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Ebselen, an Active Seleno-Organic Compound, Alleviates Articular Cartilage Degeneration in a Rat Model of Knee Osteoarthritis

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Abstract

Osteoarthritis (OA) is a prevalent articular disease mainly characterized by extracellular matrix degradation, apoptosis, and inflammation, which lead to cartilage destruction and abnormal bone metabolism. With undesirable side effects, current limited symptomatic treatments are aimed at relieving pain and improving joint mobility in patients with OA. Intra-articular (IA) hyaluronic acid (HA) injection, as a nonsurgical therapy, is commonly used in the clinical management of knee OA, but the efficacy of this therapeutic option remains controversial. Ebselen has tremendous pharmacological importance for some diseases due to its antioxidant, antiapoptotic, and anti-inflammatory features. However, there is no research examining the therapeutic effect of Ebselen in OA using the rat OA model. Therefore, we aimed to investigate the therapeutic effect of Ebselen on cartilage degeneration and its role in bone morphogenetic protein 2 (BMP2) and nuclear factor kappa B (NF-κB) signaling in the molecular pathogenesis of OA. We induced a knee OA model in rats with an IA injection of monosodium-iodoacetate (MIA). After the treatment of Ebselen, we evaluated its chondroprotective effects by morphological, histopathological, and immunohistochemical methods and an enzyme-linked immunosorbent assay. We report for the first time that Ebselen treatment alleviated articular cartilage degeneration in the rat knee OA model and reduced MIA-induced BMP2 and NF-κB expressions. In addition, our results unveiled that Ebselen decreased IL-β and IL-6 levels but did not affect COMP levels in the rat serum. Ebselen could be a promising therapeutic drug for the prevention and treatment of OA by alleviating cartilage degeneration and regulating BMP2 and NF-κB expressions.

Keywords Osteoarthritis · Ebselen · Cartilage Degeneration · Monosodium iodoacetate · BMP2 · NF-κB

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Introduction

Osteoarthritis (OA) is a prevalent and progressive articular disease which is mainly characterized by cartilage destruction, abnormal bone metabolism, and pain. OA affects millions of people worldwide and causes disability, well-being loss and an enormous burden on the healthcare system [1]. With undesirable side effects, current limited symptomatic treatments are aimed at ameliorating pain and improving joint mobility in OA patients. Among current therapeutic approaches, intra-articular (IA) drug therapy is standard and safe for OA patients. Due to its decreased cost, reduced systemic exposure, and low side effects, IA hyaluronic acid (HA) injection, as a nonsurgical therapy, is commonly used in the clinical management of knee OA. However, the efficacy of this therapeutic option remains controversial [2, 3]. Therefore, many researchers worldwide focus on identifying novel therapeutic molecules that maintain cartilage integrity, slowing or stopping the progression of OA [4, 5]. OA is a multifactorial disease, and some risk factors, including genetics, aging, and trauma, accelerate the onset and development of the disease; however, its molecular pathophysiology is still not fully clarified [6]. Articular cartilage degeneration caused by apoptosis, extracellular matrix (ECM) degradation, and oxidative stress is the main feature of OA, and cartilage integrity is maintained by the anabolic and catabolic activity of chondrocytes [7, 8]. Reactive oxygen species (ROS), which lead to oxidative stress, contribute to OA progression by promoting catabolic activity and the apoptosis process in chondrocytes [9]. Nuclear factor-kappa B (NF-κB) is a well-known signaling pathway which is implicated in OA pathogenesis via stimulating inflammatory cytokine releases, ECM degrading enzymes, and apoptosis [10]. Bone morphogenetic protein-2 (BMP2), which is highly expressed in OA chondrocytes, is related to the severity of the disease [11–13].

Ebselen is an active seleno-organic compound implicated in various cellular mechanisms such as apoptosis, inflammation, genomic stability, and transport [14–16]. Moreover, Ebselen acts as a potent scavenger of ROS, and its chemical structure and activity are similar to glutathione peroxidase, which is an antioxidant enzyme [17]. Previous studies have revealed that Ebselen has many beneficial features such as antioxidant, anti-inflammatory, antimicrobial, and antimutagenic [14, 16, 18]. Furthermore, numerous studies have emphasized that Ebselen has the importance of great pharmacological due to its cardioprotective, neuroprotective, chemotherapeutic, and antiviral effects, and it can be used as a drug in clinical settings [16, 19–21]. In addition, some studies reported that Ebselen has a protective effect on cartilage degradation induced by IL-1 [22, 23]. Although these possible effects of Ebselen on cartilage tissues have been known, no research has been performed to evaluate its effect on OA pathogenesis using the rat OA model.

Considering these potential useful impacts of Ebselen on cartilage and chondrocytes, we speculated that Ebselen could be a novel therapeutic molecule for OA. Therefore, we aimed to examine the therapeutic effect of Ebselen on cartilage degeneration and its role in BMP2 and NF-κB signaling in the molecular pathogenesis of OA.

Materials and Methods

Experimental Design and MIA-Induced OA Rat Model

The animal care and experimental protocol in the present study were approved by the local Animal Ethics Committee (approval number: 2020/04–15) at Hatay Mustafa Kemal University before beginning the project. We strictly abided by the Institutional Animal Care and Use Committee Guidelines during the experiment (8th edition, National Academies Press). In the present study, we employed 42 male *Wistar albino* rats weighing ~ 250 g obtained from Hatay Mustafa Kemal University, Experimental Researches Center. All rats were housed in controlled environmental conditions (12 h light/dark cycle, 60–80% humidity, 23 ± 2 °C temperature) and had access to water and food ad libitum. All rats were fed a commercial complete maintenance diet with a metabolizable energy value of 65% carbohydrates, 24% protein, and 11% fat (Altromin 1324, Lage, Germany) during the study, and they were acclimatized for one week before starting the experiment. We randomly divided all rats into 6 groups ($n = 7$) as follows: 1-Sham (S), 2-Monosodium iodoacetate (MIA) (Sigma–Aldrich, St. Louis, MO, USA) + Vehicle-1 [Saline (ia: intraarticularly)] (M + V1), 3-MIA + Vehicle-2 [Ebselen Solvent (ip: intraperitoneally)] (M + V2), 4-MIA + Ebselen (ip) (M + E), 5-MIA + Hyaluronic acid (M + H) (ia), 6-MIA + Ebselen + Hyaluronic acid (M + E + H).

We purchased Ebselen with more than 99% purity from Cayman Chemical (Ann Arbor, MI, USA) and used dimethyl sulfoxide to dissolve it. As previously described [4, 24], we created the rat OA model using MIA. We dissolved 3 mg MIA in 50 µl saline solution and administered it into the intra-articular space of all rats' right knee in 2–6 groups under anesthesia using a 26.5-G needle at day 0 (zero) [25]. We administrated 50 µl saline solution into the intra-articular space of all rats' right knees in the sham group at day 0 (zero). The rats in sham group 1 were intraperitoneally injected with the equivalent volume of saline at 3-day intervals for 21 days. The rats in groups 2 and 3 were treated

with an equivalent saline volume (ia) and solvent dimethyl sulfoxide (ip) at 3-day intervals for 21 days. The rats in groups 4 and 6 were treated with Ebselen (15 mg/kg, ip, Cayman, USA) at 3-day intervals for 21 days. The rats in groups 5 and 6 were treated with hyaluronic acid (0.4 mg/kg, ia, INARTRO 48 mg/3 ml, Regenyal Laboratories, Italy) at 3-day intervals for 21 days. All rats were euthanized on the 23rd day of treatment. At the end of the experiments, approximately 5–6 ml whole blood samples of all rats were collected in tubes containing separator gel by cardiac puncture and allowed blood samples to clot for 30–40 min at room temperature. Subsequently, we centrifuged the tubes containing blood at 3500 rpm for 10 min at 4 °C to obtain serum, and these samples were stored at—80 °C until later use. We presented the design of the experimental procedure in Fig. 1.

Gross Morphology

At the end of the experiments, we evaluated the gross morphology of the joint tissues of the rats after all rats were euthanized. We carefully removed the soft tissue surrounding the joint, and we photographed all joint tissues after rinsing the joints with phosphate-buffered saline.

Histopathological Examination

All knee joints from rats were harvested and fixed in 4% buffered formalin for 3 days and subsequently decalcified in 10% Ethylenediaminetetraacetic acid for 12 h. After that, all knee joint samples were processed with ethanol and xylene and embedded in paraffin in frontal orientation and taken sections at a thickness of 5 µm. All sections were stained with Hematoxylin and Eosin (H-E), Safranin O and Fast Green FCF, and toluidine blue stainings for histopathological examination of knee joints. Histological sections were analyzed with an Olympus CX41 microscope and photographed with image analysis software. The pathological changes such as structure, matrix staining, and cellular abnormalities in the medial tibial plateau of knee joints were evaluated utilizing the Mankin scoring system (Table 1) [26].

Immunohistochemical Analysis of BMP2 and NF-κB

We utilized the avidin–biotin-peroxidase complex method for immunohistochemical analysis of BMP2 and NF-κB in the knee joints of rats as previously described [27]. After deparaffinization and rehydration of all sections, we incubated all sections in 3% H_2O_2 in methanol for 15 min to suppress endogenous peroxidase activity. Then, we incubated the sections with primary antibodies against BMP2 (YL Biont, YID0638) and NF-κB (Novus Biologicals, NB100-56,055) in a humidified chamber for 60 min. As a detection system, the biotinylated secondary antibody-streptavidin-peroxidase was employed as per the manufacturer's instructions (Ultra Vision Detection System-HRP kit, Thermo Scientific). We utilized the 3-amino-9-ethylcarbazole as a chromogen in order to visualize the positive signals and Mayer's hematoxylin for counterstaining. We assessed the expression levels of BMP2 and NF-κB with a Kameram Gen III, Argenit image analysis software and used an integrated

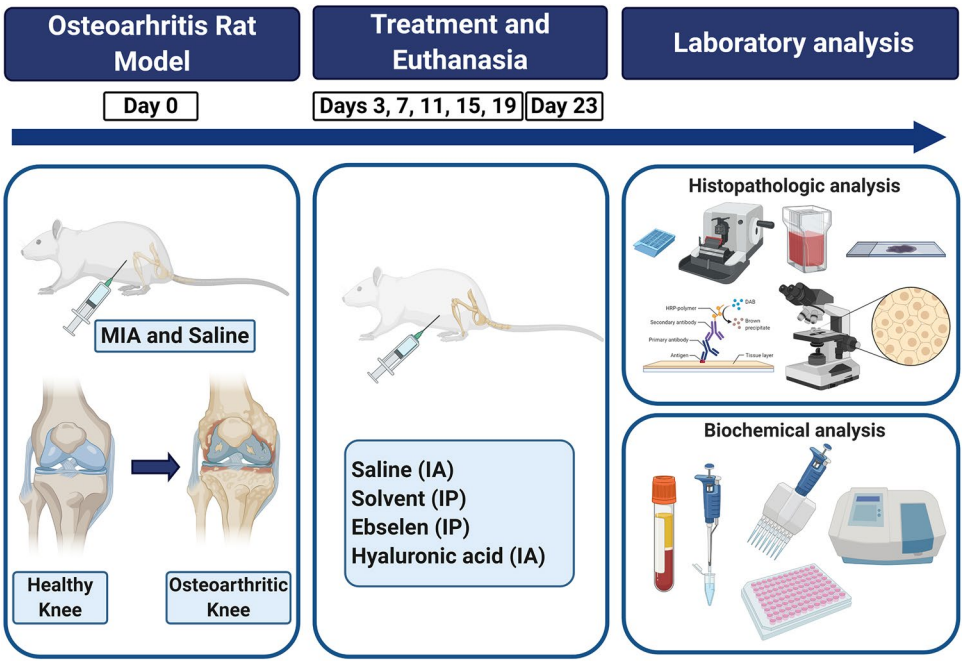


Fig. 1 Study design (Created with BioRender.com). MIA: monosodium iodoacetate, IA: intraarticularly, IP: intraperitoneally

Table 1 Mankin's score grading system for osteoarthritic articular cartilage [26]

Category	Subcategory	Score
Structure	Normal	0
	Surface irregularities	1
	Pannus and surface irregularities	2
	Clefts to transitional zone	3
	Clefts to radial zone	4
	Clefts to calcified zone	5
	Complete disorganization	6
Cells	Normal	0
	Diffuse hypercellularity	1
	Cloning	2
	Hypocellularity	3
Safranin O staining	Normal	0
	Slight reduction	1
	Moderate reduction	2
	Severe reduction	3
	No dye noted	4
Tidemark integrity	Intact	0
	Crossed by blood vessels	1
Total		0–14

optical density measurement method to quantitatively detect the intensities of BMP2 and NF-κB.

Enzyme-Linked Immunosorbent Assay (ELISA)

Interleukin 1 beta (IL-1β), interleukin 6 (IL-6), and cartilage oligomeric matrix protein (COMP) protein levels from serum samples of rats were measured using commercial ELISA kits as per the manufacturer's protocols. The intra-assay coefficients of variation for IL-1β, IL-6, and COMP were 4.49, 5.25 and 4.95, respectively. The inter-assay coefficients of variation for IL-1β, IL-6, and COMP were 4.9, 5, and 4.38, respectively.

Statistical Analysis

All data analyses were conducted utilizing GraphPad Prism (v6.0). We checked the distribution of normal of data with the Kolmogorov–Smirnov test and compared the groups with Kruskal–Wallis and Mann–Whitney *U* test. We presented the results as mean ± standard deviation, and $p < 0.05$ was considered significant for the statistical analyses.

Results

Macroscopic Analyses

The morphological comparisons of rat knee joint tissues are presented in Fig. 2. We observed undamaged, intact, and sleek femoral condyles and tibial plateaus of rats in sham groups whereas we detected the degenerations of articular cartilage and subchondral bone in rat knee joints induced by MIA. Also, we detected that articular cartilage degradation and joint deterioration were seen less after the Ebselen treatment.

Ebselen Ameliorates MIA-Induced Cartilage Destruction in the Rat Knee OA Model

Histopathological changes were examined for the general evaluation of articular cartilage tissue in our rat OA model. Our H&E findings indicated that the general view and surface of cartilage tissues of rats were intact and smooth in sham groups. Furthermore, the numbers of chondrocytes in these groups were markedly declined compared to the sham groups. In contrast to alterations of M + V1 and M + V2, cartilage degenerations were considerably reduced in M + E, M + H, and M + H + E groups (Fig. 3, left panel). Safranin O & Fast Green FCF staining provides essential knowledge about proteoglycan density in articular cartilage. We observed significantly decreased staining intensity in the

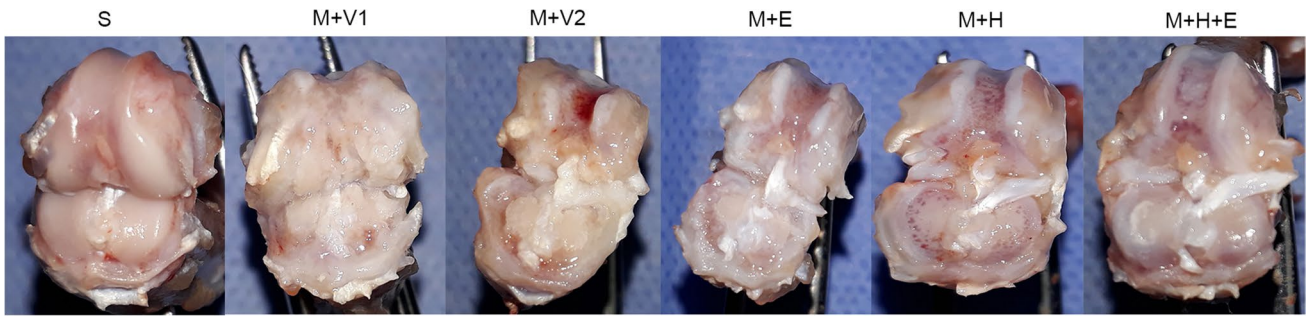


Fig. 2 Macroscopic comparison of the effect of Ebselen on femoral condyles and tibial plateaus of rat knee joins. S: sham, M: monosodium iodoacetate, V: vehicle, E: Ebselen, H: hyaluronic acid

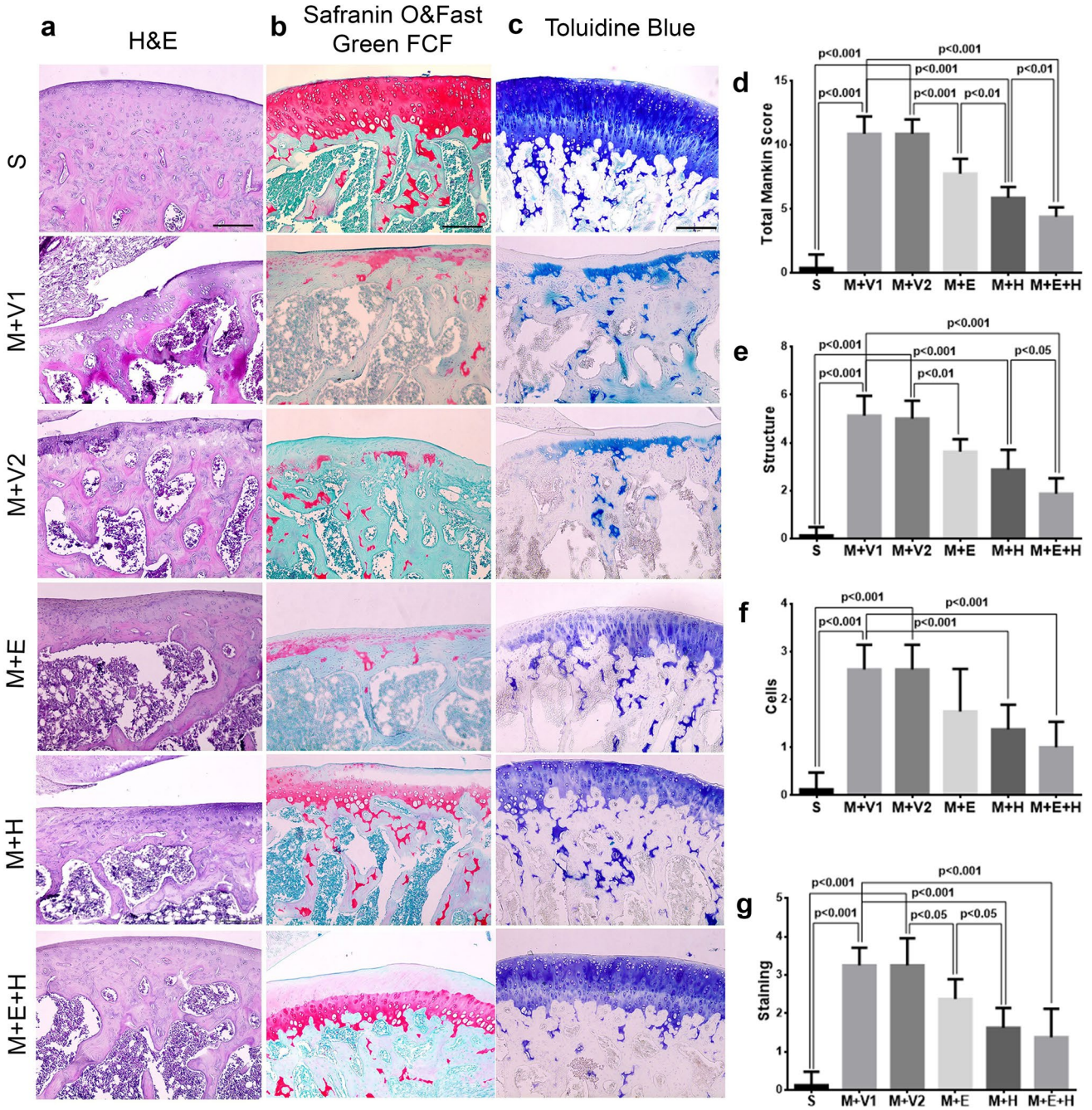


Fig. 3 The effect of Ebselen and Hyaluronic acid treatment on articular cartilage tissues in the MIA-induced Osteoarthritis model in rats. The panel demonstrates histopathological changes in the knee joint tissues that are stained with H&E (**a**), Safranin O&Fast Green FCF (**b**), and toluidine blue (**c**). The histopathological evaluation of the severity of the OA was performed using Mankin's scoring system; total Mankin score (**d**), structure (**e**), cell abnormalities (**f**), and staining (**g**). Data are presented as mean ± SD. Severe subchondral bone and articular cartilage damage were observed in the M + V1 and M + V2 groups. Ebselen and hyaluronic acid ameliorate the articular cartilage destruction in M + E, M + H, and M + E + H (scale bar; 100 µm). S: sham, M: monosodium iodoacetate, V: vehicle, E: Ebselen, H: hyaluronic acid

M + V1 and M + V2 groups compared to the sham group, whereas considerably enhanced staining was observed in the M + H, M + E, and M + H + E groups compared to the sham group (Fig. 3, middle panel). We also used the toluidine blue staining to assess glycosaminoglycan content and cartilage condition in the rat OA model (Fig. 3, right panel). We observed diminished toluidine blue Staining in the M + V1 and M + V2 groups compared to the sham group.

The structure of cartilage tissue integrity in the M + H, M + E, and M + E + H groups was protected compared to the sham group (Fig. 3, right panel). We utilized Mankin's scoring system to evaluate and compare the protective effect of Ebselen and Hyaluronic acid on the severity of cartilage degeneration in the rat OA model. Our findings showed that Mankin scores in the M + V1 and M + V2 groups were considerably more than those in the sham group, whereas Mankin scores in the M + E, M + H, and M + E + H groups were significantly diminished compared to those in the M + V1 and M + V2 groups. These findings unveiled that Ebselen may have chondroprotective effects in the rat OA model.

Ebselen Treatment Reduces BMP2 and NF-κB Expressions in the Rat Knee OA Model

We investigated whether Ebselen's potential therapeutic effect is associated with BMP2 and NF-κB, which are involved in the pathogenesis of OA [4]. Our immunohistochemistry analyses revealed that BMP2 and NF-κB expressions were increased in M + V1 and M + V2 compared to the sham group (Fig. 4a and b). Further, the expressions of BMP-2 were significantly decreased in the M + E, M + H, and M + E + H groups compared to the M + V1 and M + V2 groups (Fig. 4a). Similar to BMP2, NF-κB expressions in the Ebselen and hyaluronic acid treatment groups are significantly lower than those in the M + V1 and M + V2 groups (Fig. 4b). These results unveiled that Ebselen may have BMP2- and NF-κB-mediated therapeutic effects.

While Ebselen Decreases Serum IL-1β and IL-6 Levels, It Does Not Affect COMP Levels in the Rat Knee OA Model

IL-1β and IL-6 are known as inflammatory mediators that contribute to chronic inflammation and joint destruction the initiation and development of OA [28]. Thus, we also evaluated the effect of Ebselen on serum IL-1β and IL-6 levels in the rat OA model [29]. These proinflammatory cytokines, which are highly expressed in OA, contribute markedly to OA progression [29]. IL-1β and IL-6 levels were significantly elevated in the M + V1 and M + V2 groups compared to the sham group (Fig. 5a and b) ($p < 0.01$). IL-1β and IL-6 levels in the M + E and M + H + E groups were significantly lower than those in the M + V1 and M + V2 groups (Fig. 5a and b) ($p < 0.05$). Our data revealed that Ebselen's administration might have an impact on inflammatory

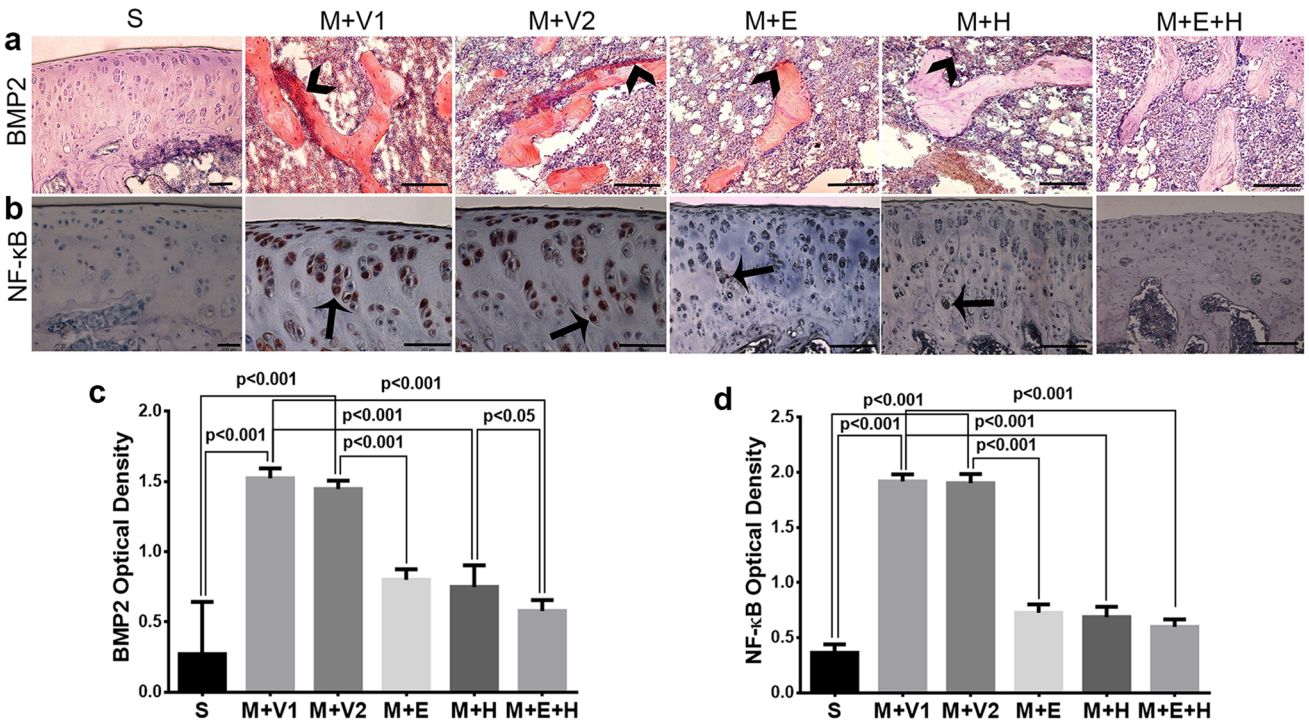


Fig. 4 The effect of Ebselen and hyaluronic acid on BMP-2 and NF-κB expressions in the MIA-induced osteoarthritis model in rats. The panel demonstrates immunostaining of BMP2 (**a**) and NF-κB (**b**) expressions in the rat joint tissue. BMP-2 (**c**) and NF-κB (**d**) expressions in joint tissues were increased in the M + V1 and M + V2 groups. Ebselen and Hyaluronic acid inhibited BMP-2 and NF-κB expressions in joint tissues (scale bar; 100 μm). Data are presented as mean ± SD. S: sham, M: monosodium iodoacetate, V: vehicle, E: Ebselen, H: hyaluronic acid

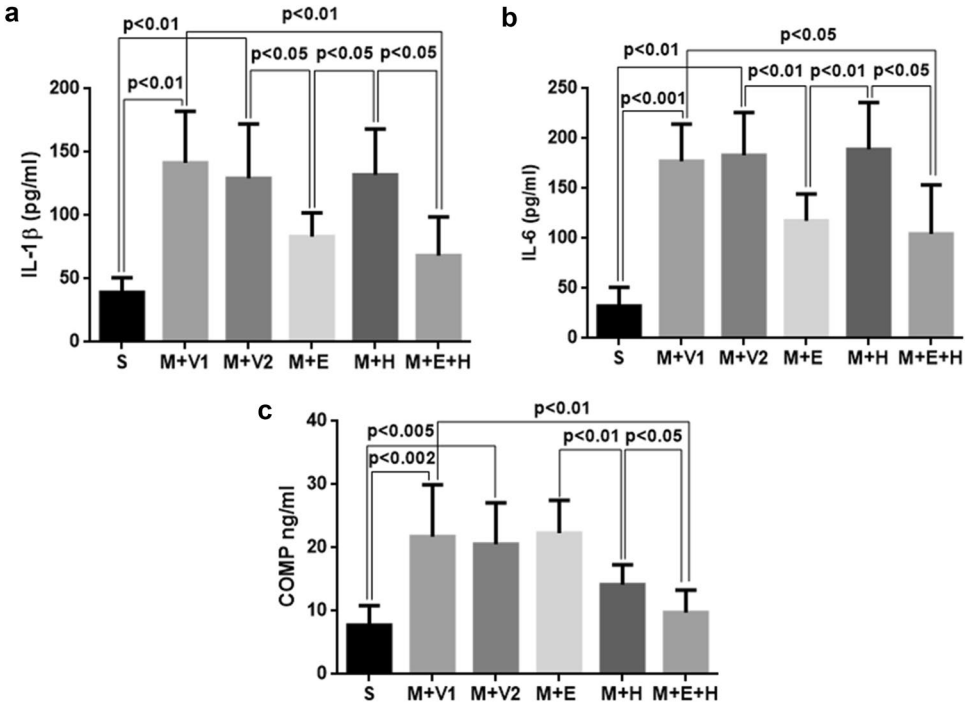


Fig. 5 The effect of Ebselen on serum IL-1β, IL-6 and COMP levels in the MIA-induced osteoarthritis model in rats. Data are represented as the mean ± SD. IL-1β: interleukin 1 beta, IL-6: interleukin 6, COMP: cartilage oligomeric matrix protein, S: sham, M: monosodium iodoacetate, V: vehicle, E: Ebselen, H: hyaluronic acid

cytokines such as IL-1β and IL-6 in the rat OA model. There was no significant difference in IL-1β and IL-6 levels between the M + H group and M + V1 ($p > 0.05$), indicating that hyaluronic acid does not affect these molecules. COMP is a well-known molecule that can be measured in the serum of patients with OA and is involved in cartilage degeneration [30]. Hence, we also investigated whether Ebselen's effect on serum COMP expressions in our in vivo OA model. Serum COMP concentrations were significantly increased in the M + V1 and M + V2 groups compared to the sham group. There was no significant difference in the M + E and M + V2 groups. These findings indicated that Ebselen has no impact on COMP expressions in the rat OA model.

Discussion

OA is prominently characterized by pathophysiological changes such as chondrocyte apoptosis, ECM destruction, oxidative stress, and inflammation, which contribute considerably to cartilage destruction and impaired bone metabolism [7–9]. Although IA HA injection is extensively used to alleviate pain and improve joint function in OA patients, its efficacy is controversial [2]. Since there is currently no effective treatment for OA, it is crucial to design treatments to prevent and inhibit the progression of the disease [3]. Due to Ebselen's antioxidant and anti-inflammatory properties, it is seen as a promising drug candidate for many human diseases [14, 18]. Furthermore, a few studies emphasize that Ebselen has a protective role in cartilage degradation and bone fracture [22, 23, 31]. However, these works did not examine the antiosteoarthritic role of Ebselen in the development of knee OA using the in vivo OA model yet. Therefore, we investigated whether the treatment of Ebselen inhibits the progression of OA.

Here, we for the first time report that the administration of Ebselen alone or in combination with Hyaluronic acid alleviates articular cartilage degeneration in the rat knee OA model. Our data suggest that Ebselen might support hyaluronic acid treatment in the rat OA model. A previous study indicated that Ebselen suppresses cartilage destruction stimulated by IL-1 [22], which is consistent with our results. The results of this study showed that while Ebselen blocks the IL-1-triggered proteoglycan degradation, it had no impact on the suppression of proteoglycan synthesis caused by IL-1. Furthermore, Matsushita and colleagues have unveiled that Ebselen has a chondroprotective effect on IL-1-induced degradation in cartilage tissues and chondrocyte cultures [23]. Moreover, their findings revealed that Ebselen reversed the suppression of proteoglycan synthesis in cartilage and chondrocyte cultures. Consistent with these data, our Safranin O staining analyses demonstrate that the treatment of Ebselen in rats slows down the development of OA induced by MIA and reduces proteoglycan loss. These data suggest that Ebselen delays the degradation of ECM during OA progression.

Due to the essential roles of NF-κB in pathological conditions, such as the synthesis of matrix-degrading enzymes and inflammatory cytokines and induction of apoptosis, which contribute to cartilage destruction and abnormal

subchondral bone metabolism, it has been highlighted that the NF-κB pathway mediators might be an important therapeutic target for OA treatment [10]. More importantly, Kang and colleagues recently reported that NF-κB-stimulated-miR-204 contributes to cartilage destruction by targeting the proteoglycan biosynthesis mechanism [32]. Therefore, considering the chondroprotective effect of Ebselen on cartilage destruction in this study, we speculated that Ebselen might have a regulatory effect on NF-κB levels. Our immunostaining analyses revealed that Ebselen reduces NF-κB protein levels in degenerative cartilage caused by MIA, suggesting that Ebselen may have a chondroprotective effect on cartilage tissue in OA development through inhibition of NF-κB. However, more detailed studies should focus on elucidating the possible relationship of Ebselen with NF-κB expressions in OA.

BMP2, an effective molecule in cartilage metabolism, has a potential diagnostic and therapeutic value for preventing OA [11, 12]. BMP2 has an anabolic effect on articular cartilage by stimulating proteoglycan synthesis, suggesting that BMP-2 might repair OA progression functions [33, 34]. Therefore, we also investigated whether Ebselen has an effect on BMP2 concentrations in the rat OA model. The immunostaining analyses revealed that Ebselen declines BMP2 expressions in the MIA-induced degenerative cartilage. These data suggest that there may be a relationship between Ebselen and BMP2.

IL-1β is a crucial player, contributing to cartilage destruction by stimulating several mechanisms such as ECM synthesis, oxidative stress, and inflammatory cytokine releases [28]. IL-6, another critical factor actively involved in the pathogenesis of OA, is upregulated in synovial fluids and serum of patients with OA, and its synthesis is stimulated by IL-1B in the course of OA [28, 35]. Moreover, it has been shown that IL-1β and IL-6 concentrations are upregulated in the rat OA model [36]. Thus, we investigated whether Ebselen affects the synthesis of OA-related inflammatory factors such as IL-1β and IL-6. Our results revealed that the treatment of Ebselen considerably decreases serum IL-1β and IL-6 levels in the MIA-induced OA model.

Conclusions

Here, we report for the first time that Ebselen treatment ameliorated articular cartilage degeneration and reduced BMP2 and NF-κB expressions in the rat knee OA model. Furthermore, we report that cotreatment of HA and Ebselen has more therapeutic effects in ameliorating the pathological findings of OA. Moreover, we indicate that Ebselen treatment decreased serum IL-1β and IL-6 levels in the rat knee OA model. Our data emphasize the potential therapeutic importance of Ebselen for inhibiting the progression of OA. Further experimental research should focus on the protective effect of Ebselen in bone and cartilage disorders.

Author Contribution H.M.O., Z.Y. and A.K. designed the study. H.M.O., Z.Y., I.K. and F.K. carried out experiments. H.M.O., Z.Y., I.K. and A.K. wrote the manuscript and interpreted the results. H.M.O., Z.Y., I.K., F.K. and A.K. conducted statistical analyses, prepared graphs and revised the paper. All the authors of the paper have contributed to the present study and approved the final version of this manuscript.

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Data Availability The data obtained after analysis in the current study can be obtained from the relevant authors upon reasonable request.

Declarations

Ethics Approval The animal care and experimental protocol in the present study were approved by the local Animal Ethics Committee of Hatay Mustafa Kemal University (Approval number: 2020/04–15).

Consent to Participate All authors contributed voluntarily to this study.

Consent for Publication All authors have consent for the publication of the manuscript.

Conflict of Interest The authors declare that they have no conflict of interest.

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