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TRAUMA SURGERY

The effect of zymosan and the protective effect of various antioxidants on fracture healing in rats

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Abstract
Aim To investigate the effects of free oxygen radicals and various antioxidants on bone healing after experimental formation of fracture.
Materials and Methods Fifty male rats were used and divided into five groups (ten rats in each). The right forelimbs of the rats were broken by bimanual compression method. One hour before this procedure, 5 ml/kg of intraperitoneal (i.p.) physiologic saline were given to the control Group 1. All 40 rats in the experimental Groups 2, 3, 4 and 5 were treated with i.p. zymosan at a dosage of 100 mg/kg to induce the production of free radicals by stimulating NADPH oxidase in polymorphonuclear leukocytes. Zymosan induction was stopped on the fifth post-fracture day. In addition to the zymosan, i.p. 1 g/kg/day of dimethyl sulfoxide were given to the animals in Group 3, 50 mg/kg/d of Ginko biloba Extract (EGb 761) in Group 4 and 500 mg/kg/day of vitamin C in Group 5. Radiographs of the fractures of all animals were obtained to assess callus formation, remodeling and bridging bone formation under ether anesthetics on postfracture day 7, 14 and 21. All rats were euthanized on day 22, and sections of the radius and ulna were examined both histologically with light and electron microscopy and ultrastructurally. Statistical analysis was made with Kruskal-Wallis variance analyze test and comparison between groups was performed by Dunn's multiple comparision test.
Results An impairment of bone healing was observed in Group 2 inducted with purely zymosan. Variable results were obtained for bone healing in the groups treated with various antioxidants. There was very significant difference of fracture healing between Groups 1 and 2 both histologically and radiologically ($P < 0.001$). There was significant difference between Groups 2 and 5 radiologically ($P < 0.05$).
Conclusion Free oxygen radicals demonstrate a negative effect on fracture healing and vitamin C (an antioxidant) partially prevents the negative effect of zymosan on fracture healing.

Keywords Free-oxygen radicals · Zymosan · Antioxidant therapy · Fracture healing

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Introduction

Oxidative stress defines the breakdown of the preoxidant-antioxidant balance toward the preoxidant side in the tissues and in the body. In the aerobic life, the continuously formed preoxidants should be regularly captured and be

balanced with consuming by the antioxidants. Otherwise, oxidative damage will form and result in physiopathological events. Most of the preoxidants that create oxidative stress are free radicals. Being short surviving molecules, oxygen free radicals can produce cellular damage by causing various metabolic disarrangements in the cell and can eventually produce cell death [23, 27, 28].

Oxygen free radicals have been implicated as inducers of tissue injury in several pathologic conditions such as inflammation, circulatory shock injury, irradiation, respiratory distress syndrome and ischemic/reperfusion events in different tissues [39, 45–48].

Today, there are evidences for the roles of free radicals in appearance of many pathologic events including cancer and aging. Their roles in ischemia-reperfusion injury, in stress and in gastric mucosal damage by drugs have become precise. Besides these, their negative effects on wound healing, on granulation tissue, on collagen and cartilage tissue have been shown [9, 19, 20, 24].

Bone fractures are associated with inflammation and ischemia, stimulating free radical oxidation [47]. A free radical is defined as a molecular species capable of independent existence and contains one or more unpaired electrons [21]. Oxygen free radicals cause subcellular abnormalities in the tissues [38]. Oxygen-derived free radicals are believed to initiate a chain reaction leading to cell membrane damage via lipid peroxidation, thereby causing cell death [42, 50].

Although the mechanism of free radical formation is well known, there are a limited number of histopathological and ultrastructural studies regarding the effects of free radicals on fracture healing [16, 26, 27, 49]. This study aimed to investigate the effects of free oxygen radicals on fracture healing. In addition, the effects of antioxidants such as dimethyl sulfoxide (DMSO), Ginkgo biloba extract (EGb.761) and vitamin C were also evaluated on fracture healing under oxidative stress.

Materials and methods

The ethics committee of Erciyes University reviewed the study, and the experiments conformed to the Principles and Guidelines for the Use of Animals in Research, Testing and Education issued by the Committee on Educational Programmes in Laboratory Animal Science [11]. Rats were obtained from the Medical Sciences Experimental Research Unit of the University. All procedures were performed in the same unit.

Animal and tissue preparation

Fifty Wistar albino male rats weighting 150–250 g were used in this study. The rats were acclimated to caged laboratory conditions for 2 weeks. The rats were randomly divided into five equal groups (ten rats in each group), consisting of one control and four experimental groups. They were permitted to take water and standard laboratory diet. After rats were anesthetized intraperitoneally with ketamine (40 mg/kg Ketalar®, Eczacibasi, Istanbul, Turkey) and xylazine (5 mg/kg Rompum®, Bayer, Leverkusen, Germany), right forelimbs of the rats were broken by bimanual compression method using the standardized technique [17]. The fractures were detected to be simple transverse fractures in the radius and ulna by radiography taken just after production of fracture, while the rats were under anesthesia. All groups except control rats (Group 1) were inducted with intraperitoneal (i.p.) zymosan at 09:00 a.m. to produce free oxygen radicals (Zymosan A, MO, USA, Sigma chemical co.) at a dosage of 100 mg/kg [27]. The rats in Group 1 were treated with 5 mg/kg of i.p. saline 1 h before the fracture procedure. Injection of Zymosan and saline was continued once in a day until the fifth day of fracture.

In addition to zymosan, i.p. dimethyl sulfoxide (DMSO, Sigma chemical co.) was administered at a dosage of 1 g/kg/day for 21 days in Group 3 [25]. Ginkgo biloba extract (EGb.761) (Tebokan®, Abdi İbrahim Drug Co.) was administered at a dosage of 50 mg/kg/day in Group 4 for 21 days [43]. Group 5 rats were treated with 500 mg/kg/day of vitamin C (Redoxon®, Roche) for the same period [51].

Radiographs of the fractures of all animals were obtained to assess callus formation, remodeling and bridging bone formation under ether anesthetics on postfracture day 7, 14 and 21. Modified Lane and Sandhu [34] method was used for radiological scoring system (Table 1). When mineralized bridging was radiographically detected in the fractures of control group, rats were euthanized by high dose ether anesthesia and evaluated under histological examination to perform the difference of complete and incomplete bone healing on day 22. The tissues were fixed in 10% neutral buffered formalin.

Forearm bones were decalcified in 10% ethylene diamine tetra acetic acid (EDTA). Paraffin blocks were prepared and sections of 5–6 mm around each callus were obtained [1]. Histological sections of 5 μm thick were stained with hematoxylin-eosin (HE) and were examined under light microscope [30]. All preparations were blindly examined by the same pathologist under light microscopy. The histological grading of fracture healing was performed according to the 5-grade system (Table 3) [1]. The least healed bone (radius or ulna) was considered in the grading of recovery. In case of technical irregularity in sections, grading was done by evaluating callus of both fractures [1]. For electron microscopic evaluation, fresh bone specimens were fixed by immersion in 2.5% gluteraldehyde and then post fixed in 1% osmium tetroxide in 0.1 M phosphate buffer at 4°C for 1 h. After washing in phosphate buffer,

Table 1 Modified Lane and Sandhu radiological scoring system [34]

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 (proximal and distal evaluated separately)	
Nonunion	0
Possible union	1
Radiographic union	2
Remodeling	
No evidence of remodeling	0
Remodeling of medullary cana	1
Full remodeling of cortex	2
Total point per category	
Bone formation	4
Proximal union	2
Distal union	2
Remodeling	2
Maximum score	10

they were dehydrated in graded series of ethanols to absolute ethanol in preparation for embedding in araldite Cy 212 Agar-R1030 (epoxy resin kit). Thin sections were subsequently stained with uranyl acetate and lead citrate and evaluated with LEO 906 E transmission electron microscope.

Statistical analysis

Data are expressed as median (min–max). Comparison of histopathological and radiological scores between groups was made using the Kruskal–Wallis one way analysis of variance on Ranks test (KW). Post-hoc comparisons on parameters were performed using Dunn's procedure. Statistical significance was set at $P < 0.05$. All analyses were performed with the statistical package for scientist (SİGMASTAT) Windows version 3.10.

Results

Radiographical evaluation

Simple transverse fracture was detected in all radius and ulna radiographically after production of fracture (Fig. 1).

At the end of the first week, there was significant difference between the mean scores ($P < 0.05$). The multiple comparison of the groups showed that there was a statistical difference between Group 2 and Group 5 ($P < 0.001$).

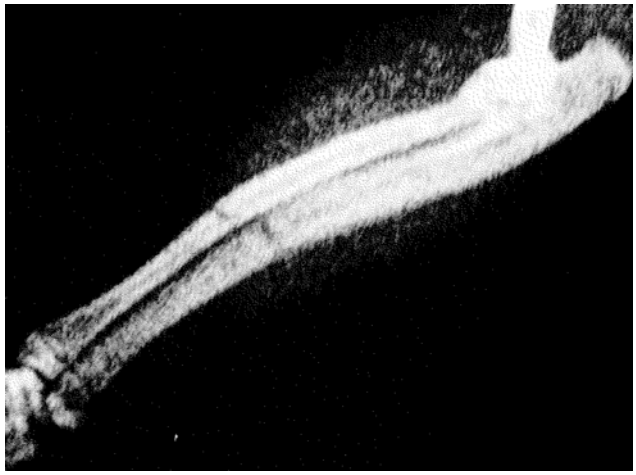

Fig. 1 Simple transverse fracture on rat's radius and ulna in Group 5 subject

There was significant difference between the mean scores at the end of the second week ($P < 0.001$). The multiple comparison of the groups showed that there was significant difference between Group 2 and Group 5 ($P < 0.01$).

At the end of the third week, variable *healing* of fractures were observed between the groups. The most significant fracture healing was observed in Group 5 (Table 2) (Fig. 2) when compared with the second group, showing enhanced consolidation after 22 days. Six of the animals in Group 2 were found to have "no bridging" upon radiological examination. There was statistical difference between the mean scores of the groups ($P < 0.001$). The multiple comparison of the groups showed that there were significant differences between Group 1 and Group 2 ($P < 0.01$) and Groups 2 and 5 ($P < 0.05$).

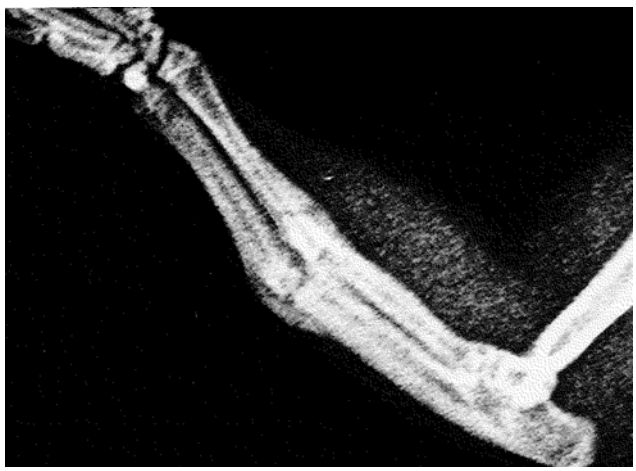

Fig. 2 Complete bone union and callus formation at the end of third week of the same subject in Group 5

Table 2 Statistical findings of the groups radiologically

Group		Control $n = 10$	Zymosan $n = 10$	Zymosan and DMSO $n = 10$	Zymosan and EGb 761 $n = 10$	Zymosan and Vitamin C $n = 10$	P
Week 1	Median (min–max)	0 (0–1)^{ab}	0 (0–0)^{a}	0 (0–1)^{ab}	0 (0–1)^{ab}	1 (0–2)^{b}	$P < 0.05$
Week 2	Median (min–max)	7 (0–8)^{a}	2 (0–5)^{b}	4 (0–7)^{ab}	5.5 (0–7)^{ab}	7 (0–9)^{a}	$P < 0.001$
Week 3	Median (min–max)	9 (1–9)^{a}	4 (0–6)^{b}	6 (1–9)^{ab}	6 (1–9)^{ab}	8 (1–10)^{a}	$P < 0.001$

Kruskal-Wallis test to compare radiological scores between groups; statistically significant ($P < 0.05$) different radiological scores between groups were labeled with different letters

Table 3 Grading system of fracture recovery scores

Histological evaluation	Grade
Pseudoarthrosis formation	0
Incomplete cartilaginous union	1
Complete cartilaginous union	2
Incomplete bony union	3
Complete bony union	4

Histopathological evaluation

Complete bony union was detected in nine rats in control group (Group 1). Pseudoarthrosis formation was observed in one rat.

In rats of zymosan administered group (Group 2), six were observed to have cartilaginous union, two were observed to have incomplete bony union and two were observed to have pseudoarthrosis formation. In animals which were administered DMSO with zymosan (Group 3), four were observed to have incomplete bony union, one was observed to have complete bony union, three were observed to have complete cartilaginous union and two were observed to have pseudoarthrosis formation. In animals that were administered EGb 761 with zymosan (Group 4), five were observed to have complete cartilaginous union, three were observed to have incomplete bony union, one was observed to have complete bony union and one was observed to have pseudoarthrosis formation. In animals that were given vitamin C (Redoxon) with zymosan (Group 5), seven were observed to have incomplete bony union, one was observed to have complete bony union, one was observed to have complete cartilaginous union and one was observed to have pseudoarthrosis formation.

Histopathological evaluation showed that there was complete bony union in H.E stained preparations in control group with a healthy development of the cartilage, bone and the bone marrow (Fig. 3). Complete cartilaginous union was predominant in H.E stained sections of Group 2. Destruction was observed in bony tissue (bone resorption) (Fig. 4). Incomplete bony union and complete cartilaginous union were predominant in H.E stained sections of Group 3 (Fig. 5).

Complete cartilaginous union and incomplete bony union were predominant in H.E stained sections of Group 4 (Fig. 6). Incomplete bony union was predominant in H.E stained sections of animals in Group 5 (Fig. 7).

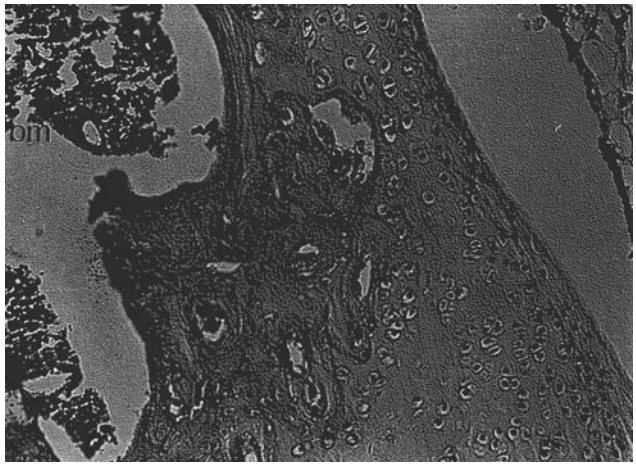

Fig. 3 Hyaline cartilage (*h*), bone (*b*) and bone marrow (*bm*) in control group (stain, hematoxylin and eosin; original magnification, ×50)

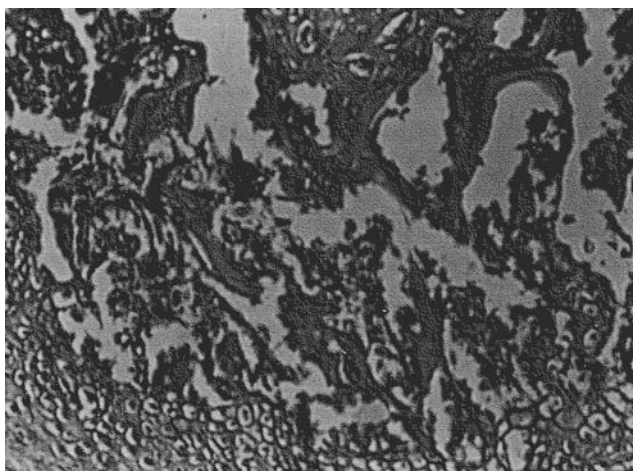

Fig. 4 Bone resorption in only zymosan administered group (Group 2) (stain, hematoxylin and eosin; original magnification, ×50)

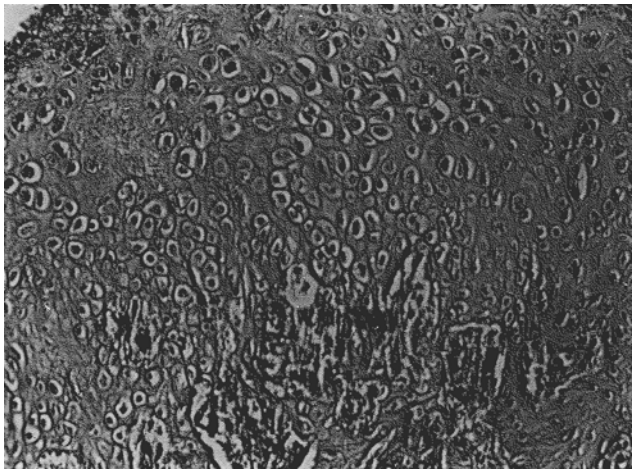

Fig. 5 Incomplete bony union and complete cartilaginous union in DMSO administered group with zymosan (Group 3) (stain, hematoxylin and eosin; original magnification, ×50)

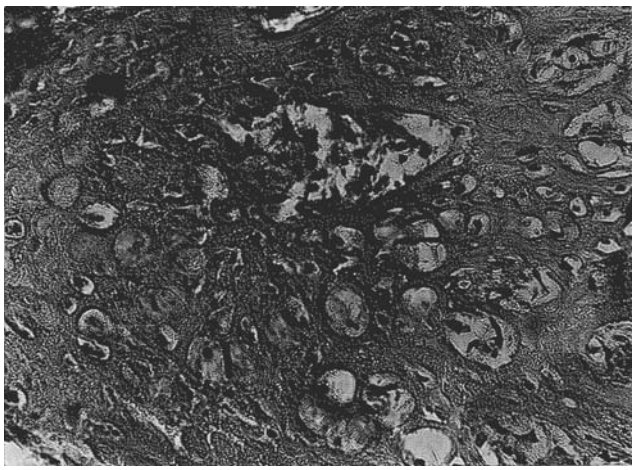

Fig. 6 Complete cartilaginous union and incomplete bony union in EGb.761 administered group with zymosan (Group 4) (stain, hematoxylin and eosin; original magnification, ×100)

Statistical analysis showed that there was significant difference between mean histological scores of the groups at the end of the third week ($P < 0.001$) (Table 4). There was significant difference between Groups 1 and 2 ($P < 0.001$), and Groups 1 and 3 and Groups 1 and 4 ($P < 0.05$) with multiple comparisons. There were similar histological scores between Groups 1 and 5 ($P > 0.05$).

Electron microscopic evaluation

Findings of bone healing such as macrophages, fibroblasts, undifferentiated mesenchymal cells and collagen fibrils were observed in the control group (Fig. 8). In Group 2, bone destruction and undifferentiated migrating mesenchymal cells were observed (Fig. 9). On the other hand, collagen fibrils were observed in Group 5 treated with vitamin C (Fig. 10).

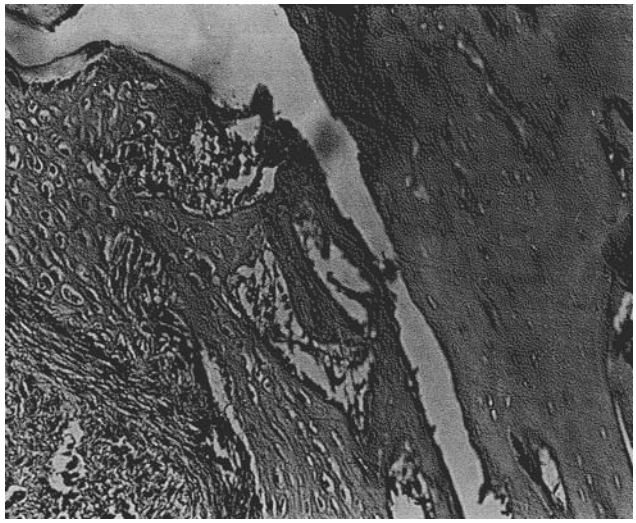

Fig. 7 Incomplete bony union in vitamin C administered group with zymosan (Group 5) (stain, hematoxylin and eosin; original magnification, ×50)

Discussion

The administration of zymosan to the rats in Group 2 significantly inhibited the fracture healing. The negative effects of zymosan on fracture healing were reduced by the addition of vitamin C in Group 5. Histological and radiological evaluation demonstrated that zymosan inhibited fracture healing in rats and vitamin C prevented this effect at least partially.

Inflammation is a step in fracture healing and involves just 5 days after fracture. Inflammation is an event occurring in all injured tissues. Blood, lymph and tissue exudate accumulates in between tissues as a result of destruction of endosteum, periosteum and surrounding soft tissue when bone is fractured. This accumulate named fracture hematoma involves the basic elements for fracture healing. After this first step, inflammatory changes like vasodilatation in soft tissues and leukocyte exudation from plasma appear. Soon, as a response, polymorphonuclear leukocytes (PNLs), histiocytes and macrophages increase in number. The early period of fracture healing is very important. Most of the biological insufficiencies appear at the first week after fracture [12, 22].

Since we obtained the autopsy materials on the 22nd day of production of fracture, we were unable to observe the inflammatory stage. We observed that there was a significant decrease in the callus formation in rats treated with zymosan. These changes were also partially prevented by administration of vitamin C in Group 5.

Oxygen free radicals are ubiquitous compounds that occur naturally in all biological tissues. They contain reactive unpaired electron which can attack susceptible chemical

Table 4 Statistical findings of the groups histopathologically

Groups	Control $n = 10$	Zymosan $n = 10$	Zymosan and DMSO $n = 10$	Zymosan and EGb 761 $n = 10$	Zymosan and Vitamin C $n = 10$	P
Median (min–max)	4 (0–4)[a]	2 (0–3)[b]	2.5 (0–4)[b]	2 (0–4)[b]	3 (0–4)[ab]	$P < 0.001$

Kruskal-Wallis test to compare histopathological scores between groups; statistically significant ($P < 0.05$) different histopathological scores between groups were labeled with different letters

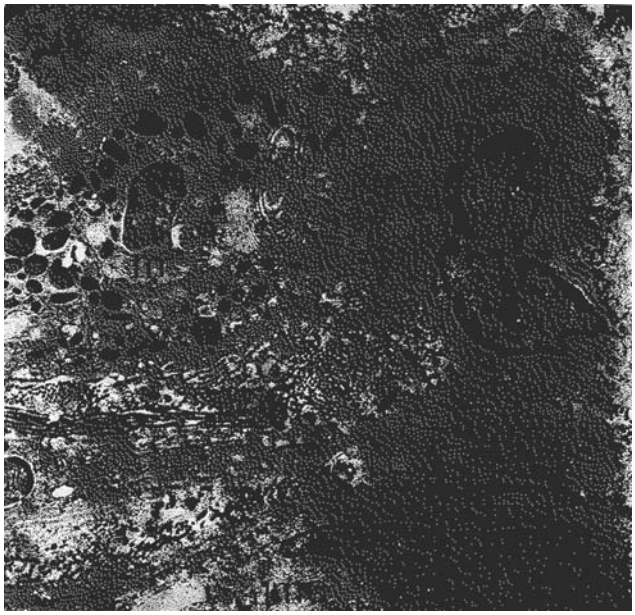

Fig. 8 Picture showing macrophage (*m*), fibroblast (*f*), undifferentiated mesenchymal cell (*imc*) and collagen fibrils (*c*) in control group (original magnification, ×7,750)

Fig. 9 Picture showing undifferentiated mesenchymal cell (*imc*) and bone damage in zymosan administered group (original magnification, ×6,000)

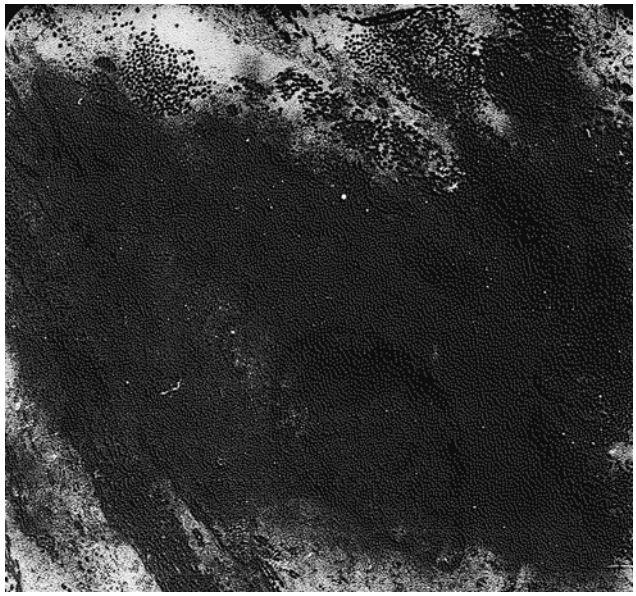

Fig. 10 Picture showing collagen fibrils (*c*) produced by osteoblasts in vitamin C administered group (original magnification, ×7,750)

groups on all classes of macromolecules in the cell. Under aerobic conditions, the damage to macromolecules caused by free radicals is minimized by endogenous scavengers which convert the free radicals to less reactive and therefore less toxic forms [30]. Oxygen free radicals, the superoxide anion, the hydroxyl radical and their intermediary, hydrogen peroxide, are believed to be generated during ischemia and at the time of reperfusion. These reactive oxygen species can be cytotoxic by attacking fatty acids, which leads to lipid peroxidation of membranes, and reacting with proteins, including destruction of amino acids, oxidation of sulfhydryl groups and polypeptide chain [23, 30].

When free oxygen radicals were generated in the bone environment, osteoclasts were formed and bone resorption occurred. It is possible that the generation of oxygen-derived free radicals may be particularly important in the bone resorption that occurs in association with inflammatory diseases [10, 54]. Oxygen-derived species are produced by activated phagocytes including monocytes, macrophages and neutrophils. Since these cells accumulate adjacent to bone surfaces in chronic inflammatory diseases, radical production by these cells could be responsible for stimulating osteoclast formation or activation to resorb bone [23, 54].

Antioxidants are essential molecules in preventing the cellular damage caused by free radicals. Oxygen-related free radicals are produced in inflamed and ischemic tissues. These free radicals are responsible for tissue destruction by lipid peroxidation of biologic membranes. In normal metabolism, there is a balance between the generation of free radicals and antioxidant defense mechanism [20, 52]. These findings have led us to speculate if free radicals play an important role in inflammation and induction of reperfusion-induced muscle and tendon damage as well as fracture healing.

Several compounds are able to prevent the damage induced by oxygen free-radicals [20, 23, 27, 32, 43, 53]. We have used DMSO, Ginkgo biloba extract and vitamin C to prevent the negative effects of zymosan on fracture healing process.

It is known that free radicals formed by activated polymorphonuclear cells (PNL) disrupt wound healing and granulation tissue. In the studies of Foschi et al. [19, 20] on the effects of free oxygen radicals on wound healing and granulation tissue, they used zymosan to produce free oxygen radicals in PNL by stimulation of NADPH oxidase. They used zymosan with a dose of 100 mg/kg for 5 days and showed that this dosage produced enough free oxygen radicals to produce a pathological condition. We also observed such findings of destruction in bone tissue in light and electron microscopy in Group 2 (Figs. 4, 9).

Zymosan, being a 3–5 μm cell wall fragment of *Saccharomyces cerevisiae*, is formed by polysaccharide, protein and lipid [14, 18]. Glucan, a polysaccharide compound, forms 60% of the dry weight of zymosan. A second polysaccharide mannan forms 20% of zymosan. Although glucan being the main component in the activation of PNL, mannan also plays an important role in the transmembrane activation of respiratory burst in these cells [31, 55]. Zymosan is a xenobiotic known to stimulate oxygen free radicals production by stimulation of NADPH oxidase of the polymorphonuclear cells. It increases the anion superoxide radical (with formation of such secondary toxic products as H_2O_2, the singlet oxygen, OH• and hypochloric acid) and by this way necrotic effects are induced in the target tissues [14, 44].

Dimethyl sulfoxide (C_2H_6OS), first found in 1866, has the ability of using H• atoms in unsaturated sulfur atom in methyl group, and is a strong scavenger of hydroxyl radical [27, 29]. It traps the hydroxyl radical (OH•), dimethyl sulfide (DMS) and the oxygen radical [7]. In addition, it inhibits thrombocyte aggregation, protects cell membrane components and mitochondrial oxidative phosphorylation, decreasing inflammatory response. DMSO possesses many in vivo properties that mediate its effect on musculoskeletal trauma. Besides these, DMSO has prostaglandin and fibroblast inhibiting, immune modulating and pain relieving effects [28, 40].

There was a significant difference histopathologically when Group 3 animals were compared to the control group. The difference was not significant when it was compared to the other groups. Therefore, DMSO has not preventive effect on reversing the detrimental effects of zymosan on fracture healing.

Both classic drug–receptor interactions and certain enzyme inhibition must be considered to explain the therapeutic effects of EGb 761 [13]. It may prolong the half-life of endothelium-derived relaxing factor by scavenging superoxide anions (O_2•–), and thus stimulate the relaxation of contracted blood vessels. In addition, the ginkgolide constituents of the extract, especially ginkgolide B, may inhibit platelet aggregation and O_2 generation induced by platelet-activating factor [6].

In vitro and in vivo experimental studies in humans and animals have indicated that EGb 761 has significant free radical scavenging activity [2]. It leads to the inhibition of the formation of lipid peroxides in the brain and liver microsomes, and could protect the retina against lipoperoxidation, reperfusion-induced injury and argon laser (photocoagulation) damage [25, 32, 53]. Indeed, we found a significant difference histologically between Group 4 animals and the control group. The difference was not significant when it was compared to the other groups. Therefore, EGb 761 has not preventive effect on reversing the detrimental effects of zymosan on fracture healing. When Group 2 and Group 4 have been compared for litic lesions of cartilage and bone tissue histopathologically, Group 4 showed significant improvement. There were also histopathological findings of callus tissue formation to bone tissue in Group 4.

Vitamin C is a water-soluble free radical scavenger and is found in many fruits and vegetables, acting as an antioxidant. It is required for the optimal function of a number of enzymes. Its deficiency causes scurvy and poor wound repair [3, 33, 43]. Matsuda et al. extensively investigated the effects of high-dose vitamin C therapy on dermal burns [35, 36] and found that it stops the progression of vascular permeability after burns and, therefore, reduces the microvascular leakage of fluid and protein. It also reduces lipid peroxidation after burns [37]. Lipid peroxidation damages the microvascular endothelial cells, thereby increasing capillary permeability [15]. The lipid peroxide in the cell membrane can only be scavenged by vitamin E, the primary lipid-soluble small-molecule antioxidant, [8] producing a vitamin E free-radical complex [4, 41]. In the extracellular fluid vitamin C, the terminal water-soluble small-molecule antioxidant, acts on this complex and removes the free radical moiety, regenerating vitamin E. Vitamin C is a natural antioxidant that can scavenge hydroxyl radicals [5] and superoxide radicals that produce hydroxyl radicals [56]. By scavenging these radicals, vitamin C stops free-radical reactions and prevents the propagation of chain reactions,

protecting the capillary endothelium and circulating cells such as erythrocytes and leukocytes [4, 41].

When the animals in Group 5 were compared to the animals with control group, the difference was not found both histologically and radiologically. When Group 5 was compared to the second group, there was significant difference radiologically. The difference was not significant when it was compared to the other groups. Even though histopathological results showed that there was significant difference among the second, third, fourth groups and the control group, the fifth group's and control group's histopathological scores were similar. This result demonstrates that vitamin C administered group prevented the detrimental effects of zymosan and showed similar histopathological results as control group.

We conclude that free oxygen radicals have a role in the disruption of fracture healing, and vitamin C can partially prevent the negative effects of zymosan on fracture healing. Further investigations are needed to clarify the mechanism of the prevention of negative effects of oxidants.

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