Biochemistry and Biophysics Reports 36 (2023) 101559

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

# Antioxidant and anti-inflammatory effects of selenomethionine promote osteogenesis via Wnt/β-Catenin pathway

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ARTICLE INFO

*Keywords:*
Osteoporosis
Wnt/β-Catenin pathway
Selenomethionine
MC3T3-E1 cells
Oxidative stress
Inflammation

ABSTRACT

*Background:* Recently, the antioxidant properties of the natural compound, selenomethionine (Se-Met), have been recognized. However, its effect on the osteogenic mineralization of the Wnt/β-Catenin pathway under conditions of oxidative stress and inflammation remain unclear.
*Methods:* This study utilized *tert*-butyl hydroperoxide (TBHP) to simulate oxidative stress and inflammation. Se-Met was then subsequently used to inhibit these effects *in vitro*.
*Results:* TBHP induces oxidative stress and inflammatory responses by increasing the expression of reactive oxygen species and NLRP3, whereas decreasing the expression of GPX4, thereby inhibiting the viability of MC3T3-E1 cells. TBHP further promotes lipid peroxidation and damages the ultrastructure of mitochondria. Furthermore, TBHP inhibits the expression levels of β-Catenin, thereby reducing the activity of the Wnt pathway, which in turn suppresses the osteogenic differentiation and mineralization capacity. Importantly, Se-Met significantly alters the aforementioned responses to enhance expression levels of Wnt pathway-related proteins and improving the osteogenic differentiation and mineralization capacity of the cells.
*Conclusion:* Se-Met enhances antioxidant and anti-inflammatory responses in MC3T3-E1 cells via the Wnt/β-Catenin signaling pathway to promote osteogenesis. Thus, Se-Met plays a crucial role in the field of bone homeostasis, and presents an opportunity for the future development of novel drugs for treating osteoporosis and maintaining bone stability. However, further detailed preclinical animal studies are required to generate solid and reliable data to aid this development.

## 1. Introduction

Osteoporosis (OP) is a widespread disorder of bone metabolism that causes loss of bone mass and thinning of bone tissue, leading to an increased risk of fracture [1]. OP affects a significant portion of the global population, with rates ranging from 10 % to 50 %. This condition is even more prevalent among individuals who are 65 years and older, with rates exceeding 50 % [2]. OP can result in height loss, spinal curvature, and skeletal deformities, which can seriously affect the patient's quality of life [3]. At present, the medications prescribed for OP primarily consist of antiresorptive and bone-forming agents. However, their efficacy is not satisfactory, and prolonged usage may result in adverse effects.

The Wnt/β-Catenin signaling pathway is the most important pathway in bone biology and plays a key role in promoting osteoblast differentiation and bone formation. Activation of this pathway has been shown to promote bone formation and inhibit bone resorption, making it a promising target for the treatment of OP. The importance of the Wnt/β-Catenin signaling pathway in bone health has led to extensive research into its mechanisms of action and potential therapeutic applications [4–6]. However, this pathway is susceptible to direct regulation by various factors, including oxidative stress and inflammation, which can have a negative impact on its function. Therefore, further study of the

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https://doi.org/10.1016/j.bbrep.2023.101559
Received 14 July 2023; Received in revised form 19 August 2023; Accepted 13 October 2023

**Abbreviation**

| | |
|---|---|
| OP | osteoporosis |
| Se-Met | selenomethionine |
| TBHP | *tert*-butyl hydroperoxide |
| TEM | transmission electron microscopy |
| ROS | reactive oxygen species |
| FITC | Fluorescein isothiocyanate isomer |
| DCFH | 2′,7′-Dichlorodihydro fluorescein |
| NLRP3 | NLR family pyrin domain containing 3 |
| ALP | alkaline phosphatase |
| ARS | Alizarin Red S |
| IL-6 | interleukin-6 |
| GPX4 | glutathione peroxidase 4 |
| BCA | bicinchoninic acid |
| PVDF | poly-vinylidene fluoride. |

regulatory role of the Wnt/β-Catenin signaling pathway in bone metabolism is of great significance to better understand its role in bone diseases and develop potential therapeutic strategies [7–9]. Previous studies have shown that oxidative stress can stimulate the formation of NLRP3 inflammasomes, which in turn activate inflammatory responses and result in the oxidation and degradation of crucial proteins in this signaling pathway. This ultimately leads to reduced activity of this signaling pathway [10–12]. As a result, regulation of oxidative stress and inflammatory responses to encourage the activation of the Wnt/β-Catenin signaling pathway and enhance bone formation may emerge as a novel therapeutic approach for treating OP.

Selenomethionine (Se-Met) is a natural organic selenium compound that exhibits improved biocompatibility compared to inorganic selenium. Se-Met has been shown to have various roles in the body, including antioxidant, anti-inflammatory, and immune promoting functions [13–15]. Recent studies have shown that Se-Met can also promote osteogenesis and differentiation of osteo-association cells to promote formation of bone tissue [16–18]. These data suggest a specific relationship between Se-Met and the Wnt/β-Catenin signaling pathway. Moreover, previous studies have also shown that Se-Met can inhibit the occurrence of Alzheimer's disease in mice by inhibiting the negative regulatory factor, GSK-3β, in this pathway [19,20]. However, the mechanism behind the effects of Se-Met on the Wnt/β-Catenin signaling pathway under oxidative stress and inflammation in osteoblasts remains unclear.

Herein, to study the protective effects of Se-Met on the Wnt pathway under conditions of oxidative stress and inflammation, MC3T3-E1 cells were induced to establish a model of inflammation and oxidative damage using *tert*-butyl hydroperoxide (TBHP). Flow cytometry, immunofluorescence staining, and protein immunoblotting were employed to illustrate TBHP-induced upregulation of reactive oxygen species (ROS), NLR family pyrin domain containing 3 (NLRP3), and interleukin-6 (IL-6) expression, with concurrent downregulation of glutathione peroxidase 4 (GPX4) at the protein level. These changes induced oxidative stress and inflammation, resulting in reduced MC3T3-E1 cell viability. Additionally, we used immunofluorescence and transmission electron microscopy (TEM) to show that TBHP further promotes lipid peroxidation and causes damage to mitochondrial ultrastructure. Importantly, Se-Met was also able to reverse TBHP-induced oxidative damage and inflammatory responses. Furthermore, immunoblotting data confirmed that TBHP inhibits expression of alkaline phosphatase (ALP), Human Collagen Type I Protein (Col-1), Runt-related Transcription Factor 2 (Runx-2), and β-Catenin, thereby reducing the activity of the Wnt pathway. This inhibition was further confirmed through ALP staining and Alizarin Red S (ARS) staining, which demonstrated TBHP-mediated inhibition of osteogenic differentiation and mineralization in MC3T3-E1 cells. Encouragingly, Se-Met significantly altered the aforementioned responses, thereby increasing the expression of Wnt pathway-related proteins and improving the osteogenic differentiation and mineralization capacity of MC3T3-E1 cells.

## 2. Materials and methods

### 2.1. Cell culture

MC3T3-E1 Subclonal 14 cells are one in a series of mouse preosteogenic cell lines (GNM15) taken from a mouse neonate and obtained from Chinese Academy of Sciences (Shanghai, China). Cells were grown at 37 °C in 5 % carbon dioxide, with 10 % (v/v) fetal bovine serum (FBS, Hyclone). The culture medium was changed 1–2 times, and the cell fusion was frozen once the cells reached 80 % confluency.

### 2.2. Cell viability

Cell viability was evaluated using the Cell-Quanti-Blue assay kit from Bioassay Systems(Texas, US). MC3T3-E1 cells were pretreated with Se-Met (100 nM) and then exposed to TBHP (0, 100, 200, 250, 300, and 400 μM), followed by a 3 h incubation with the Cell-Quanti-Blue reagent. To determine cell survival, absorbance at 540 nm was measured on an ELISA plate reader.

### 2.3. TEM

After digestion with trypsin, the cell suspension was centrifuged and the supernatant was discarded. The cells were then fixed with glutaraldehyde, stored at 4 °C, and then analyzed under an electron microscope to assess MC3T3-E1 cell mitochondrial morphology.

### 2.4. Determination of ROS generation

Intracellular ROS levels were detected using the 2′,7′-Dichlorodihydro fluorescein (DCFH) probe (Sigma). Cells were incubated in serum-free medium containing 10 μM DCFH for 30 min at 37 °C. Next, the medium was replaced with regular culture medium and incubated for approximately 10 min. Finally, images were obtained on a fluorescence microscope at excitation wavelength of 488 nm.

### 2.5. Flow cytometry

Flow cytometry was used in parallel to measure intracellular ROS levels. The cells were collected and stained with DCFH using the aforementioned method and following a 10-min incubation at 37 °C, cells were analyzed by flow cytometry, and date were analyzed using FlowJo 10.0.7 software.

### 2.6. Lipid oxidation measurement

The amount of oxidized lipids was assessed using the C11-BODIPY 581/591 probe (Amgicam). MC3T3-E1 cells were induced and grown to approximately 80 % confluency in the presence of TBHP. Subsequently, cells were fixed at room temperature with 4 % paraformaldehyde and stained with 10 μM C11-BODIPY 581/591 probe for 10 min. Additionally, Hoechst 33342 (Beyotime) was used for nuclear staining for 5 min before images were acquired under a fluorescence microscope.

### 2.7. Quantification of total superoxide dismutase (T-SOD) and glutathione (GSH) analysis

MC3T3-E1 cells grown to 80 % confluency were then harvested by cell scraping, followed by homogenization and centrifugation. T-SOD and GSH concentrations were measured using commercial assay kits

(Elabscience and Sigma, respectively). GSH was measured using an ELISA microplate reader at excitation and emission wavelengths of 550 nm and 405 nm, respectively.

### 2.8. ALP staining

ALP staining of osteoblasts was performed using an ALP kit (Beyotime). After 7 days of osteogenic induction in MC3T3-E1 cells, the cells were fixed with paraformaldehyde at room temperature for 30 min. Subsequently, cells were stained using the ALP staining solution before cell images were acquired under a light microscope. Staining images were quantified using Image J software.

### 2.9. ARS

Mineralization was evaluated using ARS staining (Beyotime). To encourage osteogenic mineralization, MC3T3-E1 cells were stained with ARS solution for 50 min following fixation with 4 % paraformaldehyde at room temperature for 30 min. Cells were then washed twice with phosphate buffered saline (PBS) to remove extra dye before mineralization was examined under a microscope. Staining images were quantified using Image J software.

### 2.10. Western blotting

Cells were first washed three times with PBS before addition of cell lysis buffer and subsequent ultrasonication. Protein quantification was performed using the bicinchoninic acid (BCA) assay kit (Beyotime). After gel electrophoresis of self poured (Beyotime), the proteins were transferred onto a Polyvinylidene fluoride (PVDF) membrane. Non-specific antibody binding sites on the membrane were blocked with 5 % (w/v) skimmed milk (Abcam) in 10 × tris buffered saline with 0.01 % Tween-20 (TBS-T) (Abcam) for 2 h at room temperature with constant agitation. Next, primary antibodies against NLRP3, IL-6, GPX4, ALP, Col-1, Runx-2, β-Catenin and β-actin were incubated overnight at 4 °C. Following a thorough wash step (three washes for 5 min each), the membrane was then incubated with goat anti-rabbit IgG. After another thorough was step as before, blot images were acquired on a Image Lab imager and protein expression was quantified by densitometry using Image J software.

### 2.11. Data analysis

All experiments were performed in triplicate and the data are presented as the mean ± standard deviation. Statistical analyses were conducted using *t*-tests for comparisons between two groups, and analysis of variance (ANOVA) for comparisons between multiple groups. Post-hoc multiple comparisons were conducted only when significant differences were determined by ANOVA analyses.

The study primarily conducted literature searches in the Pubmed and Web of Science databases. The keywords we focused on were Wnt pathway, oxidative stress, ROS, inflammation. The referenced articles mainly span from 2000 to 2023.

## 3. Results

### 3.1. Se-Met reduces TBHP-mediated reduction of MC3T3-E1 cell viability

The effect of various concentrations of TBHP on MC3T3-E1 cell viability after 24 h exposure was assessed using the Cell-Quanti-Blue assay. The results showed a notable inverse relationship between MC3T3-E1 cell viability and TBHP concentration. Additionally, treatment with 100 nM of Se-Met for 3 days dramatically enhanced TBHP-induced reduction in cell survival at TBHP concentrations between 200 μM and 250 μM. High concentrations of TBHP (i.e., 300 μM and 400 μM) dramatically reduced cell viability and Se-Met had no statistically significant effect on this outcome (Fig. 1A and B). Thus, in subsequent experiments, we used 200 μM of TBHP to cause oxidative stress and inflammation in MC3T3-E1 cells.

### 3.2. Se-Met alleviates TBHP-induced oxidative stress in MC3T3-E1 cells

#### 3.2.1. Se-Met alleviates TBHP-induced ROS in MC3T3-E1 cells

Although Se-Met successfully suppressed this response, DCFH staining revealed that TBHP significantly increased ROS formation in osteoblasts (Fig. 2C and D). The fraction of DCFH-positive cells and the geometric mean Fluorescein isothiocyanate isomer (FITC) significantly increased after TBHP treatment but these effects were successfully inhibited by Se-Met pretreatment (Fig. 2A and B) as illustrated by our flow cytometry data. These findings suggest that Se-Met can successfully reduce TBHP-induced ROS generation in MC3T3-E1 cells. Additionally, TBHP treatment greatly reduced T-SOD and GSH enzyme activity in MC3T3-E1 cells, whereas Se-Met significantly increased the activity of these enzymes (Fig. 2E and F). Furthermore, western blotting showed that TBHP decreased expression of the antioxidant enzyme GPX4 (Fig. 2G), thereby supporting the notion that TBHP can cause oxidative stress in some organisms.

#### 3.2.2. Se-Met alleviates TBHP-induced lipid oxidation in MC3T3-E1 cells

In this study, we investigated the mechanism by which oxidative stress affected MC3T3-E1 cells, with a focus on the effect of lipid peroxidation on cell death. We achieved this this by studying the lipid peroxidation response and mitochondrial morphology of MC3T3-E1 cells. The findings demonstrated that treatment with TBHP resulted in aberrant mitochondrial morphology and lipid peroxidation responses, including smaller mitochondria, increased mitochondrial membrane density, and fewer or absent mitochondrial cristae. However, results from TEM experiments showed that Se-Met pretreatment improved the mitochondrial morphology of MC3T3-E1 cells (Fig. 3B). Green fluorescence is an indication of lipid oxidation. We found that TBHP stimulation produces significantly higher levels of green fluorescence than Se-Met protection (Fig. 3A), thereby suggesting that Se-Met decreases MC3T3-E1 cells responses to lipid peroxidation.

### 3.3. Se-Met effectively inhibit the inflammatory response of MC3T3-E1 cells induced by TBHP

A crucial component of the inflammatory response is the NLRP3 inflammasome, which can be activated by high ROS in several ways. Our western blotting data showed that TBHP increased protein expression of IL-6 in the NLRP3 inflammasome, whereas Se-Met successfully repressed it (Fig. 4).

### 3.4. Se-Met effectively alleviates the inhibitory effect of TBHP on the osteogenic differentiation and mineralization of MC3T3-E1 cells

ALP staining data revealed that osteoblasts that had been pretreated with Se-Met had deeper staining and an increased number of stained cells after 7 days of mineralization induction. In comparison, TBHP treatment resulted in lighter staining with fewer stained cells. After 21 days of mineralization induction, ARS staining revealed that TBHP-stimulated cells had fewer and smaller mineralization nodules, whereas Se-Met pretreated cells had an increased number of larger mineralization nodules. These data suggest that Se-Met can improve the inhibitory effect of TBHP on the early osteogenic activity and late mineralization capacity of osteoblasts (Fig. 5A–C).

In this work, we evaluated the importance of Se-Met in regulating osteoblast proliferation, differentiation, and migration in the Wnt/β-Catenin signaling pathway. Using Western blot analysis, we evaluated the expression of key associated proteins in this pathway, including ALP, Runx-2, Col-1, and β-Catenin. Our data show that TBHP significantly inhibits the expression of these proteins in MC3T3-E1 cells, whereas the

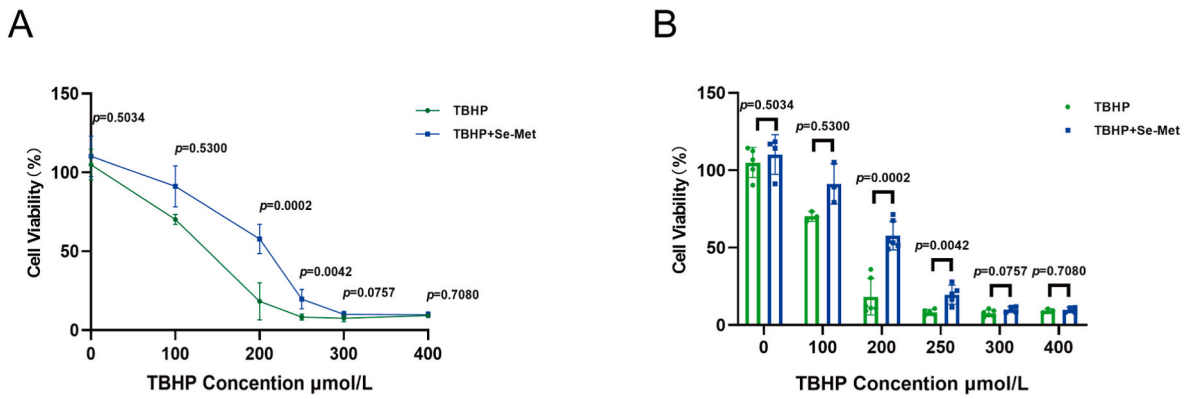


**Fig. 1.** Se-Met increases the survival rate of MC3T3-E1 cells after 24 h of TBHP culture. (A) The effect of different concentrations of TBHP (0, 100, 200, 250, 300, or 400 μM) on MC3T3-E1 cell viability after 24 h; (B) MC3T3-E1 cells were pretreated with 100 nM Se-Met for 72 h and then exposed to 0, 100, 200, 250, 300, or 400 μM TBHP for 24 h. Cell-Quanti-Blue results showed that Se-Met increased the viability of MC3T3-E1 cells at concentrations of 200 μM and 250 μM TBHP. $p < 0.05$ indicate significant differences, whereas $p \geq 0.05$ indicates no significant differences. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

addition of Se-Met increased their expression levels. This suggests that Se-Met can effectively alleviate the inhibitory effect of TBHP on osteogenic differentiation and mineralization of MC3T3-E1 cells by regulating this pathway (Fig. 5D). These findings highlight the potential value of Se-Met in promoting bone formation and treating bone-related diseases such as OP.

## 4. Discussion

OP is a complex disease with a difficult diagnosis and unsatisfactory treatment outcomes. Although the Wnt/β-Catenin signaling pathway plays a crucial part in OP, oxidative stress and inflammation are unfavorable factors that activate this pathway. There is an urgent need to identify anti-osteoporotic drugs that can target this pathway while resisting oxidative stress and inflammation. The importance of the Wnt/β-Catenin signaling pathway cannot be overlooked as it plays a crucial part in various biological processes and its regulation has significant implications in neurodegenerative diseases, bone disorders, and cancer, among others [21,22]. Similarly, in bone disorders, this pathway plays a crucial part in regulating osteoblast proliferation and differentiation, bone formation, and maintenance of bone homeostasis [23,24]. However, the Wnt pathway is susceptible to various factors that can impair its function, such as oxidative stress and inflammation. Targeting this pathway for therapeutic intervention holds significant promise for preventing a range of diseases, including neurodegenerative and bone disorders.

Selenium is a crucial trace element for the human body, and a shortage can result in insufficient antioxidant protection, which can harm multiple tissues and organs and cause a variety of disorders [25,26]. Selenium can be classified as inorganic or organic, with selenomethionine, an example of organic selenium, exhibiting improved biocompatibility and biosafety compared to inorganic selenium. Selenomethionine, an active form of selenium, can be extracted from selenium-rich plants, and compared to selenomethionine from other sources, the one extracted from selenium-rich plants have lower toxicity, higher bioavailability, and better stability [27]. After absorption by the human body, selenomethionine can form a selenium reservoir within the body [28], which has been shown to play a crucial part in the prevention of Keshan disease and osteoarthritis [29,30]. As a result, there has been increased interest in the study of potential benefits of selenomethionine. In 2020, Ahmadov et al. synthesized environmentally friendly silver nanoparticles (AgNPs) from plant extracts, which exhibited significant antioxidant and antibacterial properties [31]. This suggests that nanomaterials derived from plant sources have significant potential for application in disease treatment due to their high biological activity and low toxicity. Se-Met has been shown to eliminate ROS and lessen inflammatory responses, making it a natural antioxidant [32]. In a previous study by Cheff et al., 100 nM selenium was used to investigate iron death [33]. Next, we assessed cell viability following treatment with TBHP (100, 200, 250, 300, and 400 μM) in the presence of 100 nM Se-Met as previously described. Our data showed that, 200 μM TBHP considerably decreased MC3T3-E1 cell viability, therefore this dosage was suitable for subsequent investigations. Se-Met may protect osteoblasts from oxidative damage and inflammation, however the full impact of this effect remains unclear. Therefore, further studies are required to investigate the potential of using Se-Met in the management of OP.

The simplest and most straightforward biomarker of intracellular oxidative stress is ROS [34]. In addition, GSH and T-SOD are also important indicators for evaluating cellular oxidative stress levels [35]. Therefore, ROS, GSH, and T-SOD measurements can be utilized to assess the degree of cellular oxidative stress and to gauge the severity of ROS-induced cell damage. In 2018, Eftekhari et al. discovered that berberine could alleviate oxidative stress by reducing GSH depletion in paraquat-treated liver cells [36]. Mitochondria are the main site of cellular metabolism and the primary regulator of oxidative stress. They are the major source of ROS within cells and play a crucial part in oxidative stress and ferroptosis [37]. Studies have shown that ROS are mainly produced as byproducts of the respiratory chain reactions in mitochondria, consuming approximately 1%–5% oxygen content to generate ROS. Excessive ROS can lead to mitochondrial membrane hyperpolarization, accumulation of lipid peroxides, and ferroptosis [38]. Therefore, targeting the elimination of ROS within mitochondria has become an important focus for some researchers. A study obtained biogenic selenium nanoparticles from *Bacillus subtilis* and found that it reduced intracellular ROS levels, decreased mitochondrial membrane potential, and provided protection to the mitochondrial structure [39]. These findings provide additional options for the development and application of green nanomaterials. Lipid peroxidation, the mechanism by which ROS oxidize biological membranes in cells (e.g., mitochondrial and cell membranes) is another crucial marker for ROS-related cell damage [40]. This process has the potential to alter the permeability and fluidity of cell membranes, ultimately harming the structure and function of the cell. In this study, we showed that the ROS content dramatically increased after stimulation with 200 μM TBHP for 24 h, along with a significant decrease in GSH and T-SOD activity [41]. This shows that oxidative stress can be induced by TBHP stimulation in MC3T3-E1 cells and that these effects are reduced following Se-Met pretreatment. Moreover, we also showed that TBHP activation decreased expression of GPX4, which is strongly associated to oxidative stress. However,

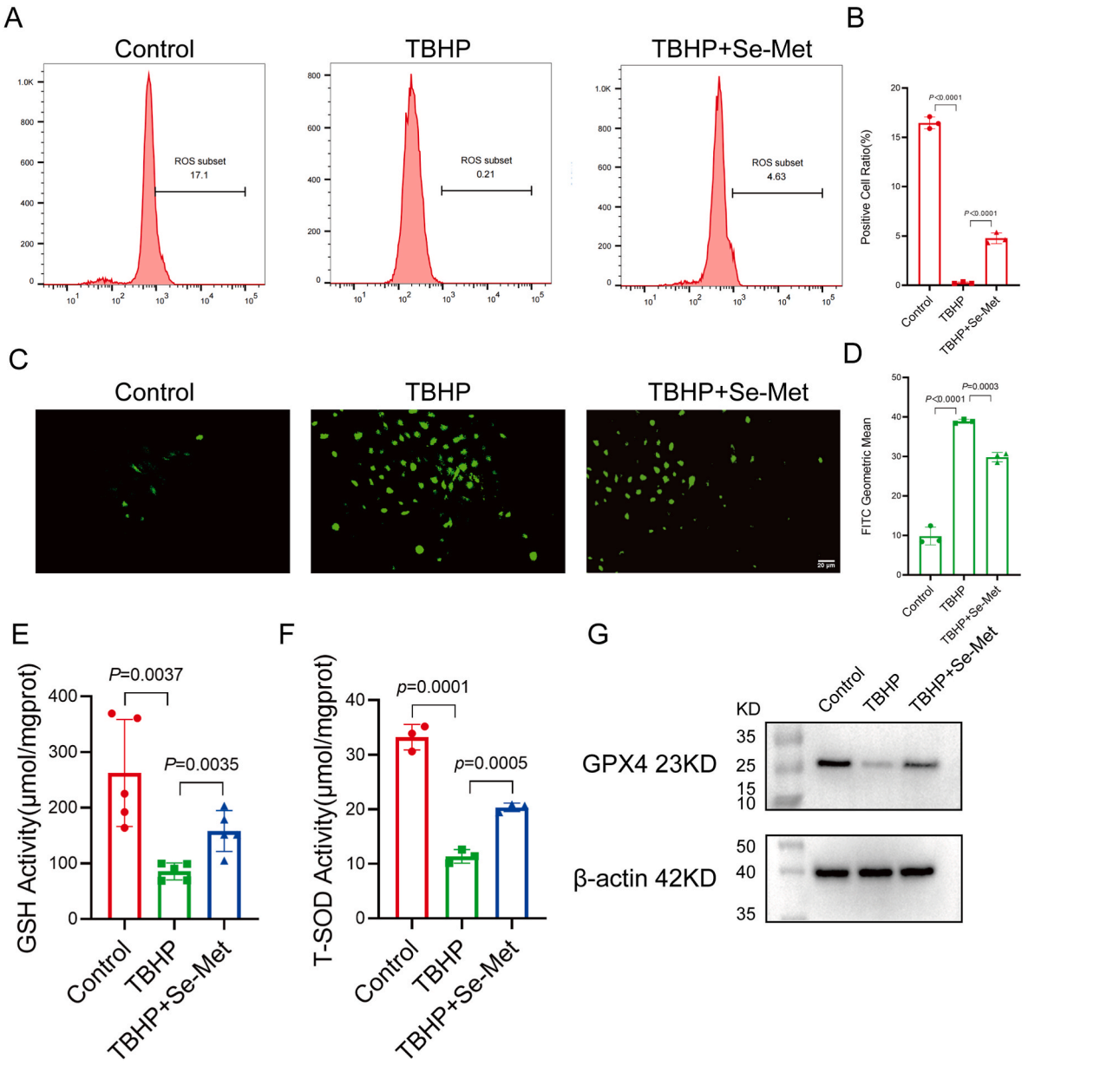


**Fig. 2.** Se-Met increases the survival rate of MC3T3-E1 cells following 24 h TBHP treatment. (A,B) Flow cytometry analysis was conducted to determine the levels of ROS in MC3T3-E1 cells; (C,D) Representative images of DCFH staining in MC3T3-E1 cells. Scale bar = 20 μm. Se-Met was able to reduce the percentage and geometric mean of DCFH-positive cells in TBHP-treated MC3T3-E1 cells; (E,F) ELISA results showing that Se-Met increased levels of T-SOD and GSH in MC3T3-E1 cells under TBHP induction; (G) Representative Western blot images of GPX4. Indicating that Se-Met plays a role in preventing excessive ROS generation in MC3T3-E1 cells. $p < 0.05$ indicate significant differences, whereas $p \geq 0.05$ indicates no significant differences.

pretreatment with Se-Met had the opposite effect. In TBHP-stimulated MC3T3-E1 cells, the mitochondria were typically smaller, the density of the mitochondrial membrane was concentrated, the mitochondrial cristae were diminished or even nonexistent, and lipid oxidation was dramatically elevated. Se-Met can also alleviate lipid oxidation and changes in mitochondrial ultrastructural. We hypothesize that Se-Met improves the MC3T3-E1 cells redox balance in the presence of oxidative damage, leading to protection from oxidative damage. However, the exact mechanism behind this effect remains unclear.

The NLRP3 inflammasome is an important mediator in the inflammatory response and is significantly related to the occurrence of many systemic diseases. It is currently the most extensively studied member of the neutrophil-lymphocyte ratio (NLR) family. Studies have suggested that NLRP3 can be activated by various endogenous danger signals and exogenous non-microbial risk factors, especially ROS activation, which can lead to cell death [42,43]. For example, high glucose levels have been shown to promote osteoclast differentiation and bone resorption through the NLRP3 pathway [44], whereas bisphenol A has been shown to activate Caspase I through the NLRP3 inflammasome, promote IL-1β expression, and cause osteoblast pyroptosis [45]. In this study, we used TBHP to simulate oxidative stress and found that NLRP3 protein expression significantly increased, thereby suggesting that NLRP3 activation may be due to excessive ROS production following TBHP stimulation. To clear the NLRP3 inflammasome in the cells, we used Se-Met. Compared to the TBHP group, the Se-Met group showed significantly reduced NLRP3-associated cell inflammation, suggesting that Se-Met can improve the aforementioned inflammatory response. This further supports our hypothesis that Se-Met can improve oxidative stress and inflammation in MC3T3-E1 cells.

Several studies have suggested that the Wnt/β-Catenin signaling

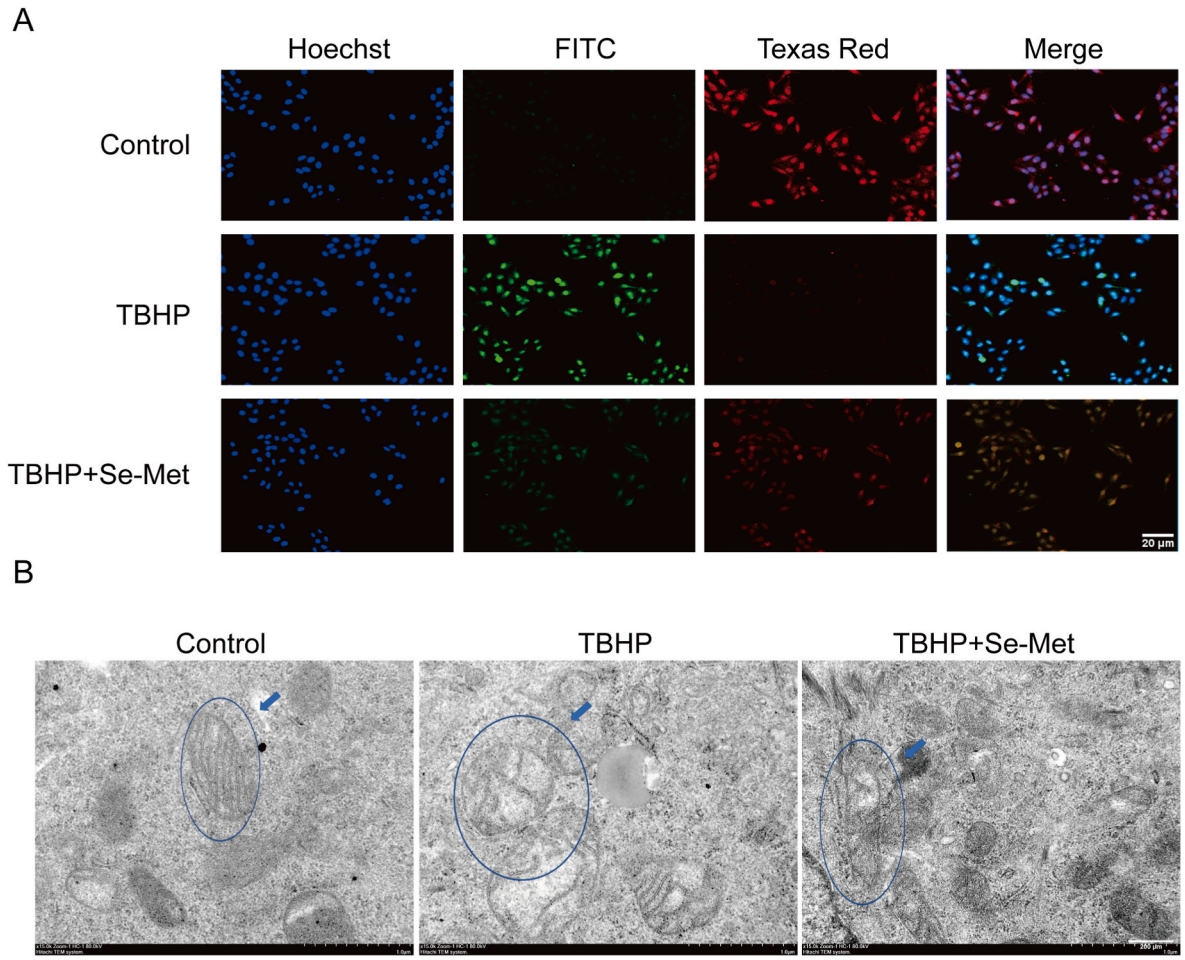


**Fig. 3.** Se-Met alleviates lipid peroxidation in TBHP-induced MC3T3-E1 cells. (A) Representative images of MC3T3-E1 cells stained with C11-BODIPY 581/591 and Hoechst. Scale bar = 20 μm; (B) Representative transmission electron microscopy images of MC3T3-E1 cells. Scale bar = 200 μm.

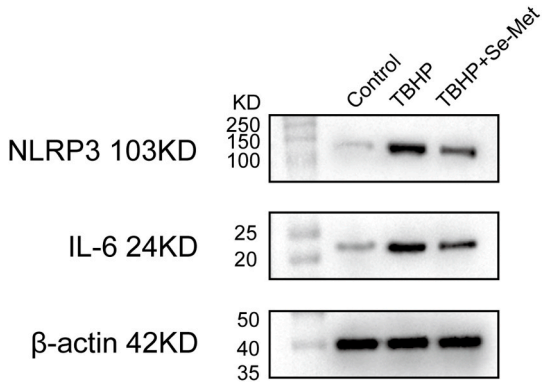


**Fig. 4.** Se-Met alleviates inflammatory response in TBHP-induced MC3T3-E1 cells. Representative Western blot images of IL-6 and NLRP3 inflammasome expression.

pathway is associated with the regulation of osteoblast proliferation, differentiation, and migration, and can promote the expression of osteogenic factors such as Runx-2 [46]. This signaling pathway can be regulated by oxidative stress and inflammatory factors, thus participating in the regulation of osteogenic function [47,48]. Thus, ALP staining was used to detect early osteogenic ability, whereas ARS staining was used to detect late osteogenic mineralization ability. Western blotting was used to detect the protective effect of Se-Met on osteogenic differentiation and mineralization of the Wnt/β-Catenin signaling pathway. According to previously published experimental conditions, we performed ALP staining after 7 days of cell osteogenic induction and ARS staining after 21 days. ALP staining showed that after 7 days of mineralization induction, the staining of TBHP (200 μM) stimulated cells was significantly lower than that of the control group. Similarly, the ARS staining results showed that the number and area of mineralized nodules produced by TBHP-induced cells significantly decreased after 21 days of osteogenic induction, whereas the results after Se-Met pretreatment were satisfactory. The results showed that the staining increased after 7 days of osteogenic induction, and the number and area of mineralized nodules increased significantly after 21 days of osteogenic induction. Similarly, in western blotting results, we found increased expression levels of Runx-2, Col-1, and β-Catenin proteins in Se-Met treated MC3T3-E1 cells after TBHP stimulation. These results suggest that Se-Met may promote osteogenic differentiation of MC3T3-E1 cells by protecting the Wnt/β-Catenin signaling pathway from oxidative stress and inflammation.

In conclusion, we found that selenomethionine effectively alleviates TBHP-induced oxidative stress and inflammation induced in MC3T3-E1 cells, while protecting the Wnt pathway and promoting osteogenic differentiation and mineralization. Thus, TBHP shows potential as a promising alternative for the prevention and treatment of OP. However, one key limitation of this study is the lack animal experiments. In subsequent studies, we plan to supplement our findings by constructing a New Zealand White rabbit model with bone defects. Further research is needed to determine whether these conclusions apply to other aspects of the Wnt pathway (e.g., neurodegenerative diseases and diabetes).

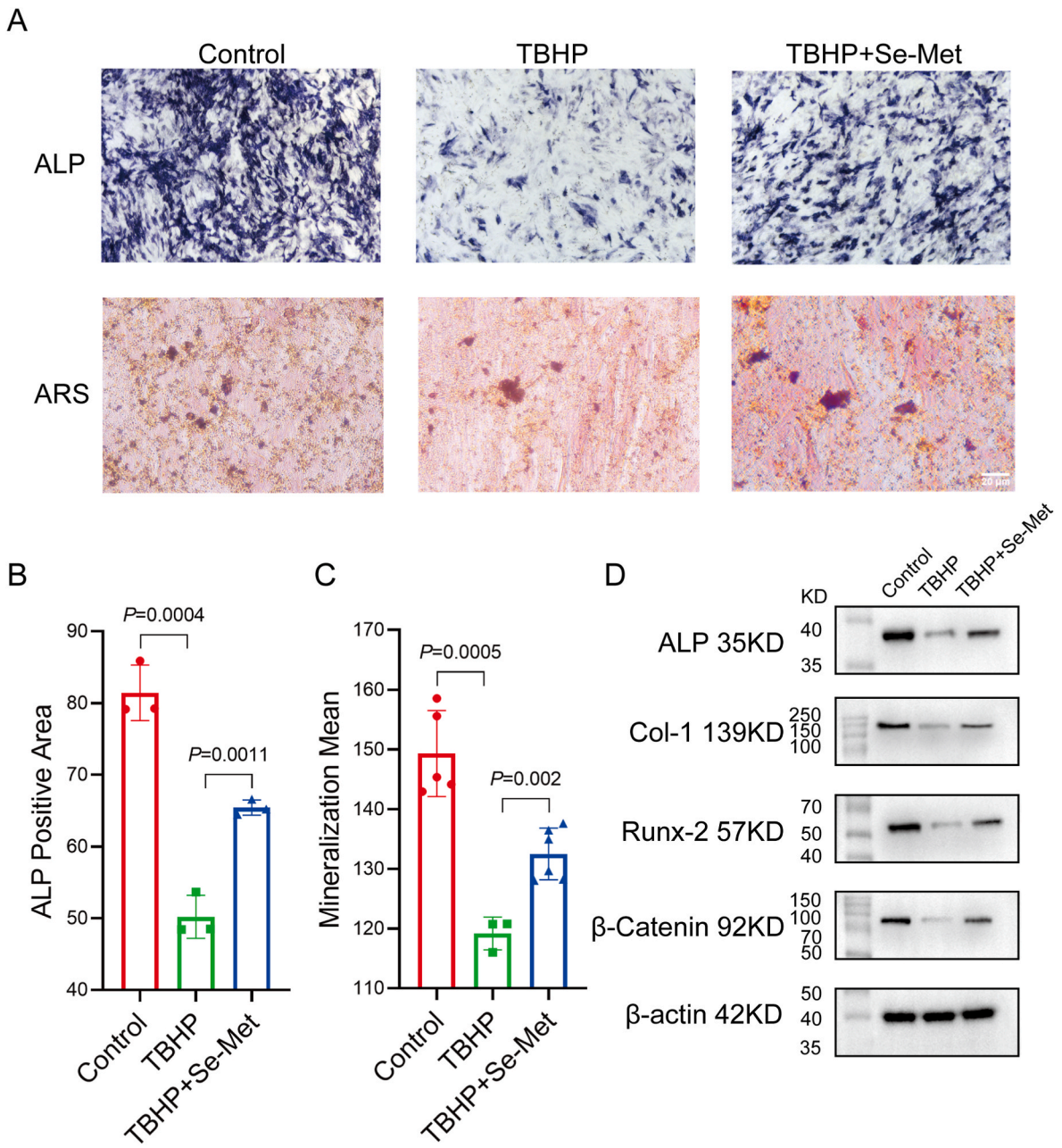


**Fig. 5.** Se-Met improves osteogenic differentiation and mineralization of MC3T3-E1 cells against TBHP. (A) Representative images of alkaline phosphatase (ALP) and Alizarin Red S (ARS) staining. Scale bar = 20 μm; (B) Quantitative analysis of calcium deposition normalized to the control group; (C) Quantitative analysis of ALP-positive area normalized to the control group; (D) Representative Western blot images of ALP, Runx-2, Col-1, and β-Catenin. $p < 0.05$ indicate significant differences, whereas $p \geq 0.05$ indicates no significant differences. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

## 5. Conclusions

This study demonstrates that Se-Met can enhance antioxidant enzyme activity, maintain mitochondrial morphology, and reduce ROS levels, thereby inhibiting oxidative stress and suppressing lipid peroxidation induced by oxidative stress. We also showed that Se-Met can inhibit inflammation by reducing the expression of NLRP3 inflammasome and IL-6. Finally, ALP staining, ARS staining and western blotting experiments proved that Se-Met can protect osteogenic differentiation and mineralization of MC3T3-E1 cells by reducing oxidative stress and inflammation to promote activation of this pathway. Further, we confirmed that Se-Met regulates osteogenic differentiation of MC3T3-E1 cells through the Wnt/β-Catenin signaling pathway. These findings indicate that Se-Met may play a significant role in regulating and maintaining appropriate levels of ROS and inflammation in OP. However, this study only characterized biological effects *in vitro*, and further evaluation is needed to determine its suitability for *in vivo* applications. The next step will be to conduct *in vivo* studies of Se-Met on animal models of OP, which will provide further experimental basis and ideas for the development of novel anti-osteoporotic drugs.

## Author contributions

The following statements should be used “Conceptualization, methodology, software, validation,G.D.Z. and Y.T.Z.; formal analysis, P.Y.G and Y.T.Z.; investigation, Y.P.T.; resources, Z.B.Y.; data curation, Q.Z.;

writing—original draft preparation, G.D.Z.; writing—review and editing, J.H.; visualization, J.H.; supervision, Q,Z.; project administration, Z.B.Y.; funding acquisition, Z.B.Y. All authors have read and agreed to the published version of the manuscript.

## Availability of data and materials

Not applicable.

## Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## Declaration of generative AI usage

Not applicable.

## Ethics and dissemination

This study will not involve the individual patient and any ethical problems since its outcomes are based on published data.

## Consent for publication

Authors are responsible for correctness of the statements provided in the manuscript.

## Institutional review board statement

Not applicable.

## Declaration of competing interest

The authors declare no conflict of interest.

## Acknowledgments

Not applicable.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2023.101559.

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