Original Article

DOI: 10.5152/eurasianjmed.2019.18443

Is Ebselen A Therapeutic Target in Fracture Healing?

Kose A[1], Kose D[2], Halici Z[3], Aydin A[4], Ezirmik N[5], Karsan O[6] Toktay E[7]

[1] Orthopedics surgeon, Department of Orthophedics and Traumatology, Erzurum Regional Training and Research Hospital, Erzurum, Turkey

[2] Medical Doctor, Department of Pharmacology, Faculty of Medicine, Ataturk University, Erzurum, Turkey

[3] Medical Doctor, Professor, Department of Pharmacology, Faculty of Medicine, Ataturk University, Erzurum, Turkey

[4] Orthopedics surgeon, Associate professor, Department of Orthophedics and Traumatology, Faculty of Medicine, Ataturk University, Erzurum, Turkey

[5] Orthopedics surgeon, Professor, Department of Orthophedics and Traumatology, Faculty of Medicine, Ataturk University, Erzurum, Turkey

[6] Orthopedics surgeon, Professor, Department of Orthophedics and Traumatology, Faculty of Medicine, Ataturk University, Erzurum, Turkey

[7] PhD student, Department of Histology and Embryology, Faculty of Medicine, Ataturk University, Erzurum, Turkey

Running title: **Ebselen in Fracture Healing**

Corresponding author: Ali Aydin, Orthopedics surgeon, Associate professor, Department of Orthophedics and Traumatology, Faculty of Medicine, Ataturk University, Erzurum, Turkey

aliaydin@atauni.edu.tr

Acknowledgements : This work was supported by the Ataturk University Medical Research Council (grant number 2011/274).

You may cite this article as: Kose A, Kose D, Halici Z, et al. Is Ebselen A Therapeutic Target in Fracture Healing? Eurasian J Med 2019; 10.5152/eurasianjmed.2019.18443.

Is Ebselen A Therapeutic Target in Fracture Healing?

Abstract

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

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

Results: According to the radiographic fracture healing scores, ebselen treatment increased the percentage 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 such as vascular endothelial growth factor and osteocalcin compared to the control group in the ebselen treatment group. **Discussion:** We demonstrated that ebselen

treatment increases the formation of new bone in femur in experimentally created femoral fracture model.

Conclusion: Ebselen has been shown to improve bone fracture healing in a radiological and histopathological manner and more detailed studies are needed.

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

Introduction

Fracture healing process is a serious health problem due to reduced quality of life. [1]. They often require surgical procedures. In order to accelerate bone healing, avascular necrosis, infection, prevent osteomyelitis and reduce complications such as nonunion, new medical treatments recommended after surgical treatment have been developed [2, 3].

Bone tissue can repair and renew itself. The fracture healing process divide three stages: 1. reactive phase 2. repair phase and 3. remodeling phase [4]. The reactive phase lasts for approximately one week and occurs shortly after fracture. It is defined by 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 like osteocalcin, hydroxyproline and bone alkaline phosphatase. Growth factors such as vascular endothelial growth factor [VEGF], fibroblast growth factor and insulin-like growth factor take important roles in these

overcoming. In this process, antioxidant application cleans free radicals and reduces oxidative stress and facilitates 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 reactions 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 ; 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, anti-thrombotic, detoxifying, cytoprotective and anti-mutagenic [12] properties due to its antioxidant behavior and 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 bone tissue [23].

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

Matarials and Methods

Chemicals

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

All drugs was administered intraperitonealy and calculated on the weight of the each rat and was administered intraperitonealy.

Animals

Twelve male albino Wistar rats 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 bed in an air-conditioned room at 22 ± 1 ° C under 12 h light and 12 h dark cycle lighting controls. Standard rat feed and tap water were given as ad libitum.

Dose Selection

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

Bone fracture model in rat femur

All surgery was performed by an orthopedist and under sterile conditions. We divided rats into two groups of 6 rats in each group: experimental femur fracture control group and ebselen treatment group with 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. Femoral fracture model was created with transverse femur midsection. After manual reduction, the fractured femur was fixed with intramedullary Kirschner wires. The wounds were watered and closed using 4-0

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as: Kose A, Kose D, Halici Z, et al. Is Ebselen A Therapeutic Target in Fracture Healing? Eurasian J Med 2019; 10.5152/eurasianjmed.2019.18443.

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 first day of experimental femur fracture in the Ebselen treatment group. Rats were anesthetized with sodium thiopental [20 mg / kg] one mounth after surgery and euthanized for tissue collection. For histopathological analysis, femurs were stored in 10% buffered formalin.

X-ray imaging

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 five-point radiographic scoring system [25] [Table 1]. For X-ray analyzes, a researcher blind to treatment groups evaluated the x-ray films.

Histopathological procedures

For histopathological analysis, rats were euthanized on the 30th day after the operation. The femurs were collected, placed in 10% buffered formaline. Paraffin-embedded longitudinal 4-5 µm sections were taken and stained with toluidine blue. Histopathological examination performed at 94 magnification. Cartilage area and newly formed bone region, as a 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

Immunohistochemistry

Randomized sections were obtained for all rats and immunohistochemical staining was performed. Paraffin block sections were cut to 4-5 µm thickness 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]. Epitope recovery was performed for 20 minutes, followed by osteocalcin and VEGF primary antibody [Novocastra, UK]. VEGF is a necessary mediator during angiogenesis. The systemic and local effects of osteocalcin are potentially caused by bone remodeling. VEGF and osteocalcin expression scores were compared in Ebselen treatment and control groups. Immunohistochemical staining was observed under light microscopy [9100 magnification] [BX51; Olympus, Japan].

Results

X-ray imaging

In anteroposterior and lateral X-ray images, bone healing was assessed using a modified five-point radiographic scoring system [25] [Figure 2, Tab 1]. Accordingly, the fracture healing score; 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 [$p < 0.05$].

Histological evaluation of fracture healing

The percentage of new bone formation and cartilage callus in the ebselen treatment group were significantly higher than the control group [$p = 0.05$] [Figure 2A, 2B, Figure 4A-B]. There was a statistically significant difference in the group treated with ebselen according to the histopathological recovery scores [Table 3].

Immunohistochemistry

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as: Kose A, Kose D, Halici Z, et al. Is Ebselen A Therapeutic Target in Fracture Healing? Eurasian J Med 2019; 10.5152/eurasianjmed.2019.18443.

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 [Tab 4, Figure 4C,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 modarate immunopositivity was observed for osteocalcin [Figure 4F].

Discussion

In our study, we investigated the 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 the control group at the end of one mounth. The radiographic improvement score was significantly higher in the ebselen treatment group than the control group at the end of one mounth. Ebselen treatment was found to be very effective in improving both new bone and callus formation.

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

VEGF is important for vascularity and angiogenesis formation and plays 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 one mounth. 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 is important 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.

Conclusion

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 by immunohistochemistry in addition to radiographic scores. We have concluded that Ebselen accelerate the healing of bone fractures. Detailed studies on this subject are necessary.

Conflict of interest

There is no conflict of interest in this study.

REFERENCES

1. Aydin A, Halici Z, Akpinar E ,et al. What is the role of bosentan in healing of femur fractures in a rat model? J Bone Miner Metab. 2015 Sep;33[5]:496-506.

2. Giannoudis PV, Kontakis G, Christoforakis Z, Akula M, Tosounidis T, Koutras C. Management, complications and clinical results of femoral head fractures. Injury. 2009 Dec;40[12]:1245-51.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as: Kose A, Kose D, Halici Z, et al. Is Ebselen A Therapeutic Target in Fracture Healing? Eurasian J Med 2019; 10.5152/eurasianjmed.2019.18443.

3. Papakostidis C, Bhandari M, Giannoudis PV. Distraction osteogenesis in the treatment of long bone defects of the lower limbs: effectiveness, complications and clinical results; a systematic review and meta-analysis. Bone Joint J. 2013 Dec;95-B[12]:1673-80.

4. Giannoudis PV, Jones E, Einhorn TA. Fracture healing and bone repair. Injury. 2011 Jun;42[6]:549-50.

5. Xu H, Watkins BA, Seifert MF. Vitamin E stimulates trabecular bone formation and alters epiphyseal cartilage morphometry. Calcif Tissue Int. 1995 Oct;57[4]:293-300.

6. Sarisozen B, Durak K, Dincer G, Bilgen OF. The effects of vitamins E and C on fracture healing in rats. J Int Med Res. 2002 May-Jun;30[3]:309-13. PubMed PMID: 12166348.

7. Estai MA, Suhaimi FH, Das S ,et al. Piper sarmentosum enhances fracture healing in ovariectomized osteoporotic rats: a radiological study. Clinics [Sao Paulo]. 2011;66[5]:865-72. PubMed PMID: 21789393.

8. Sakurai T, Kanayama M, Shibata T ,et al. Ebselen, a seleno-organic antioxidant, as an electrophile. Chem Res Toxicol. 2006 Sep;19[9]:1196-204.

9. Ullrich V, Weber P, Meisch F, von Appen F. Ebselen-binding equilibria between plasma and target proteins. Biochem Pharmacol. 1996 Jul 12;52[1]:15-9.

10. Zhao R, Masayasu H, Holmgren A. Ebselen: a substrate for human thioredoxin reductase strongly stimulating its hydroperoxide reductase activity and a superfast thioredoxin oxidant. Proc Natl Acad Sci U S A. 2002 Jun 25;99[13]:8579-84.

11. Antony S, Bayse CA. Modeling the mechanism of the glutathione peroxidase mimic ebselen. Inorg Chem. 2011 Dec 05;50[23]:12075-84.

12. Miorelli ST, Rosa RM, Moura DJ ,et al. Antioxidant and anti-mutagenic effects of ebselen in yeast and in cultured mammalian V79 cells. Mutagenesis. 2008 Mar;23[2]:93-9.

13. Nakamura Y, Feng Q, Kumagai T ,et al. Ebselen, a glutathione peroxidase mimetic seleno-organic compound, as a multifunctional antioxidant. Implication for inflammation-associated carcinogenesis. J Biol Chem. 2002 Jan 25;277[4]:2687-94.

14. Imai H, Masayasu H, Dewar D, Graham DI, Macrae IM. Ebselen protects both gray and white matter in a rodent model of focal cerebral ischemia. Stroke. 2001 Sep;32[9]:2149-54.

15. Noguchi N, Yoshida Y, Kaneda H, Yamamoto Y, Niki E. Action of ebselen as an antioxidant against lipid peroxidation. Biochem Pharmacol. 1992 Jul 07;44[1]:39-44.

16. Schewe T. Molecular actions of ebselen--an antiinflammatory antioxidant. Gen Pharmacol. 1995 Oct;26[6]:1153-69.

17. Chew P, Yuen DY, Stefanovic N, et al. Antiatherosclerotic and renoprotective effects of ebselen in the diabetic apolipoprotein E/GPx1-double knockout mouse. Diabetes. 2010 Dec;59[12]:3198-207. PubMed PMID: 20823099.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as: Kose A, Kose D, Halici Z, et al. Is Ebselen A Therapeutic Target in Fracture Healing? Eurasian J Med 2019; 10.5152/eurasianjmed.2019.18443.

18. Lindenblatt N, Schareck W, Belusa L, Nickels RM, Menger MD, Vollmar B. Anti-oxidant ebselen delays microvascular thrombus formation in the rat cremaster muscle by inhibiting platelet P-selectin expression. Thromb Haemost. 2003 Nov;90[5]:882-92.

19. Nogueira CW, Rotta LN, Zeni G, Souza DO, Rocha JB. Exposure to ebselen changes glutamate uptake and release by rat brain synaptosomes. Neurochem Res. 2002 Apr;27[4]:283-8.

20. Kasraee B, Nikolic DS, Salomon D, Carraux P, Fontao L, Piguet V, et al. Ebselen is a new skin depigmenting agent that inhibits melanin biosynthesis and melanosomal transfer. Exp Dermatol. 2012 Jan;21[1]:19-24.

21. Morin D, Zini R, Ligeret H, Neckameyer W, Labidalle S, Tillement JP. Dual effect of ebselen on mitochondrial permeability transition. Biochem Pharmacol. 2003 May 15;65[10]:1643-51.

22. Chen J, Li H, Addabbo F ,et al. Adoptive transfer of syngeneic bone marrow-derived cells in mice with obesity-induced diabetes: selenoorganic antioxidant ebselen restores stem cell competence. Am J Pathol. 2009 Feb;174[2]:701-11.

23. Baek JM, Kim JY, Yoon KH, Oh J, Lee MS. Ebselen Is a Potential Anti-Osteoporosis Agent by Suppressing Receptor Activator of Nuclear Factor Kappa-B Ligand-Induced Osteoclast Differentiation In vitro and Lipopolysaccharide-Induced Inflammatory Bone Destruction In vivo. Int J Biol Sci. 2016;12[5]:478-88..

24. Bubolz AH, Wu Q, Larsen BT, Gutterman DD, Liu Y. Ebselen reduces nitration and restores voltage-gated potassium channel function in small coronary arteries of diabetic rats. Am J Physiol Heart Circ Physiol. 2007 Oct;293[4]:H2231-7.

25. Shuid AN, Mohamad S, Muhammad N, et al. Effects of alpha-tocopherol on the early phase of osteoporotic fracture healing. J Orthop Res. 2011 Nov;29[11]:1732-8.

26. Kim SH, Kim JH, You DG, Saravanakumar G, Yoon HY, Choi KY, et al. Self-assembled dextran sulphate nanoparticles for targeting rheumatoid arthritis. Chem Commun [Camb]. 2013 Nov 14;49[88]:10349-51.

27. Huo MH, Troiano NW, Pelker RR, Gundberg CM, Friedlaender GE. The influence of ibuprofen on fracture repair: biomechanical, biochemical, histologic, and histomorphometric parameters in rats. J Orthop Res. 1991 May;9[3]:383-90.

28. Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med. 1999 Jun;5[6]:623-8.

29. Huh JE, Kwon NH, Baek YH, Lee JD, Choi DY, Jingushi S, et al. Formononetin promotes early fracture healing through stimulating angiogenesis by up-regulating VEGFR-2/Flk-1 in a rat fracture model. Int Immunopharmacol. 2009 Nov;9[12]:1357-65.

30. Park CG, Joo MW, Jeong J, Kang YK, Lee DR. Evaluation of the effects of the combination of autologous mesenchymal stem cells and platelet-rich plasma on structural bone allograft healing. Cell Tissue Bank. 2017 Feb 24.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as: Kose A, Kose D, Halici Z, et al. Is Ebselen A Therapeutic Target in Fracture Healing? Eurasian J Med 2019; 10.5152/eurasianjmed.2019.18443.

31. Ingram RT, Clarke BL, Fisher LW, Fitzpatrick LA. Distribution of noncollagenous proteins in the matrix of adult human bone: evidence of anatomic and functional heterogeneity. J Bone Miner Res. 1993 Sep;8[9]:1019-29.

32. Hauschka PV, Lian JB, Cole DE, Gundberg CM. Osteocalcin and matrix Gla protein: vitamin K-dependent proteins in bone. Physiol Rev. 1989 Jul;69[3]:990-1047. PubMed PMID: 2664828.

33. Goldstone AP, Howard JK, Lord GM, Ghatei MA, Gardiner JV, Wang ZL, et al. Leptin prevents the fall in plasma osteocalcin during starvation in male mice. Biochem Biophys Res Commun. 2002 Jul 12;295[2]:475-81.

34. Ngueguim FT, Khan MP, Donfack JH, Tewari D, Dimo T, Kamtchouing P, et al. Ethanol extract of Peperomia pellucida [Piperaceae] promotes fracture healing by an anabolic effect on osteoblasts. J Ethnopharmacol. 2013 Jun 21;148[1]:62-8.

Figure Legends

Figure 1 Schematic representation of the properties of ebselen

Figure 2 A.Fracture control B. Ebselen treatment

Figure 3 X-Ray control scores, Ebselen treatment group. * means p <0.05 according to the control group

Figure 4 A. No treatment group. Toluidine blue. Magnification is 100X. B. Ebselen treatment group histology. Toluidine blue. Magnification is 100X. C. Immunohistochemistry shows 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 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.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as: Kose A, Kose D, Halici Z, et al. Is Ebselen A Therapeutic Target in Fracture Healing? Eurasian J Med 2019; 10.5152/eurasianjmed.2019.18443.

Table Legends

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

Tab. 2 Histomorphometric analysis measurements

The data represent mean values standard ± deviation

* means p <0.05 according to the control group

Tab. 3 Histological fracture healing scores

The data represent mean values standard ± deviation

* means p <0.05 according to the control group

Tab. 4 VEGF and OC expression scores in groups

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as: Kose A, Kose D, Halici Z, et al. Is Ebselen A Therapeutic Target in Fracture Healing? Eurasian J Med 2019; 10.5152/eurasianjmed.2019.18443.