Is Ebselen A Therapeutic Target in Fracture Healing?

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ABSTRACT

Objective: We investigated the effect of ebselen on fracture healing in an experimental fracture model.

Materials and Methods: We divided rats into two groups, 6 rats in each: the experimental femur fracture control group and the ebselen treatment group with an experimental femur fracture. In the experimental femur fracture control group, we created only experimental femur fracture. In the ebselen treatment group, we administered ebselen treatment with creating an experimental femur fracture. We administered ebselen intraperitoneally at 5 mg/kg once daily for 1 month after the 1st day of experimental femur fracture in the ebselen treatment group. We evaluated the recovery status of fractured femurs at the end of 1st month with radiographic, histopathological, and immunohistochemical methods.

Results: According to the radiographic fracture healing scores, ebselen treatment increased the extent of new bone formation and fracture cartilage callus significantly compared to the control group. According to the histopathological recovery scores, ebselen treatment significantly improved healing scores compared to the control group. Ebselen treatment increased the expression scores of bone healing markers in the ebselen treatment group, such as vascular endothelial growth factor and osteocalcin, compared to the control group.

Conclusion: We demonstrated that ebselen treatment increases the formation of new bone in the femur in an experimentally created femoral fracture model. Ebselen has been shown to improve the bone fracture healing in a radiological and histopathological manner, and more detailed studies are needed.

Keywords: Bone, ebselen, fracture healing, osteocalcin, vascular endothelial growth factor

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Introduction

The fracture healing process is a serious health problem that may lead to a reduced quality of life [1]. Fractures often require surgical procedures. To accelerate bone healing; prevent avascular necrosis, infection, and osteomyelitis; and reduce complications such as nonunion, new medical treatments that are recommended after surgical treatment have been developed [2, 3].

The bone tissue can repair and renew itself. The fracture healing process can be divided into three stages: (1) the reactive phase, (2) the repair phase, and (3) the remodeling phase [4]. The reactive phase lasts for approximately 1 week and occurs shortly after fracture. It is defined by the granulation tissue formation and injured region inflammation. The repairing phase is associated with the fracture callus formation. The remodeling phase continues for 2 months after the fracture.

Osteoblasts, osteoclasts, and the extracellular matrix work together to restore the fractured bone segment through certain local factors and with bone formation markers such as osteocalcin (OC), hydroxyproline, and bone alkaline phosphatase. Growth factors such as the vascular endothelial growth factor (VEGF), fibroblast growth factor, and insulin-like growth factor play important roles. In this process, the antioxidant application cleans free radicals and reduces oxidative stress facilitating the fracture healing [5-7].

Ebselen [C13H9NOSe] is an organocelenium compound (Figure 1) and chemically, it is an electrophile [8]. The general mechanism of action is the reaction with specific cysteine thiol

groups in proteins [9, 10]. Ebselen allows the reactive oxygen species to be catalyzed in a similar way to glutathione peroxidase, and it is a potential chemopreventative for various diseases associated with oxidative stress [11]. An important pharmacological activity of ebselen can be attributed to its antioxidant effect. It shows anti-inflammatory, antiatherosclerotic, antithrombotic, detoxifying, cytoprotective, and antimutagenic [12] properties due to its antioxidant behavior, and it shows antimicrobial activity against several microorganisms [13-21] (Figure 1). Ebselen makes a positive contribution to the bone healing process. In addition, studies have shown that ebselen increases the viability of bone marrow-derived cells by reducing oxidative stress [22]. Histological analyzes confirmed that the ebselen inhibited trabecular bone matrix degradation and osteoclast formation in the bone tissue [23].

In light of this information, we investigated the possible healing effect of Ebselen in an experimental bone fracture model in rats.

Materials and Methods

Chemicals

Drugs	Company
Ebselen	Sigma Chemical
Thiopental sodium	Ulagay
Metamizol sodium	Sanofi-Aventis

All drugs were administered intraperitoneally, and the dose was calculated based on the weight of each rat.

Main Points

- According to the radiographic fracture healing scores, ebselen treatment increased the extent of new bone formation and fracture cartilage callus significantly compared to the control group.
- According to the histopathological recovery scores, ebselen treatment significantly improved healing scores compared to the control group.
- Ebselen treatment increased the expression scores of bone healing markers in the ebselen treatment group, such as vascular endothelial growth factor and osteocalcin, compared to the control group.
- We demonstrated that ebselen treatment increases the formation of new bone in the femur in an experimentally created femoral fracture model.
- Ebselen has been shown to improve the bone fracture healing in a radiological and histopathological manner, and more detailed studies are needed.

Table 1. The 5-point radiographic scoring system [25]

Score	Description
0	No evidence of healing
1	Callus formation evident but fracture gap not bridged
2	Callus formation evident with bridging of the fracture gap but fracture line evident
3	Callus formation evident with bridging of the fracture gap with only faint fracture line
4	Fracture union

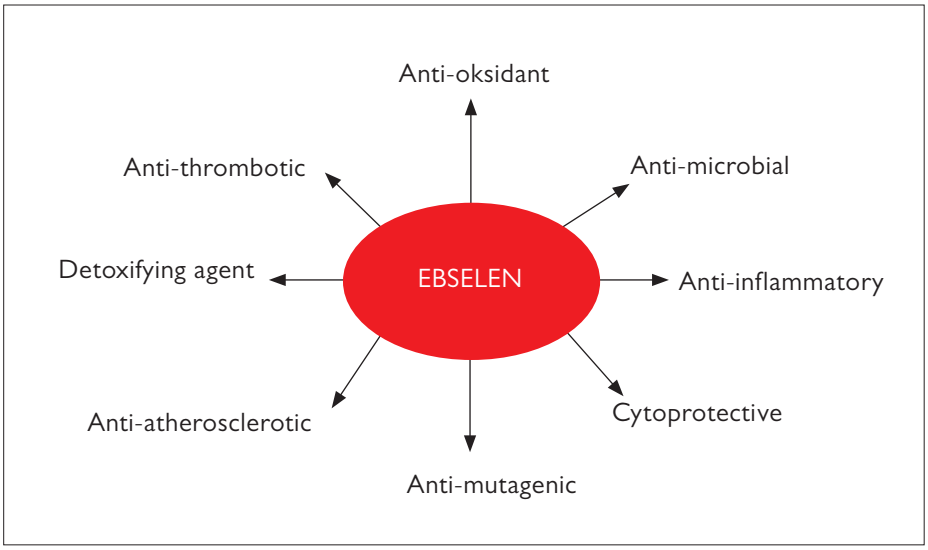

Figure 1. Schematic representation of the properties of ebselen

Twelve male albino Wistar rats weighing from 250 to 300 g were obtained from the Medical Experimental Research Center (10-12 weeks old). The rats were kept in separate groups at normal temperature conditions (22°C) prior to the experiments. The study protocol was approved by the local animal care committee. The rats were placed in standard plastic cages with sawdust bedding in an air-conditioned room at 22±1°C under the 12 h light/dark cycle lighting control. Standard rat feed and tap water were given ad libitum.

It was determined that 5mg/kg would constitute one dose of ebselen, which the literature identified the most effective dose for chronic experiments [24].

All surgical procedures were performed by an orthopedist and under sterile conditions. We divided rats into two groups, 6 rats in each: the experimental femur fracture control group and ebselen treatment group with an experimental femur fracture (5 mg/kg) (ebselen dissolved in 1 ml saline). All rats were anesthetized with intraperitoneal sodium thiopental (20 mg/kg) during the operation. Metamizol sodium (150 mg/kg) was administered intraperitoneally at the beginning of the procedure to prevent postoperative pain. The right hind limb was shaved, a 2 cm lateral parapatellar incision was made, and the patella was placed laterally to expose the distal femoral condyle of the right hind limb. The femoral fracture model was created with transverse femur midsection. After the manual reduction, the fractured femur was fixed with intramedullary Kirschner wires. The wounds were watered and closed using 4-0 nylon sutures, and the soft tissue and skin were closed with 4-0 Vicryl sutures. The rats were allowed to eat and drink freely after the operation. We administered ebselen intraperitoneally at 5 mg/kg once daily for 1 month after the 1st day of experimental femur fracture in the ebselen treatment group. Rats were anesthetized with sodium thiopental (20 mg/kg] 1 month after surgery and euthanized for tissue collection. For the histopathological analysis, femurs were stored in 10% buffered formalin.

X-ray images of the fractured femurs were evaluated to determine the stages of fracture healing. The healing of the fractured femurs was assessed by X-ray images using a modified 5-point radiographic scoring system [25] (Table 1). For X-ray analyzes, a researcher blind to treatment groups evaluated the x-ray films.

Table 2. Histomorphometric analysis measurements

Group (n=5)	Callus area (mm^2)	New bone (%)	Cartilage (%)
No treatment group	22±2.82	15±0.89	15±0.89
Ebselen 5 mg/kg treatment group	21±1.67	22±1.41*	18±1.41*

Data represent mean values standard±deviation
*means p<0.05 according to the control group

Table 3. Histological fracture healing scores

Group (n=5)	Score (1-10)
No treatment group	4.5±0.54
Ebselen 5 mg/kg treatment group	6.5±0.54

Data represent mean values standard±deviation
*means p<0.05 according to the control group

Table 4. VEGF and OC expression scores in groups

	Control	Ebselen
VEGF	+1	+3
OC	+1	+2

Grade 0: - (negative), Grade 1: +1 (mild), Grade 2: +2 (moderate), Grade 3: +3 (severe)

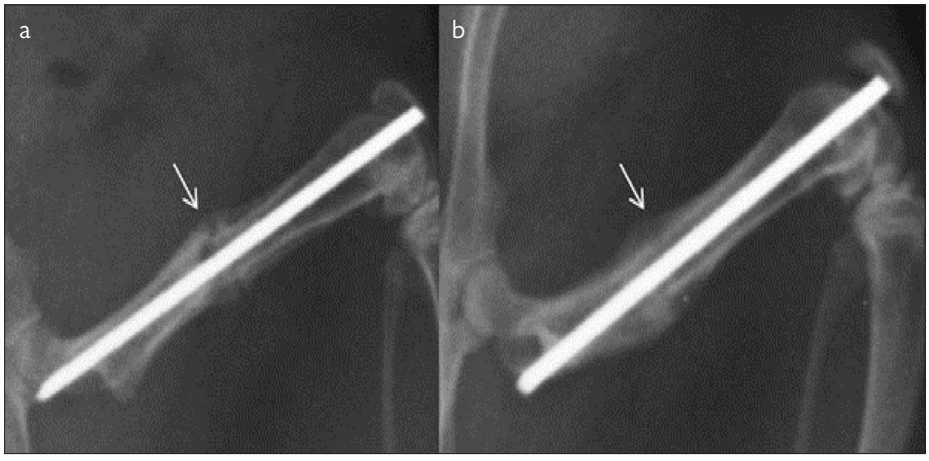

Figure 2. a, b. (a) Fracture control, (b) Ebselen treatment

For histopathological analysis, rats were euthanized on the 30th day after the operation. The femurs were collected and placed in 10% buffered formalin. Paraffin-embedded longitudinal 4-5 µm sections were taken and stained with toluidine blue. Histopathological examination was performed at a 94X magnification. The cartilage area and newly formed bone region, as the percentage of total fracture callus area, was measured by ImageJ (Version 1.46r) [26]. Fractures to describe the degree of recovery were scored using a method described previously; a score of 1 in immature healing and a score of 10 indicates an advanced mature callus [27]. A researcher evaluated the samples blinded for treatment groups for histopathological analyzes

Randomized sections were obtained for all rats, and immunohistochemical staining was performed. Paraffin block sections were cut to 4-5 µm thick slices for immunohistochemical evaluation using the Leica BOND system, Leica BOND dewax solution (AR9222), Leica BOND epitope taking solution 1 (AR9961), and Leica BOND polymer thinning detection (DS9800). The epitope recovery was performed for 20 minutes, followed by OC and VEGF primary antibody (Novocastra, UK). VEGF is a necessary mediator during angiogenesis. The systemic and local effects of OC are potentially caused by bone remodeling. The VEGF and OC expression scores were compared in ebselen treatment and control groups. Immunohistochemical staining was observed under light microscopy (9100X magnification) (BX51; Olympus, Japan).

Results

In anteroposterior and lateral X-ray images, bone healing was assessed using a modified 5-point radiographic scoring system [25] (Figure 2, Table 1). Accordingly, the fracture healing score of the rats in the control group was 2.0±0.89, and the fracture healing score of the rats in the ebselen treatment group was 3.5±0.54 (Figure 3). The fracture healing scores of the ebselen treatment group were significantly higher than the control group (p<0.05).

The percentage of new bone formation and cartilage callus in the ebselen treatment group were significantly higher than the control group (p=0.05) (Figures 2a, 2b, Figures 4a, b). There was a statistically significant difference in the group treated with ebselen according the histopathological recovery scores (Table 3).

The VEGF and osteocalcin expression scores in the groups were assessed (Table 4). Osteocalcin-producing cells were detected as positive on the periosteal surface and closed the fracture site. For VEGF, staining was found in hypertrophic chondrocytes. Immunohistochemistry of the control group animals for osteocalcin and VEGF showed mild positivity (Table 4, Figures 4c and 4d). The semi-quantitative analysis showed a higher number of positive cells in the treatment group than the control group (Figure 4). In the treatment group, mild to severe immunopositivity was observed for VEGF (Figure 4e). These cells are often seen in blood vessels. In the treatment group, mild to moderate immunopositivity was observed for osteocalcin (Figure 4f).

Discussion

In our study, we investigated possible effects of ebselen on fracture healing. In the radiographic examinations, the bone healing scores of the rats in the ebselen treatment group were significantly better than in the control group at the end of 1st month. The radiographic improvement score was significantly higher in the ebselen treatment group than in the control group at the end of one month. Ebselen treatment was found to be very effective in improving both the new bone and callus formation.

A histopathological examination showed that the callus and new bone formation were increased in the ebselen treatment group compared to the control group. According to the histopathological bone healing scores, the ebselen treatment significantly increased the bone healing. Histopathological findings have shown

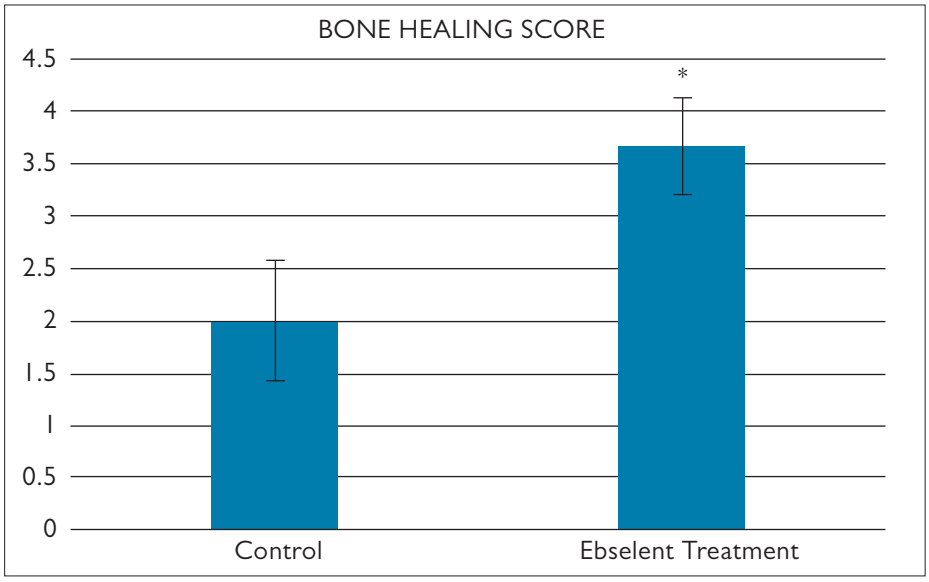

Figure 3. X-ray control scores in ebselen treatment group.
*means p<0.05 according to the control group

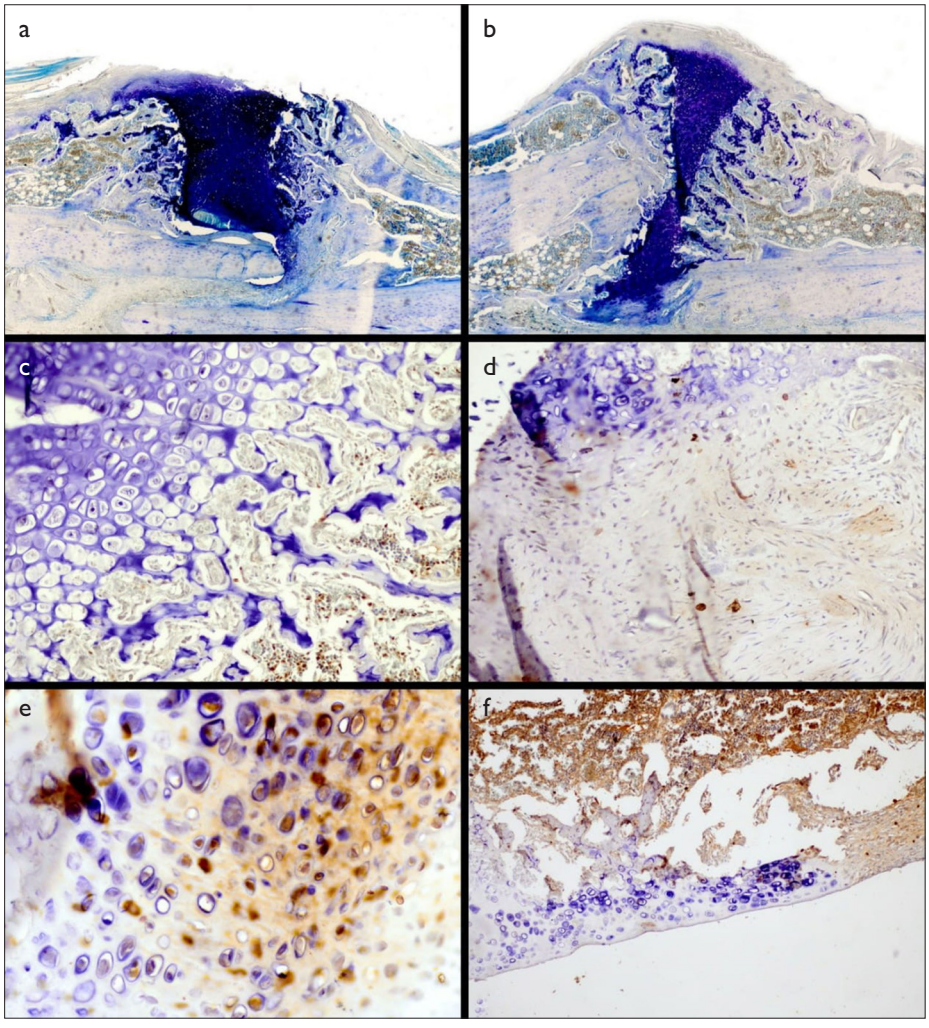

Figure 4. a-f. a) No treatment group. Toluidine blue. Magnification is 100X. b) Ebselen treatment group histology. Toluidine blue. Magnification is 100X. c) Immunohistochemistry shows the VEGF labeling in the control group animal. The magnification is 200X. d) Immunohistochemistry shows OC labeling in the control group animal. The magnification is 200X. e) Immunohistochemistry illustrates the VEGF labeling in the ebselen treatment group. The magnification is 200X. f) Immunohistochemistry shows OC labeling in the ebselen treatment group animal. The magnification is 200X.

that the ebselen treatment increases bone healing with intense callus formation.

VEGF is important for vascularity formation and angiogenesis, playing an important role in bone development [28]. Previous studies have shown that VEGF increases during bone healing, and this increase is favorable [29, 30]. In our study, VEGF levels were higher in the ebselen treatment group than in the control group at the end of 1 month. We conclude that this increase may be due to the fact that ebselen is an anti-inflammatory and antioxidant drug.

Osteocalcin (OC) is an important matrix protein obtained from osteoblasts, and it plays an important role in bone healing [31]. OC plays an important role in bone fracture healing [32]. It has been shown that OC is synthesized from osteoblasts in bone diseases such as osteoporosis and plays a role in the balance of bone mineralization and calcium ions [33]. In one study, OC levels decreased after bone fractures, and OC levels increased after treatment [34]. In our study, it was shown that OC levels increased in the ebselen treatment group compared to the control group. We conclude that this increase may be due to the fact that ebselen is an anti-inflammatory and antioxidant drug.

The limitations of our study are the small number of animals and single dose drug application.

The protective effect of ebselen was shown radiographically and histopathologically in rats. The protective effects of ebselen on bone healing were explained by VEGF and OC with immunohistochemistry in addition to radiographic scores. We concluded that ebselen accelerates the healing of bone fractures. However, further detailed studies on this subject are necessary.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Atatürk University (26102011/10-71).

Peer-review: Externally peer-reviewed.

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Conflict of Interest: Authors have no conflicts of interest to declare.

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