application of bone marrow in combination with another method for segmental defects. In the present study, defects in the bone marrow-applied group improved faster and callus formation was of higher degree than in the control group. This is consistent with the literature. Bone marrow increased healing at a higher degree than the control group, even in large defects. In the literature review, there are applications such as cell implantation following bone marrow centrifugation. Another advantage of whole bone marrow transplantation in our study is that this method supplies additionally some stromal growth factors, increasing the osteogenic effect.

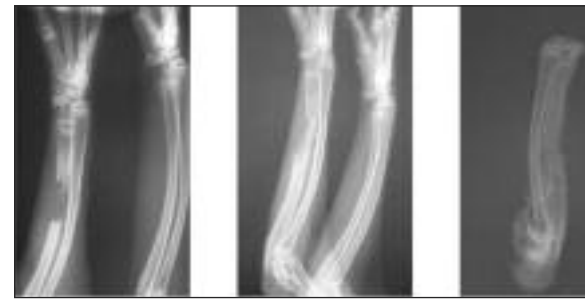

FIGURE 1d: Radiological controls of Group IV on the surgery day, third and sixth week.

The use of electrical current for fracture and defect healing began with Friedenberg and Brighton (1960), demonstrating that bioelectric potentials of cells in the bone depend on cellular chemical gradient and that they improved healing clinically.[21,22] In the literature review, we could not find any other reports on the effects of neuromuscular electrical stimulation in bone healing.

Neuromuscular electrical stimulation applied on experimental subjects is clinically used for decreasing edema, increasing muscle strength and protecting muscle tone in patients immobilized after injury or operation. Indirect and direct mechanisms

TABLE 3: Histomorphometry results for each group; mean and standard deviation values of cancellous bone surface (BS= Tb.Pm, µm), cancellous bone volume (BV/TV=Tb.Ar/Tt.Ar, %), trabecular thickness [Tb.Th= (BV/TV)/0.5 BS, µm)], trabecule number [Tb.N=(BV/TV)/Tb.Th, mm^{-1}], trabecular space [Tb.Sp= (1/TbN)-Tb.Th. µm] were illustrated.

	Control	Electrical Stimulation	Bone Marrow	EC and BM
BV/TV (%)	21.40 ± 1.69*	50.80 ± 1.16*	50.40 ± 1.86*	50.20 ± 0.58*
Tb.Th (µm)	84.25 ± 5.76**	128.80 ± 2.53**	127.3 ± 2.06**	130.8 ± 1.25**
Tb.N (mm^{-1})	2.75 ± 0.75**	5.25 ± 0.48**	5.0 ± 0.58**	5.0 ± 0.41**
Tb.Sp (µm)	71.50 ± 3.80**	122.0 ± 3.98**	122.5 ± 4.50**	125.3 ± 3.77**

All values were given as mean ± SD.* P< 0.05, **P< 0.01.

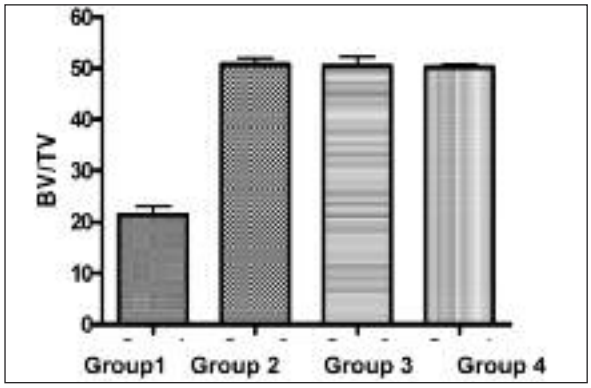

FIGURE 2a: Comparison of cancellous bone volume (BV/TV= Tb.Ar/ Tt.Ar, %) between groups.

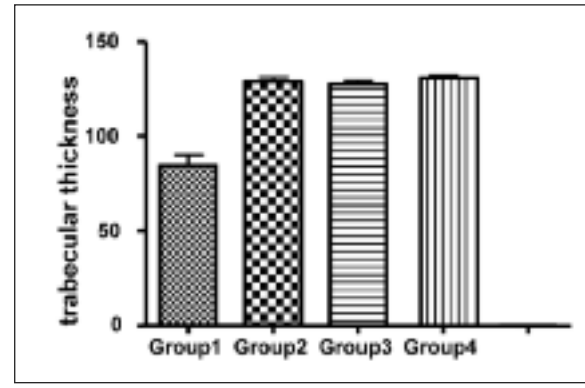

FIGURE 2b: Comparison of trabecular thickness [Tb.Th= (BV/TV)/0.5 BS, µm)] between groups.

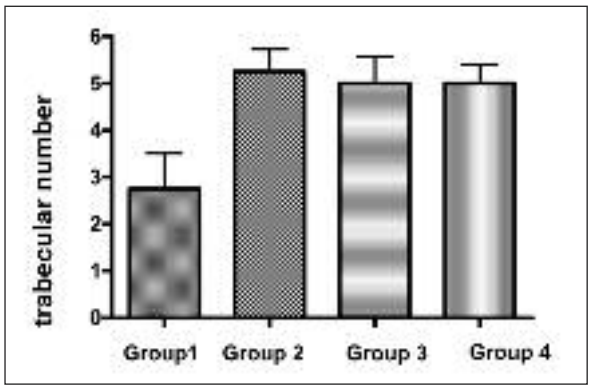

FIGURE 2c: Comparison of trabecular number [Tb.N= (BV/TV)/Tb.Th, mm^{-1}] between groups.

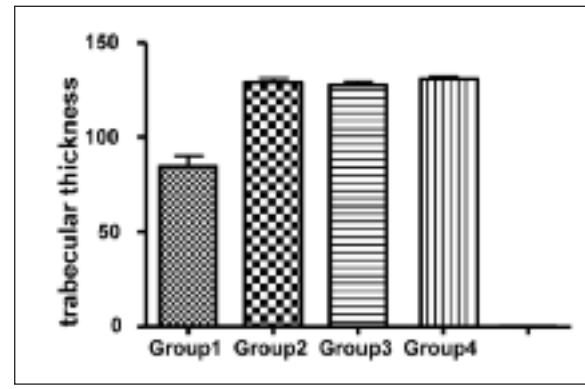

FIGURE 2d: Comparison of trabecular space [Tb.Sp = (1/TbN)-Tb. Th. µm] between groups.

are considered in clinical effects of neuromuscular electrical stimulation. As a direct effect, consecutive muscle contractions are thought to increase blood filling in the vascular bed, increase vascular permeability, accelerate venous return, and increase neovascularization. Besides these effects of the electrical current, it is also known that it increases osteoblast formation from mesenchymal stem cells directly, which promotes osteogenesis.[9,11,23-29] Although this effect is thought to be limited in neuromuscular electrical stimulation, a 1 cm interval between electrodes and bone fragments may cause a magnetic effect. As an indirect effect, it was thought that it enhanced vascular flow and increased metabolic activity, and improved bone density by minimal movements on fracture lines. In the literature, neuromuscular electrical stimulation applied on a 3 mm tibial defect improved fracture healing. In our study, it is observed that neuromuscular electrical current increased fracture healing even in larger defects. However, it showed no advantage when compared with Group III, which was the bone marrow-injected group. In histological investigations, a higher rate of vascularization than in other groups suggested that the accelerative effect of neuromuscular electrical stimulation on defect healing depended on increased local blood flow and venous return, rather than precipitating stem cell differentiation (data not shown).

Although there are many experimental and clinical studies related with the use of autologous bone marrow and electrical current on fracture and defect healing separately, to our knowledge, there are no reports using these methods in combination.

In the group in which electrical current and bone marrow injection were combined, healing

started earlier than in the control group and histologically there was more callus tissue; however, the difference was not significant. The shortest follow-up for defect healing is eight weeks in the literature. In the light of this information; we draw a conclusion that studies with combined marked cell culture and electrical current, and a follow-up period longer than six weeks are needed. Use of neuromuscular electrical current and bone marrow are promising for clinical effects on non-union, fracture and accelerating healing. Future stem cell studies combined with electrical current may be used to demonstrate and to confirm that electrical current enhances in vivo cellular differentiation.

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