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Effect of dl-alpha-tocopherol-acetate on the Fracture Healing of Experimental Radial Diaphysis Fracture in Dogs

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This experiment was conducted to determine the effect of vitamin E (dl-alpha-tocopherol acetate) on healing of radial diaphysis fracture in dogs.

Eighteen adult (mean 3 -y-old), mongrel female dogs were divided into two equal groups. Fixation was performed with bone plate after fracture constituted on radial diaphysis. Postoperatively, dl-alpha-tocopherol acetate (20 mg/kg/day) was injected intramuscularly to the first group (treatment group) for one week. The second group was kept as control group. The result of the study was evaluated clinical and radiographically on postoperative days of 15th, 30th and 60th. At each examination time tissue samples of three cases of each group were harvested and these cases were freed.

Dl-alpha-tocopherol acetate being more effective in early stages (first fifteen days) was evaluated due to its antioxidant effect on free oxygen radicals in the fracture region. Consequently, it was understood that dl-alpha-tocopherol acetate given for a week had a positive effect on the fracture healing in dogs in case of applying immediately (in early stages).

Key Words: *Fracture healing, free oxygen radicals, dl-alpha-tocopherol acetate, dog.*

Köpeklerde Deneysel Diyafizer Radius Kırıklarında dl-alfa-tokoferol-asetat'ın Kırık İyileşmesi Üzerine Etkisi

Bu deneysel çalışma köpeklerde radiusun diyafizer kırıklarında, kırık iyileşmesi üzerine E vitamininin (dl-alfa-tokoferol asetat) etkisini araştırmak amacıyla gerçekleştirdi.

Onsekiz adet, erişkin (ortalama 3 yaşında), melez dişi köpekler iki eşit gruba ayrıldılar. Radiusun diyafizinde kırık oluşturulduktan sonra fiksasyon plaka ile gerçekleştirildi. Operasyondan sonra birinci gruptaki (deneme grubu) köpeklere dl-alfa-tokoferol asetat (20 mg/kg/gün) intramusküler olarak bir hafta süre ile enjekte edildi. İkinci gruptaki köpekler kontrol grubu olarak izlemeye alındılar. Çalışmanın sonucu operasyondan sonra 15, 30 ve 60. günlerde klinik ve radyolojik olarak değerlendirildi. Her muayene gününde her gruptan 3 olgunun doku örnekleri alındı ve bu köpekler serbest bırakıldı.

Dl-alfa-tokoferol asetat'ın erken dönemde (ilk 15 günde) daha etkili olması kırık bölgesinde oluşan serbest oksijen radikalleri üzerine antioksidan etki göstermesine bağlandı. Ancak bu farkın uzun dönemde (30 günden sonra) kapandığı saptandı. Sonuç olarak kırık oluştuktan hemen sonra (erken dönemde) bir hafta süreyle verilen dl-alfa-tokoferol asetat'ın köpeklerde kırık iyileşmesi üzerinde olumlu etkisinin olduğu kanısına varıldı.

Anahtar Kelimeler: *Kırık iyileşmesi, serbest oksijen radikalleri, dl-alfa-tokoferol asetat, köpek.*

Introduction

Free oxygen radicals are reactive chemical species with an unpaired electron that are produced through a variety of physiological and pathological processes (1-3). Toxic levels of free oxygen radicals are observed in inflammation, wound healing, ischemia-reperfusion, and ionized radiation (4-6). Oxygen radicals may also directly damage cell membrane, apparently through the peroxidation of structurally important polyansatured fatty acids within the phospholipid structure of the membrane itself (7). Lipid peroxidation have recently been shown to play a role in bone metabolism especially in osteoclast activation and resorption activity (8,9). Free radicals are also found to be cytotoxic to osteoblast cells (9). Antioxidants inhibit lipid peroxidation by means of blocking of peroxidation or scavenging reactive oxygen species (10, 11).

Vitamin E is a natural biological antioxidant, which prevents peroxides from accumulating and protects cells from damaging effects of free radicals (2, 12). Vitamin E also ensures the stability and integrity of biological membranes (13, 14). It has been demonstrated that vitamin E protects against cellular lipid peroxidation in cartilage to sustain normal bone growth and modelling (14, 15), and results from animal experiments argue for an osteo-protective effect of vitamin E (16,17). Therefore, the aim of this study was to determine the effect of vitamin E (alpha tocopherol acetate) administration on the healing processes of radial diaphyseal fracture clinically, radiologically and histopathologically in dogs.

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Materials and Methods

Eighteen adult mongrel female clinically healthy dogs aged between 2 and 4-y-old (mean 3-y-old) were used in this study. The dogs were provided by Elazig Municipality, Turkey. The dogs were treated against antiparasite and vaccined. The animals were housed freely in three separate rooms, all cases were left free walk in the room and had free access to water and standard feed throughout follow up periods.

Preoperatively the dogs were left hungry for 12 hours. General anaesthesia was induced in animals by intramusculer administration of a combination of xylazine HCl 2 mg/kg (Rompun, Bayer, 23.32 mg/ml), and ketamine HCl 15 mg/kg (Ketalar, Parke-Davis, 50 mg/ml). Dogs were divided into two equal groups. Radial diaphysis was opened appropriately to surgical rules (18). Diaphyseal fracture was performed on radial diaphysis by a Gigli saw. Fixation of fractures was performed by means of a bone plate and screws. Operation wound was closed appropriately to routine surgical procedures. Postoperatively, 20 mg/kg/day dl-alpha-tocopherol acetate (Evigen ampul, Aksu Farma, 2 ml x 5, 300 mg/ml dl-alpha-tocopherol acetate) was injected intramuscularly to treatment group for one week. The second group was kept as control group. The fractured limbs were supported with a bandage reinforced by plastic casts, and the bandages were kept for a month by renewing every week. Postoperatively, 3 ml penicilline and streptomycine (Strepto-veticilline, Eczacıbaşı, procaine penicilline G 15 000 000 IU, crystalise penicilline G 500 000 IU and streptomycine sulphate 2000 mg/10 ml) were administered parenterally for 5 days. Neither restriction in walking nor bandage were applied to the cases after 30th days.

Clinical and radiographical evaluations were carried out at the 15th, 30th and 60th days after operation. Clinical examinations were performed evaluating by grading (if present) of lameness, utilizing the classification system (Table I). At the end of 15th, 30th and 60th day, three dogs in each group were reoperated under general anaesthesia to harvest a piece of bone including fracture region for histopathological examinations. The samples were placed in 10% neutral buffered formalin immediately after reoperation. The section was fixed in 10% neutral buffered formalin for 5 days and decalcifed in an decalcification solution (15 ml HNO_3+10 ml 10% neutral buffered formalin + 85 ml distilled water). The samples were cut into 5 μm sections after complete decalcification and stained with hematoxylin and eosin (H&E) and examined by light microscopy. In the histopathological examinations of the entire lenght of longitudinal bone sections, bleeding in the fracture area, osteoblastic activity, fibroblast proliferation, cartilage production, encondral ossification, bone marrow formation and bone union parameters were assessed and the differences between the groups were investigated (19, 20).

Table 1. Grading of lameness.

Degree of lameness	
Normal	No lameness
Slight	Lameness during full weight bearing
Moderate	Walking on finger point
Severe	Operated leg lifted up

The dogs were released following taking tissue samples at the time points mentioned before.

Number of the cases examined at 15th, 30th and 60th days are shown in Table II.

Table 2. Number of the cases examined at 15th, 30th and 60th days.

Days of Examination	**Clinical and Radiographical Examinations (n)**		**Histopathological Examination (n)**	
	Treatment group	Control group	Treatment group	Control group
15th day	9	9	3	3
30th day	6	6	3	3
60th day	3	3	3	3

Results

The results were evaluated under three sections as clinical, radiological, and histopathological findings.

Clinical examinations showed the presence of moderate lameness in all cases at the 15th day. At the end of the 30th day slight lameness was observed in two cases in control group, but no lameness in the other cases. All cases were free of lameness at the end of follow up period.

At the 15th day, radiological examinations showed that callus formation began in all groups except two cases of control group. At the 30th day, union was completed in all cases of the treatment group, whereas it was incompleted in 4 cases in control group. At the 60th day, union was completed in all cases (Figure 1, 2), except only one case of control group.

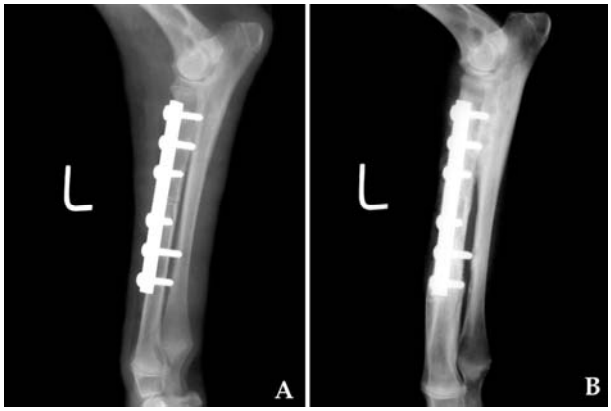

Figure 1 A. Radiographic appearance of a case of treatment group after surgery, **B.** Radiographic appearance of a case of treatment group in postoperative 60th days.

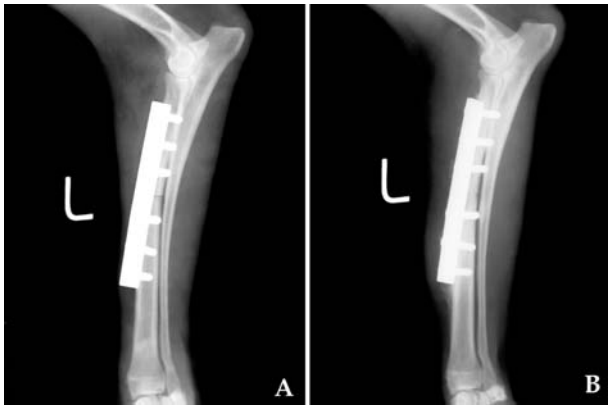

Figure 2. A. Radiographic appearance of a case of control group after surgery, **B.** Radiographic appearance of a case of control group in postoperative 60th days.

In the histopathological findings, the cases in the treatment group were seen to be better regarding condroid production and osteoblastic activity on the fifteenth day. Callus tissue was determined in the medulla and periosteum of the fracture region in treatment group; however, callus tissue was observed only periosteum of fracture region in control group. Callus tissue decreased in the 30th day according to the 15th day, and abundant bone tissue was observed in control group. Whereas bone tissue maturated in the most of areas in treatment group. At the 60th day, new bone tissue was determined in control group. There was bone union in the fracture ends, but a small focus of callus tissue continued in the fracture line. Bone tissue maturated completely in the treatment group. Bone marrow formation was better in the treatment group at the 60th day (Figure 3).

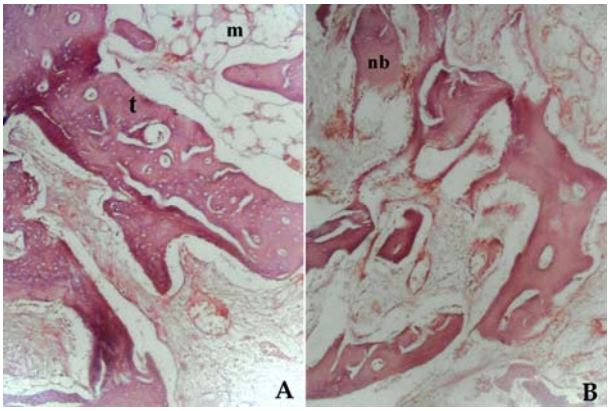

Figure 3. A. Histopathologic appearance of a case of treatment group in postoperative 60th days. It was observed that bone trabecula (t) and bone marrow (m), (H.E. x 40), **B.** Histopathologic appearance of a case of control group in postoperative 60th days. It was observed that new bone (nb), (H.E. x 40).

Discussion

Free radicals play a role in a variety of diseases and it has become apparent that pathogenesis of many diseases can be lightened by means of understanding cell sources of free radicals and defence mechanism against free radicals. Free radicals are originated from activated phagocytes, antineoplastic agents, irradiation, addictive drugs, stress, otooxidation of small molecules, enzymes, proteins, electron transport systems of mitocondrium, membran of plasma and conditions of oxidative stress (1, 2, 4, 7). Free radicals effect metabolism of cells through the damaging effects on the metabolism of protein, DNA, carbonhydrate, lipids, enzymes and other molecule groups (5, 10, 11, 21, 22).

The protective effect of Vitamin E (alpha tocopherol) might be attributed to a structural effect, however, and there is an evidence from experiments upon cell cultures that the presence of vitamin E can affect the types of fatty acids that become incorporated into membrane lipids (22). Keskin *et al.* (20) and Göktürk (23) investigated the effect of alpha tocopherol on the healing of bone in rabbits and rats respectively. In this study, it was to investigate the effect of alpha tocopherol on the healing of bone fracture in dogs.

Vasoconstruction and temporary ischemic period are developed in fracture site when a bone fractured, an arterial vasodilatation and reperfusion in fracture sites are then observed. Polymorphonuclear leucocytes, macrophages and mast cells are migrated to fracture sites in the first 5 days of fracture. This phase is important for fracture healing. It is believed that free oxygen radicals produced through the activation of polymorphonuclear leucocytes damage granulation tissue and retard wound healing (6, 20, 23-25). Negative effects of free oxygen radicals on the fracture healing were reported (8, 15, 17, 26). Norazlina *et al.* (9),

reported that Vitamin E deficiency may cause loss of bone calcium in growing female rats. Hodis (27) has mentioned that for normal antioxidant effect 400 IU vitamin E per day and for maximum antioxidant effect 800 IU per day should be administered 1000 IU per day is consider as megadose (27). Some other authors (28,29) have suggested a prophylactic dose of 1000 IU per day for 3-4 months to decrease coronary health problems. Keskin *et al.* (20) and Durak *et al.* (24) have administered 20 mg/kg/day alpha-tocopherol in rabbits. For that reason, in this study 20 mg/kg/day dl-alpha-tocopherol acetate was injected intramuscularly to treatment group for one week as the first 5 days of fracture is important for fracture healing (2, 20, 23, 24).

A role of free radicals has been proposed in the toxicity of numerous chemicals and in the pathogenesis of many diseases (30). An extensive list of disorders in which free radicals are implicated is still growing, at least in part because these reactive molecules can produce most of the tissue changes that have been identified during a variety of injurious processes (3). Some substances defined as antioxidants are used to either prevent formation of or to scavenge free oxygen radicals and their damages. Dl-alpha tocopherol is most active antioxidant among the tocopherols. Vitamin E prevents oxidation of other molecules by means of easily being oxidated (31-33). Free radicals levels were not analized in this study because the aim of this study was to investigate the effect of vitamin E administration on the healing of fracture clinically, radiologically and histopathologically.

Vitamin E deficiency would increase lipid peroxidation. It has been shown that lipid peroxidation enhance bone resorption by directly activating osteoclasts (8, 9, 34, 35). Avitabile *et al.* (36) reported that an association between low activity of antioxidant systems and demineralization of bone, consequent upon enhanced free radical levels. Yee and Ima-Nirwana (37) reported that exposure to an oxidizing agent, ferric nitrilotriacetate, reduced bone calcium content, and that this was prevented by vitamin E supplementation. Therefore, it is suggested that the vitamin E deficiency increased free radical activity, thus enhancing bone resorption and demineralization, which was seen as significantly low bone calcium content. Cohen and Meyer (38) found that vitamin E and selenium deficiency predispose rabbit bones to osteomalacia and decreased the biomechanical strength of the bones. However, vitamin E supplementation was protective against bone loss due to rotational stress in rats (38). Sergeev *et al.* (39, 40), found that rats with a vitamin E deficiency had decreased absorption of calcium through the intestines and kidneys, as well as decreased deposition of calcium in bones. Similar to these results, results of current study showed that it is clear that vitamin E plays a role in normal bone mineralization, either by its antioxidant effects or by increasing calcium availability for bone deposition.

The clinic, radiographic and histopathologic findings of the present study shows that in order to prevent negative effects free oxygen radicals on osteogenesis the administration of 20 mg/kg/day dl-alpha-tocopherol, a high antioxidant feature appeared to have a usefull effect on early healing processes (first fifteen days) of osteogenesis in experimentally induced fracture dog model.

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