Promotion of the Distraction Osteogenesis by Percutanous Injection of Allogenic Demineralised Bone Matrix and Autogenous Bone Marrow: An Experimental Study in Tibia of Dog

DİSTRAKSİYON OSTEOGENEZİSİNİN ALLOGEN DEMİNERALİZE KEMİK MATRİKSİ VE OTOJEN KEMİK İLİĞİNİN PERKUTAN ENJEKSİYONLARI İLE HIZLANDIRILMASI: KÖPEK TİBİASINDA DENEYSEL ÇALIŞMA

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Summary

The aim of this study is to investigate if percutanous injection of allogenic demineralised bone matrix and autogenous bone marrow had any effect on the velocity, duration and pattern of regenerated bone formation (distraction osteogenesis) in dog tibial defects.

20 hybrid dogs with an average weight of 23,5 kg. were divided in 4 different groups with five animals in each. A 6 cm. defect of the diaphyseal part of the right tibia was performed after it had been stabilised by a circular external fixator. Distraction was performed for 60 days with 1 mm. /day rhythm. Group one was treated by injection of 10 ml. Saline solution, group two with injection of300 mg. allogenic demineralised bone matrix, group three with injection of 2 ml. autogenous bone marrow and group four with a combination of 2 ml. of autogenous bone marrow and 300 mg. allogenic demineralised bone matrix into the distraction area.

The animals were sacrified at the end of 10th week for histopathological analysis. Best results revealing early osteogenesis on the distraction site was observed in the fourth group ($p<0.001$).

The study concluded that, combined percutenous injection of demineralised bone matrix and bone marrow can be an additive method during treatment of defective fractures of long bones with Ilizarov's circular external fixator.

Key Words: Distraction osteogenesis, Allogenic Demineralised bone matrix, Autogenous bone marrow, Tibia, Dog

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Özet

Bu çalışmada defektli köpek tibiasına perkutan yolla enjekte edilen demineralize kemik matriksi ve otojen kemik iliğinin kemik formasyonunda (distraksiyon osteogenezisi) rejenerasyon hızına etkisinin araştırılması amaçlanmıştır.

Ortalama ağırlıkları 23.5 kg olan 20 adet melez köpek her grupta 5 köpek olacak şekilde 4 gruba ayrıldılar. Tüm gruplardaki köpeklere sirküler eksternal filcsatör uygulandı ve tibianın diafizer bölgesinde 6 cm lik parça çıkarılarak defekt oluşturuldu. 60 gün boyunca günde 1 mm'lik disraksiyon yapıldı. 1. gruba 10 ml fizyolojik tuzlu su, 2. gruba 300 mg allojen demineralize kemik matriksi, 3. gruba 2 ml otojen kemik iliği ve 4. gruba 300 mg allojen demineralize kemik matriksi ile 2 ml otojen kemik iliği distraksiyon alanına enjekte edildi. Denekler 10. haftanın sonunda histopatolojik analiz için sakrifiye edildiler. En iyi sonuç 4. grubun örneklerinde alındı. Bu grubun distraksiyon alanında, erken osteogenezis gözlendi ($p<0.001$).

Bu çalışmayla demineralize kemik matriksi ve kemik iliğinin kombine perkutan enjeksiyonunun llizarov'un sirküler eksternal fiksatörünün kullanıldığı defektli uzun kemik kırıklarında iyileşmeyi hızlandırıcı ilave bir uygulama olarak kullanılabileceği ortaya konulmuştur.

Anahtar Kelimeler: Distraksiyon osteogenezisi, Allogen demineralize kemik matriksi, Otojen kemik iliği, Tibia, Köpek

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Open and fragmented fractures, malunion, delayed union, lengthening of the extremities, correction of the deformities, arthrodesis and releasing of soft tissue contractures are the main indications of external fixators (1).

Recently many different methods have been tried to enhance the healing of fractures and to

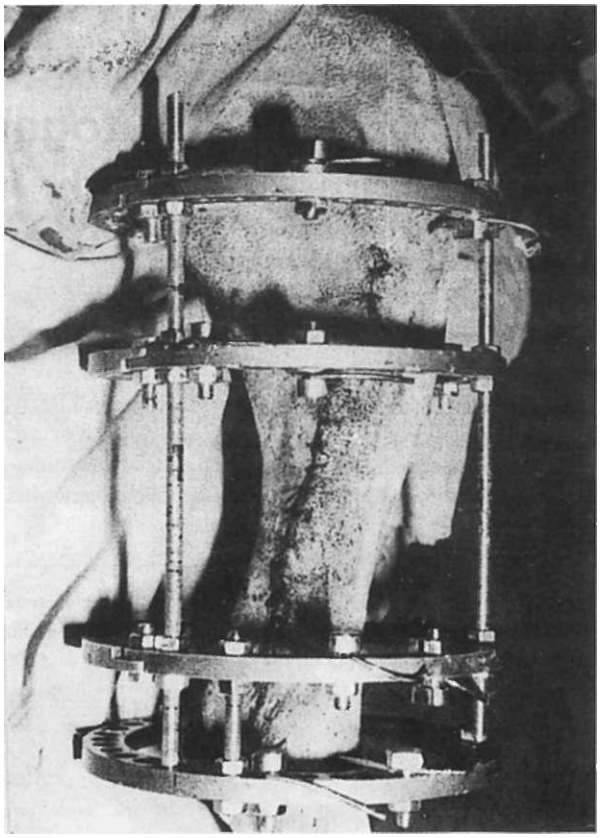
close the defects (2-7). Distraction osteogenesis for closure of defects with application of demineralised bone matrix and autogenous bone marrow for the acceleration of healing process of the fractures is a new method under consideration (8-12).

Ilizarov Technique, which depends on the principals of subperiostal cortical osteotomy and distraction osteogenesis, is being used with an increasing frequency since it is less invasive, doesn't need bone graft, doesn't affect the patients mobility. The tissue type winch fills the space after distraction, depends mainly on the stability of the apparatus. Fixators without a good stability delay healing by creating a fibrocartilagenous tissue which doesn't develop into bone (13). On the other hand, the graft having either osteoinductive or osteoconductive properties also increases the success by adding new osteogenic cells into medium (14).

The aim of this study was to investigate if percutenous injection of allogenic demineralised bone matrix and autogenous bone marrow applied to the distraction region, had any additive effect on the velocity, duration and pattern of regenerated bone formation in dog tibial defects.

Materials and Methods

20 mature, hybrid dogs with an average weight of 23.5 kg, (20-30) were used in this study. Four random groups with five experimental animals in each were made. The type of the circular external fixators was decided preoperatively according to the right tibial radiography of the animal. Their anesthesia was achieved by IM injection of 1 Omg/kg ketamin hydrocholoride following xylasin hydrochloride 0.1 ml/kg premedication. The right legs were shaved, disinfected and placed upwards on the operating table. A four-ring external fixator was fixed with suitable rods after stabilization of two 130 mm. diameter rings of the circular external fixator through transfixation of number two Stcinmann pins proximally and distally. A standard 6 cm. defect was created in mid 1/3 diaphyseal part of tibia through a medial approach, and final shape of the frame was achieved (Figure 1). A second corticotomy at the proximal metaphyseal segment was made between two rings (Figure 2). After the control of the final position of the fragments, hemostasis was obtained. Total period for the operation was approximately one hour. Local and systemic antibiotics were given to animals five days post operatively and the sutures were removed on the 7th day. Distraction was regulated as lmm/day (2 x0.5mm). At the end of first postoperative week the following were applied to the region of distraction by using a number 15 spinal needle for percutaneus injections.



Figurel. The place of osteotomy and corticotomy in animals.

Group 1) 10 cc saline (Control group).

Group 2) 300 mg allogenic demineralised bone matrix in 10 ml saline.

Group 3) 2 cc autogenous bone marrow.

Group 4) 2 cc autogenous bone marrow+300 mg allogenic demineralised bone matrix.

Demineralised bone matrix was prepared from osteotomized bone blocks according to Reddi and Huggin's method (15,16). Size of the particles were selected between 100-400 micrometer diameters through the use of special filters. The autogenous bone marrow was obtained through aspiration from iliac ala with a number 15 spinal needle. 1 mm dis-

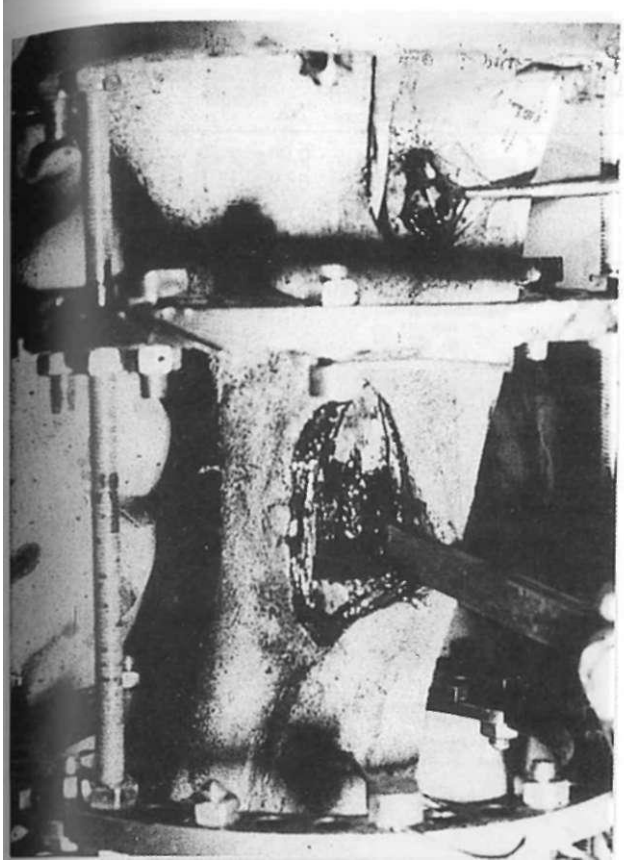


Figure 2. The apperance of tibia and apparatus following operation.

Tabic **1.** The Scale for Radiological Evaluation

Variable	Score
Bone Formation	
No evidence of bone formation	0
Bone formation covers 25%of defect	1
Bone formation covers 50% of defect	2
Bone formation covers 75% of defect	3
Bone formation in whole space	4
Union	
Complete fracture line	0
Partial	2
Absence of fracture line	4
Remodelling	
No evidence of remodelling	0
Remodelling of intramedullary channel	2
Complete cortical remodelling	4

[tra]ction was continued for sixty days. Clinical and biological controls of the animals were made at [2nd]>4th,6ih,8th, 10th weeks postoperatively.

At the end of 10th week all the animals were [Saci]'ified, and their tibias were histopathologically examined. Clinically; the status of the soft tissue, pin-tract infection, loosening of the pin, the diameter of palpable swelling of the callus, the function of the extremity, radiologically; callus formation in distraction area, progression of distraction, position of the fragments, union and remodelling, histopathologically; the amount of bone in distraction area, union and bone marrow status were examined.

For radiological evaluation modified Lane and Sandhu Scale (Table 1), for histological evaluation modified Heiple Scale were used (Table 3) (9,17).

Results

Clinical Results

Careful dissection was made, so that no neurovascular damage was observed. 4 pin tract infections occurred. According to the Paley classification 2nd degree pin tract infection in two, 3rd degree pin tract infection in three animals observed. 2nd degree infections were healed with daily dressings but 3rd degree infections needed antibiotics besides dressing.

Rotation, angulation, skin and tendon problems or refractures did not occur. The animals tolerated the circular external fixator of Uizarov very well. 130 mm rings made up of duraliminium were seen to be a very suitable model for dog tibias. With the circular external fixators the animals using started their extremities between 2nd and 7th post operative day (average 5 days) They were allowed weight bearing on 12th to 20th (average 16 day) days.

No problems were encountered during the injection of bone matrix or bone marrow. The particle diameter being between 100 and 400 micrometer not only made the injection easier but also increased the autoinduction capacity.

Radiological Results

Different results were obtained when healing models of study groups were compared.

1st Group: After 60 days, new bone formation in distraction area that is callus tissue was found not to tolerate the distraction. No uniform callus formation appeared in the saline (control group) injected area. Defect was closed with distraction os-

Table 2. The statistical results of radiological evaluations.

	1. GROUP		2. GROUP		3. GROUP		4. GROUP	
	X	SX	X	SX	X	SX	X	SX
UNION	2.00	0.00	2.00	0.00	2.00	0.00	3.60	0.30
BONE FORMATION	2.00	0.00	3.00	0.00	2.60	0.24	3.60	0.24
REMODELING	2.00	0.00	2.00	0.00	6.00	4.00	4.00,	0.00
TOTAL	6.00	0.00	7.00	0.00	10.60	4.24	11.20	0.54

Kmskal-Wallis Test was performed for the statistical Analyses. Values in the 4th Group was found to be significant at $p<0.05$ compared lo the other groups.

Table 3. Scale for Histological Evaluation

Union	
No evidence	0
Fibrous	S
Fibrocartilagcnous	2
Bony	3
Complete bone formation (Cortical and trabecular)	4
Callus	
Absent	0
Little	1
Medium	2
Wide spread	3
Bridging	4
Bone marrow	
Absent in resected area	0
Rarely seen	1
Present in 50% of defect	2
Total colonization	3
Mature fatty marrow	4

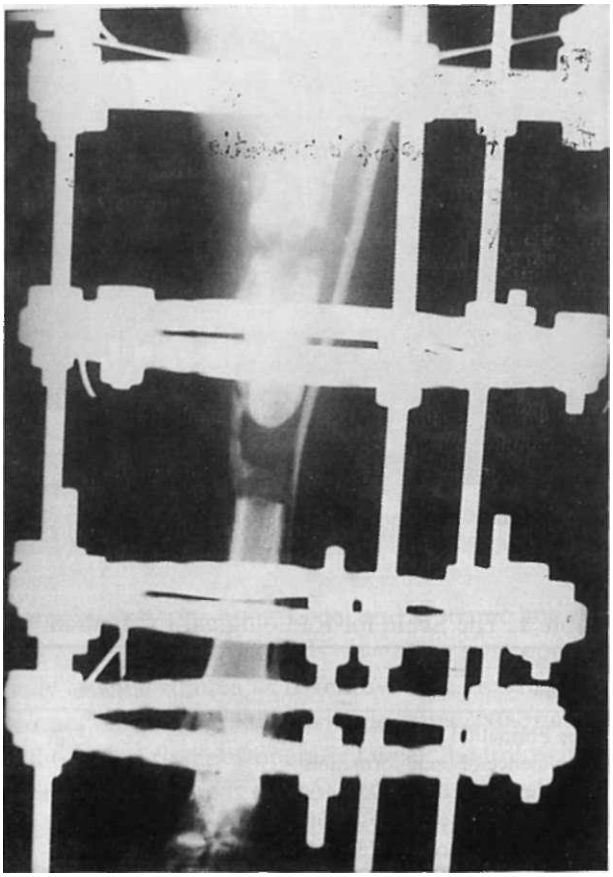


Figure 3. The radiological appearance of nonuniform callus tissue in (control) Group I.

teogenesis in each of five animals. Radiologically, the uniformity of the callus tissue in distraction area was achieved only at postoperative 10th week (Figure 3).

2nd and 3rd Group: The callus tissue in distraction area was better in both groups into which allogenic demineralized bone matrix and autogenous bone marrow were injected respectively when compared to saline injected group. More osteogenic tissue was present in the distraction area. Considering union and tissue mineralization in distraction area, a better radiological score was obtained in both groups compared to the 1st Group. There wasn't a significant difference between 2nd and 3rd Groups ($p<0.05$). Callus tissue became uniform at post operative 9th week (Figure 4-5).

4th Group: In this Group to which demineralised bone matrix and bone marrow were injected together, the radiological scores were significantly different than those in the other three groups. Not only the callus was uniform at about 8th week, but also the highest radiological score was obtained (Figure 6). The radiological evaluation results are presented in Table 2.

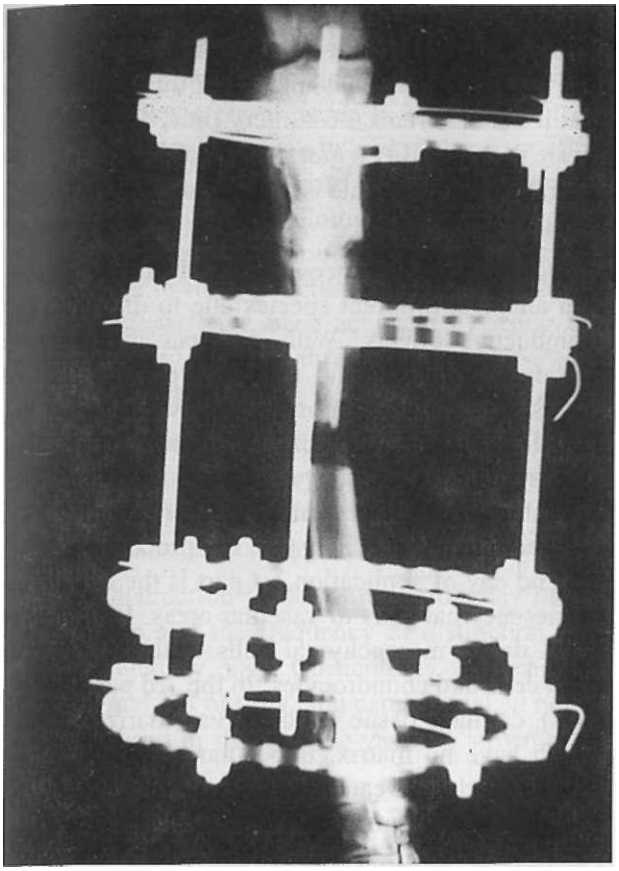


Figure 4. The incomplete radiological appearance of callus tissue in (Dcmineralised Bone Matrix) Group **II.**

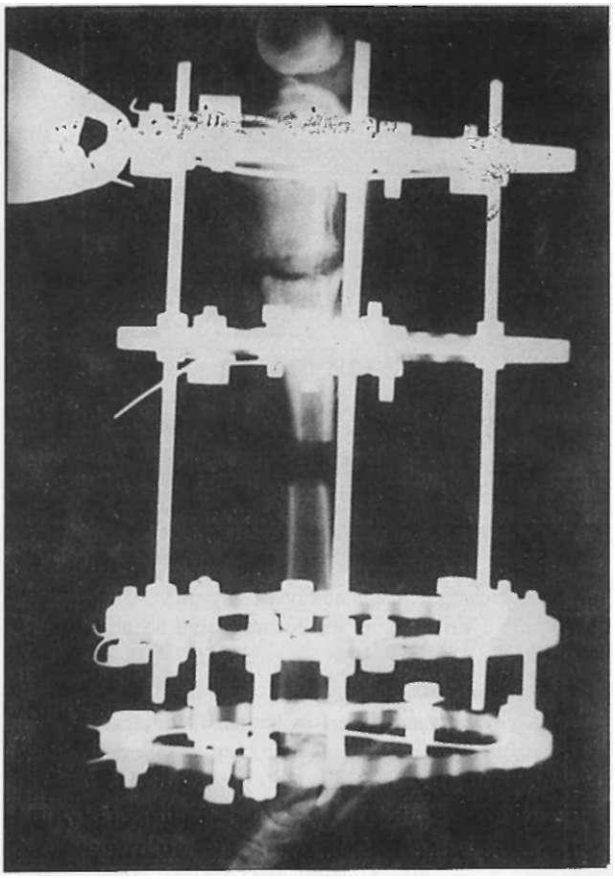


Figure 5. The incomplete radiological appearance of callus tissue in (Bone Marrow) Group **III.**

Histological Evaluation

All the examinations were made with H&E stained preparations.

1st Group: In most slides of this saline injected group insufficient bone formation, diffuse fibrous tissue islands were present. In some sections bone marrow could be observed in distraction area.

2nd and 3rd Group: In these groups to which autogenous bone marrow and allogenic demineralised bone matrix were injected respectively, more prominent callus was detectable when compared with the 1st Group. In sections, the transformation of fibrous tissue islands to fibrocartilagenous structure and association of islands in some areas were observed. Bone marrow was also dispersed. The union was significantly different than that in the 1st Group but there wasn't any statistically significant difference among the 2nd and 3rd Groups ($p<0.0T$).

4th Group: Wide spread bone formation was noticed in this group. Cortical and trabecular bone formation was much better than the first 3 Groups ($p<0.00T$). The histological evaluation results are presented in Table 4.

Discussion

Autogenous, homologous, heterologous grafts, many different inorganic materials and their combinations have been used in the treatment of defective fractures of long bones and non-unions (2-5,7,9-12,17). Although the autogenous bone graft is the most successful method, high amount of bone demand in large defects, prolongation of operation and operative trauma are its disadvantages. Allografts are being used to shorten the surgery time and minimize the trauma. Infection, resorption, and nonunion are their disadvantages. The disability to destroy the antigenic properties is an-

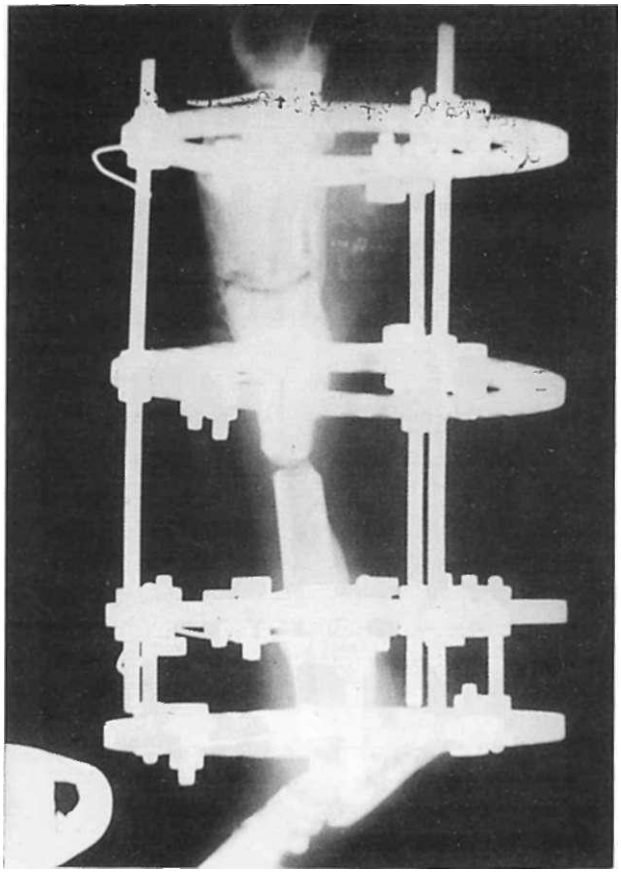


Figure 6. The radiological appearance of uniform callus tissue in (Démineraiised Bone Matrix +Bone Marrow) Group IV.

other problem (6).The reactional inflammation, which occurs during lyophylisation and freezing that are done to decrease antigenicity, diminishes vascularization rate and a fall in bone formation can be observed (6,10). The antigenic properties of xenografts are more than allografts and do not maintain enough osteogenic stimulation (3,6). Since the demineralised matrix in this study is allogenic and doesn't make any discriminance between species, no immunological reaction has occurred. The most important advantages of this method are that it both shortens the surgery time, and does not create the morbidity a graft harves of. Bone marrow aspiration from iliac ala is an easy and non-invasive method.

There are differences in bone formation duration amongst different species due to different osteoinductive effects. With different experiments this duration was found to be 8 weeks in rats, 10 weeks in rabbits and monkeys, 12 weeks in dogs. Detailed time schedule of transformation of mesenchymal cells to cartilage and bone was examined in rabbits and it was found that 98% ossification had occurred on 42nd day (6). The process starts on the 2nd day of application, 1st part is the migration of mesencymal cells to vascular areas. In 2nd part (2-18 days), mesenchymal cells differentiate into giant cells and chondrocytes. In the 3rd part (18-20 days), cartilage tissue in poorly vascularized areas, which have no matrix, trabecular tissue in richly vascularized areas can be observed. Bone tissue appears in the 4th part (20-30 days) (6,18). The total period is assumed to be between 10-12 weeks for dogs. The results of our 10-week follow up study are also similar.

The déminéralisation process performed according to Reddi and Huggins Procedure took 6 hours (15,16). The sterilisation of demineralised bone matrix was made with ethylene dioxide for 2.5 hours and stored at 4 degrees Celsius. The sterilisation technique is reported to influence the osteoinductive capacity of demineralised bone by many authors (19,20).

The particle size has also an effect on osteoinductive capacity of demineralised bone matrix

Table **4.** The statistical results of histological evaluations.

	1.GROUP		2. GROUP		3. GROUP		4. GROUP	
	X	SX	X	SX	X	SX	X	SX
UNION	1,20	0,20	2,00	0,00	1,80	0,20	3,20	0,20
CALLUS	1,40	0,24	2,00	0,00	1,80	0,20	3,20	0,20
BONE MARROW	1,00	0,00	1,20	0,20	1,00	1,00	3,20	0,20
TOTAL	3,60	0,44	5,20	0,20	4,60	1,40	9,60	0,60

Kruskal-Wallis Test was performed for the statistical Analyses. Values in the 4th Group was found to be significant at pO.OOl compared to the other groups.

(6,21-23). Injection of demineralised bone matrix as dust and particle causes a wider area of osteoinduction and a higher neovascularization . The size of the particles were prepared to be between 100-400 micrometer in our study, and it was found that particles of this size are able to induce enough osteoinduction based on radiological and histological evidence. During injection of bone marrow and bone matrix to the distraction area, a wider spread of the contents have been achieved by using the needle in many different directions.

Effect of distraction frequency and velocity have also been examined and 1 mm/day has found to be the optimal distraction velocity. The newly formed bone with this structure has similar structural properties to real bone tissue (24,25).

The distraction velocity was 1 mm/day in the study group and the frequency of distraction was well tolerated by dogs. Corticotomy was made in the proximal 1/3 of tibia, and care was taken not to injure the area or damage the vascular structure of the periosteum.

The extremity immobilization of animals was achieved by circular external fixator of Ilizarov. With this apparatus the animals started to use their extremities between 2nd and 7th days (average 5 days) postoperatively and they could bear weight on them between 12th to 20th days (average 16 days). Postoperative bandaging of fractured extremities causes many problems in fractured patients. In our study complications such as inactivation atrophy, osteopenia or osteoporosis didn't occur. Long term immobilization limits the function of joints, bones and muscles. As the blood supply of the extremity decreases, the net result is a negative effect on bone regeneration.(26,27)

In conclusion, the combined percutenous injection of allogenic demineralised bone matrix and autogenous bone marrow to distraction area significantly effected early callus formation and demonstrate clear osteoinduction. Easy sterilisation of allogenic demineralised bone matrix, simplicity of percutenous injection technique, absence of antigenic reaction, shortening of operation time, easy storage arc its advantages against all other graft materials. All these results led us to a conclusion that the percutenous injection allogenic demineralised bone matrix and autogenous bone marrow can be a solution for delayed union or non union problems when combined with Ilizarov's circular external fixator apparatus and can also maintain early union. Finally, in our opinion, combined percutenous injection of demineralised bone matrix and bone marrow can be an additional method during treatment of fractures of long bones with Ilizarov's circular external fixator.

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