Original Article

Sodium Selenite Promotes Osteoblast Differentiation via The WNT/ß-Catenin Signaling Pathway

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Abstract

Objectives: Osteoporosis is regarded as a silent disorder affecting bone slowly, leading to an increased risk of fractures. Lately, selenium has been found to be associated with the acquisition and maintenance of bone health by affecting the bone remodeling process. However, the mechanism of action of selenium on bone is poorly understood. Here, the objective of this study is to examine the protective effects and mechanism of sodium selenite on the differentiation process of osteoblasts as well as under oxidative stress-induced conditions by evaluating the expression of osteoblast differentiation markers in the sodium selenite and/or hydrogen peroxide (H_2O_2)-treated MC3T3-E1 cell line.

Materials and Methods: In this experimental study, we confirmed the inducible osteogenic effect of sodium selenite on MC3T3-E1 cells. Moreover, we investigated the recovery of expression levels of osteogenic markers of sodium selenite in H_2O_2-treated MC3T3-E1 cells.

Results: It was observed that sodium selenite could promote alkaline phosphatase (ALP) activity and collagen synthesis in pre-osteoblasts. Also, sodium selenite enhanced the mRNA expression levels of osteogenic transcriptional factors, like osterix (OSX) and runt-related transcription factor 2 (Runx2). In addition, the terminal differentiation markers, such as osteocalcin (OCN) and collagen 1α (Col1α) were also increased after the treatment of sodium selenite. Also treatment of sodium selenite recused the H_2O_2-induced inhibition of osteoblastic differentiation of pre-osteoblasts cells via the WNT signaling pathway, implicating its antioxidant activity. Furthermore, sodium selenite restored the H_2O_2 repressed β-catenin stability and axin-2 reporter activity in MC3T3-E1 cells.

Conclusion: It may be concluded that sodium selenite can stimulate bone formation and rescue the oxidative repression of osteogenesis by activating WNT signaling pathways. Further detailed studies on the role of selenium and its ability to stimulate bone formation via the WNT signaling pathway may project it as a potential therapeutic intervention for osteoporosis.

Keywords: Osteoblasts, Osteoporosis, Selenium, WNT Signaling Pathway

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Introduction

Osteoporosis is the most prominent skeletal disease that increases the risk of osteoporotic fracture by reducing bone density (1). Other complications can also be increased during the osteoporosis progression. Osteoporosis is the net outcome of an imbalance between the resorption and the regeneration of bone tissue. Osteoporosis is regarded as a 'silent disorder' as it progresses slowly and is considered as a major health issue in the world (2). This condition is distinguished by reduced bone weight and bone degeneration, leading to a tendency to fractures. Osteoporosis-related bone fracture is also considered as an age-related bone condition with a high-risk factor in approximately 33% of women and 20% of men (3). At the molecular level, many factors affect the initiation of osteoporosis. One of them is the induction of the secretion of pro-inflammatory cytokines in senescent cells during menopause. The progression of bone loss during osteoporosis has often been associated with the release of inflammatory cytokines like interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1, IL-6, IL-8, and IL-1β (4-6). Growing evidence also suggests that during aging or menopause, increased oxidative stresscontributes to the resorption of bone tissue leading to osteoporosis due to the buildup of free radicals from inflammation or mitochondrial dysfunction (5, 7).

An approach for osteoporosis treatment is the induction of osteoblastogenesis via enhancing proliferation and differentiation of osteoblast cells using anabolic agents, such as estrogens. Another approach for osteoporosis treatment is reducing osteoclastogenesis via inhibition of differentiation of bone-specific multinucleated osteoclasts cells from hematopoietic monocyte precursor cells using anti-resorptive drugs, such as bisphosphonate. Although anabolic agents and anti-resorptive agents are effective against osteoporosis, they are associated with severe side effects, including poor bone quality and carcinogenesis (3). Recent studies

on phytoestrogens having bone stimulatory effects seem promising. However, they suffer bioavailability issue and thus need efficient delivery systems (8-10). Therefore, there is a need to identify novel agents to prevent osteoporosis (3, 11).

The transcription factors, like osterix (OSX) and runt-related transcription factor 2 (Runx2) regulate osteoblast proliferation and differentiation processes at the transcriptional level. At the same time, the bone matrix proteins, such as collagen type I (Col1α), osteocalcin (OCN), alkaline phosphatase (ALP), and osteopontin (OPN), stimulate the bone mineralization process. Thus, these molecules are considered to regulate the bone development and establishment process. In addition, the proliferation and differentiation of both the osteoblast and osteoclast might be modulated by the WNT/β-catenin canonical signaling pathway at multiple levels, leading to an increase in osteoblastogenesis and a decrease in osteoclastogenesis (12). WNTs can promote the differentiation of osteoblast precursors into mature osteoblasts via β-catenin-dependent canonical pathways (13).

Reactive oxygen species (ROS) is often considered as oxidative stress and is shown to induce cellular pathology by degrading proteins, lipids, and DNA (14). Almost most of the sources having oxidative stress generate hydrogen peroxide (H_2O_2) which have the ability to penetrate cellular membranes (15, 16). Treatment of H_2O_2 is shown to exert apoptosis in osteoblasts and suppress differentiation of osteoblasts (17, 18). Hence, H_2O_2 is used to establish *in vitro* cellular model for oxidative stress and evaluate osteonecrosis, proliferation, and osteoblastic differentiation in bone-like cells (19, 20).

Selenium is a micronutrient present as a cofactor in various biologically active enzymes, thus acting as an essential antioxidant in the cellular environment. Not only can selenium reduce oxidative stress, but also it has an inverse correlation between selenium consumption and osteoporosis which has been observed (21). Inadequate selenium intake has been related to a high risk of bone disorders as it is found to be linked to increased turnover of bone and reduced bone mineral density (BMD) (22). It has been observed that sodium selenite induces apoptosis in mature osteoclasts via alterations in mitochondrial signaling pathways (23). However, the signaling mechanism associated with the role of sodium selenite in bone formation is less studied.

Here, we investigate the role of sodium selenite on the MC3T3-E1 cell proliferation and differentiation process. MC3T3-E1 is a pre-osteoblast cell linewhich has been in use to study the proliferation, differentiation process, and mineralization of osteoblasts (24). In addition, we aimed to identify the underlying molecular mechanism of sodium selenite in inducing MC3T3-E1 cell differentiation. Moreover, the antioxidant property of sodium selenium and its effect on osteogenesis in H_2O_2 treated osteoblasts was evaluated.

Materials and Methods

Materials

Cytotoxicity detection kit was purchased from Takara Bio Inc., Japan. CSPD substrate was purchased from Roche, Germany. Trizol reagent, Renilla luciferase thymidine kinase construct, and SuperScript II Reverse Transcriptase were procured from Invitrogen, USA. Phosphate-buffered saline (PBS) was procured from T&I, Korea. SYBR green qPCR MasterMix which was purchased from Bioneer, Korea. Sodium selenite (Na_2SeO_3), 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Sirius red dye, and Bouin's fluid were procured from Sigma-Aldrich, USA. Penicillin-streptomycin solution (P/S), fetal bovine serum (FBS), and α-minimum essential medium (MEM) were acquired from Gibco, USA.

Cell culture

Mice pre-osteoblast MC3T3-E1 cells (ATCC, CRL-2593) were grown in the α-MEM medium. Cells were cultured at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was supplemented with FBS (10 %), P/S (1 %), L-glutamine (2 mM), sodium pyruvate (1 mM), and non-essential amino acid (0.1 mM).

Cytotoxicity tests (MTT and LDH assay)

MTT assay was performed to evaluate the cell viability of the cells. For this, MC3T3-E1 cells (1×10^4 cells/well) were cultured in 96-wells plate and various doses (0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 μM) of Sodium selenite was treated for 24 hours. Insoluble purple MTT formazan crystal produces succinate dehydrogenase in the mitochondria of metabolically active cells. MTT solution (10 μl: 5 mg/ml in PBS) was pipetted in the wells with the cells and further incubated at 37˚C for 2 hours. Afterward, to dissolve MTT, the supernatant was removed, and DMSO (200 μl) added to each well and shaken gently. By using a UV-Vis spectrophotometer, the optical density was recorded (Molecular Devices LLC, USA) at a wavelength of 570 nm. Sodium selenite cytotoxic effect on cells was determined according to the cytotoxicity detection kit protocol.

For the lactate dehydrogenase (LDH) assay, the cells were cultured similarly as described above. The cell culture medium (10 μl) was collected from the cultured wells in a fresh 96-well plate. Next, PBS (40 μl) and LDH reagent (50 μl) was pipetted to every well of the plate and incubated (45 minutes) in the dark at 25˚C. To end the enzymatic reaction, a stop solution (50 μl) was added to each well. Using a UV-Vis spectrophotometer, optical density was noted at 490 nm. For positive control, the optical density of total cell lysate was recorded.

Alkaline phosphatase activity

In 48-wells plates, MC3T3-E1 cells were cultured at a density of 5×10^4 cells/well. Then, sodium selenite with or without hydrogen peroxide (H_2O_2) was treated with various doses (0, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 μM) to MC3T3-E1 cells. After incubating for 48 hours, cold PBS was used to wash the cells twice. The cold RIPA buffer (100 μl) was then added to each well and shaked gently. The whole-cell lysate was subjected to centrifugation for 20 minutes at 4˚C and 14,000 rpm. The supernatant (20 μl) was collected and

added to the CSPD substrate (100 µl). The reaction solution was then kept for 30 minutes at room temperature. The luminescence intensity was recorded by using a luminometer (Glomax, Promega, USA). The luminescence of the total cell lysate was used for normalization.

Sirius red staining

In a 48-well plate, the MC3T3-E1 cells were treated for 7 days with 3.2 µM of sodium selenite. The medium, along with sodium selenite was substituted every second day. After treatment, Bouin's fluid was used to fix the cells for 1 hour. Sirius Red dye (1 mg/ml in saturated aqueous picric acid) was then used to stain the cells for 1 hour. To quantitate the Sirius Red dye from the stained cells, 0.1 N sodium hydroxide was added for 30 minutes to the wells. After dissolving the dye, optical density was measured at 550 nm using a spectrophotometer in triplicate cultures (sodium hydroxide (0.1 N) was used as a blank).

Real-time reverse transcription polymerase chain reaction

MC3T3-E1 cells were cultured in a 6-wells plate at a density of 3×10^5 cells/well and were subjected to 3.2 µM of sodium selenite. The cellular RNA was collected using Trizol reagent after 48 hour of treatment. The absorbance ratio (260/280) was measured to determine the quality of the collected RNA. Also, RNA was separated on an agarose gel to observe its integrity. The real-time polymerase chain reaction (PCR) was performed as per our lab protocol (25). By using 2 µg of RNA, SuperScript II Reverse Transcriptase was used to synthesize cDNA. With 1 µl of the synthesized cDNA, real-time PCR was performed using SYBR green qPCR MasterMix and Rotor-Gene 3000 real-time PCR, Corbett, Germany. The qPCR procedure was as follows: an initial step of denaturation for 10 minutes at 95˚C as, and 40-cycles of amplification at 95˚C for 20 seconds, at 60˚C for 20 seconds, and extension at 72˚C for 25 seconds. *GAPDH* was used to standardize the relative expression of mRNAs and was quantified by the double delta CT (ΔΔCT) method. Table 1 shows the list of primer sequences used.

Western blotting

In a 6-wells plate, MC3T3-E1 cells were grown at a 3×10^5 cells/well density. Sodium selenite (3.2 µM) was treated to the cells for 12, 24, and 48 hours. The protein was isolated and loaded on sodium dodecyl sulfate (SDS)-polyacrylamide gel. The separated proteins on the gel were transferred to a polyvinylidene fluoride (PVDF) membrane. 5% skim milk was used to block the membrane for 1 hour, and then it was incubated with antibodies against OSX, β-catenin, Runx2, and β-actin overnight at 4˚C. After that, 1X TBST (Tris-buffered saline, 0.1% Tween 20) was used to wash the membranes and incubated with horseradish peroxidase-conjugated secondary antibodies for 45 minutes at room temperature. Chemiluminescence reagent was used to visualize the target protein bands. As a loading control, β-actin was used. Image J software (NIH, USA) was used to quantify the band intensities.

Table 1: Mouse primers for real-time reverse transcription polymerase chain reaction (RT-PCR)

Gene	Primer sequence (5´-3´)
OSX	F: GGAAAGGAGGCACAAAGAAGCCAT
	R: AGTCCATTGGTGCTTGAGAAGGGA
Col1a	F: TTCTCCTGGCAAAGACGGAC
	R: AGGAAGCTGAAGTCATAACCGCCA
OCN	F: TGCTTGTGACGAGCTATCAG
	R: GAGGACAGGGAGGATCAAGT
Runx2	F: AAGTGCGGTGCAAACTTTCT
	R: TCTCGGTGGCTGGTAGTGA
GAPDH	F: TCGTGGATCTGACGTGCCGCCTG
	R: CACCACCCTGTTGCTGTAGCCGTAT

Statistical analysis

The quantitative statistical analysis was performed using Graphpad Prism 5.0 (San Diego, CA) software utilizing a two-tailed Student's t test. A data value of $P<0.05$ was considered statistically significant.

Results

Sodium selenite induces osteogenic activity in osteoblasts. To assess any effect of sodium selenite on the cell viability and cytotoxicity of MC3T3-E1, various concentrations (0, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 µM) of sodium selenite was treated to the cells for 24 hours. MTT and LDH assays were used to evaluate the cell viability and cytotoxicity of MC3T3-E1, respectively. Various concentrations of sodium selenite demonstrated no effect on cell viability and cytotoxicity of osteoblasts (Fig.1A, B).

To assess any osteogenic effect of sodium selenite on osteoblasts, various concentrations (0, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 µM) of sodium selenite were treated to MC3T3-E1 cells for 48 hours, and ALP activity was analyzed. ALP is an enzyme found in osteoblasts and is regarded as a marker of osteoblast differentiation. The sodium selenite-treated osteoblasts showed significantly increased ALP activity at a concentration of 1.6 and 3.2 µM compared to control; however, a remarkable decrease in the ALP activity of MC3T3-E1 cells was observed at a dose of 6.4 µM (Fig.1C).

Further, the osteogenic effect of sodium selenite was confirmed by the mRNA expression levels of osteogenic markers. Sodium selenite (3.2 µM) was treated to the MC3T3-E1 cells for 24 hours. The mRNA levels of master regulator for osteogenesis (*OSX*), osteoblast differentiation transcriptional factor (*Runx2*), and terminal differentiation

markers *Col1α* and *OCN* were detected through real-time qRT-PCR. Sodium selenite induced the mRNA expression of *OSX* (~2 folds), *Runx2* (~2 folds), *Col1α* (~2 folds), and *OCN* (~2 folds) in comparison with control (Fig.1D). As evidenced by Sirius red staining, Collagen depositions were increased by ~1.8 folds in 3.2 μM of sodium selenite-treated osteoblasts compared to control (Fig.1E). In conclusion, sodium selenite can induce osteogenic differentiation in osteoblasts.

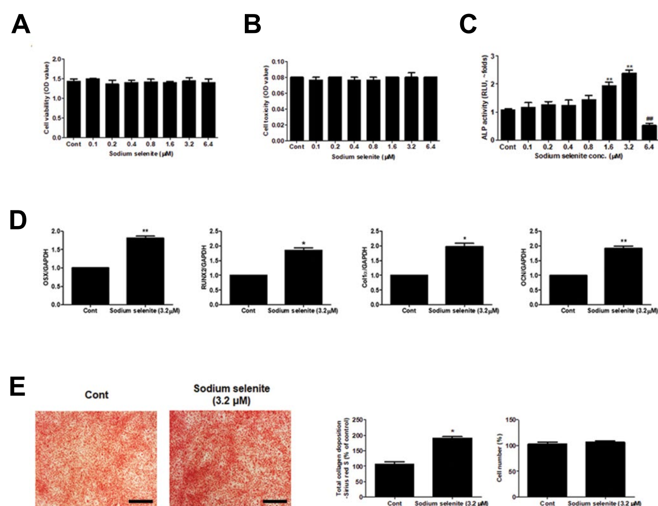
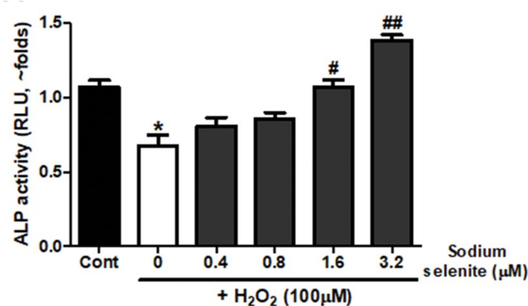


Fig.1: Effect of sodium selenite in osteoblasts. Sodium selenite was treated various concentration (0, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 μM) in osteoblasts. **A.** Cell viability and **B.** Cell cytotoxicity of sodium selenite were evaluated through MTT and LDH assay, respectively. **C.** The osteogenic activity of sodium selenite was confirmed through ALP activity. **D.** Genes expression of the osteogenic markers (*OSX*, *Runx2*, *Col1α*, and *OCN*) in sodium selenite (3.2 μM)-treated osteoblasts. Results are represented as a fold increase relative to *GAPDH* expression. All data are shown as the mean ± SD. Similar results were obtained in three independent experiments. *; P≤0.05, **,##; P≤0.01 compared to the control, MTT; 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, LDH; Lactate dehydrogenase, ALP; Alkaline phosphatase, Cont; Control, and OD; Optical density.

Sodium selenite recovered the osteoblast differentiation in H_2O_2-stimulated osteoblasts.

Previous studies have reported that H_2O_2 induces oxidative stress and contributes to the suppression of the differentiation process of osteoblasts (17). To confirm the osteogenic effect of sodium selenite in H_2O_2-stimulated osteoblasts, H_2O_2 (100 μM) was treated alone or with sodium selenite (3.2 μM) to osteoblasts at various concentrations (0, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 μM) for 48 hours. Alone treatment of H_2O_2 notably decreased the ALP activity of osteoblasts. However, decreased ALP activity was significantly recovered to a level similar to that of the control when 1.8 and 3.2 μM of sodium selenite was co-treated (Fig.2A). Further, H_2O_2 (100 μM) was treated alone or with sodium selenite (3.2 μM) to osteoblasts for 48 hours. Collected protein lysate was analyzed for the expression of osteogenic transcriptional factors for osteoblasts by western blotting. Treatment of H_2O_2 decreased the expression of osteogenic transcriptional factors (Runx2 and OSX) at the protein levels. However, decreased expression levels of Runx2 and OSX were recovered with a co-treatment of 3.2 μM of sodium selenite (Fig.2B). Our results showed that sodium selenite treatment to MC3T3-E1 cells could mask the suppressive effect of H_2O_2 on the osteogenesis process. Thus, the ability of sodium selenite to affect bone-forming signaling might be expected.

A



B

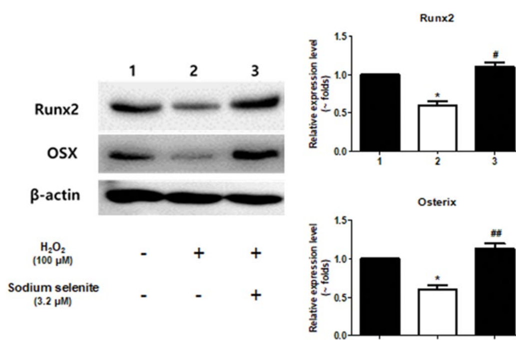


Fig.2: Effect of sodium selenite in H_2O_2-stimulated osteoblasts. **A.** Sodium selenite (3.2 μM) was pretreated to osteoblasts for 24 hours, and after that, H_2O_2 (100 μM) was treated for 48 hours. After 48 hours of incubation, ALP activity was analyzed. **B.** The osteogenic markers protein level was detected after 48 hours of sodium selenite (3.2 μM) treatment. To normalize the densitometry of western blot bands, β-actin was used as a loading control. All data are shown as the mean ± SD. Similar results were obtained in three independent experiments. *,#; P≤0.05 compared to the control, ##; P≤0.01 compared to the H_2O_2-stimulated osteoblasts, ALP; Alkaline phosphatase, and RLU; Relative luminescence units.

Sodium selenite recovers the suppressed WNT/β-catenin signaling pathway in H_2O_2-stimulated osteoblasts

WNT/β-catenin signaling pathway is crucial for the differentiation of osteoblast and bone formation (12). We next assessed the involvement of the WNT/β-catenin signaling pathway in the induction of osteogenesis in osteoblasts by sodium selenite in H_2O_2-stimulated osteoblasts. For this, *Axin-2* luciferase reporter construct was transfected to osteoblasts for 24 hours using Genefectine reagent (Genetrone Biotech, Korea), and the cells were treated with H_2O_2 (100 μM) alone or with 3.2 μM of sodium selenite for 12 and 24 hours. Compared to control, the Axin-2 luciferase activity was found decreased by H_2O_2 in both the treatments for 12 and 24 hours. While co-treatment of sodium selenite with H_2O_2 for 24 hours significantly recovered the reduced Axin-2 reporter activity, suppressed by H_2O_2 (Fig.3A).

Upon activation of the WNT signaling pathway, β-catenin gets localized to the nucleus and stabilizes. β-catenin then binds to the TCF/LEF family of DNA-binding proteins and regulates osteogenesis by targeting WNT-mediated genes (13). Therefore, osteoblasts were treated with H_2O_2 (100 µM) alone or with sodium selenite (3.2 µM) for 12 and 24 hours, and western blotting was performed to analyze the stability of β-catenin molecules. H_2O_2 decreased stabilization of β-catenin molecules after 12 and 24 hours of treatment, but sodium selenite was able to significantly recover the reduced β-catenin stabilization level by H_2O_2 after treatment of 24 hours (Fig.3B). Taken together, through WNT/β-catenin signaling, sodium selenite recovers the osteogenic activity reduced by H_2O_2 in osteoblasts.

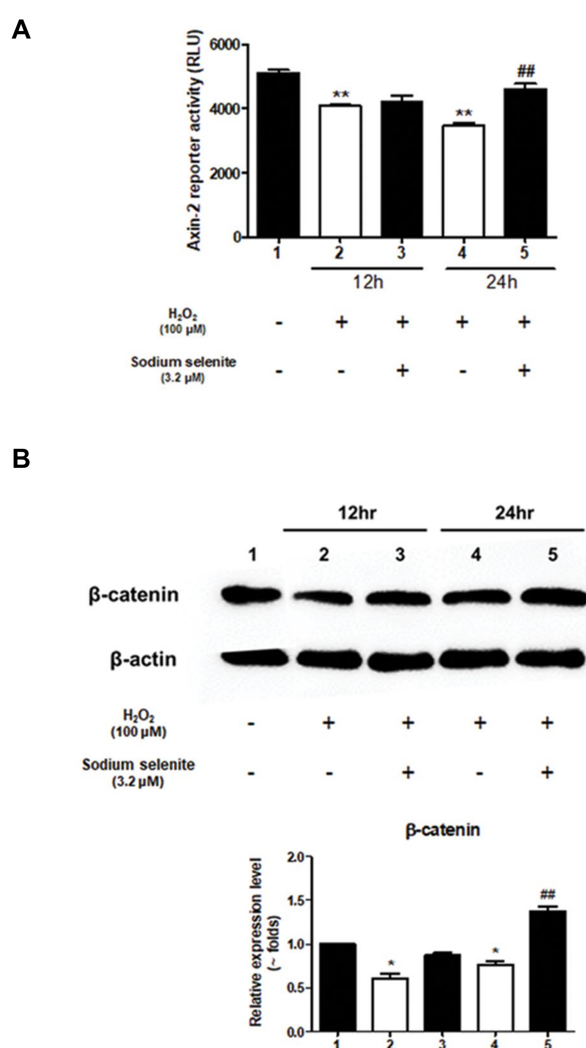


Fig.3: Sodium selenite activates the WNT/β-catenin signaling pathway in H_2O_2-stimulated osteoblasts. **A.** Axin-2 reporter plasmid was transfected to osteoblast for 24 hours. Sodium selenite (3.2 µM) was pretreated to MC3T3 E-1 cells for 24 hours followed by H_2O_2 (100 µM) treatment for 48 hours. As described in materials and methods, luciferase activities were measured in cell lysates. Renilla luciferase activity was used to normalize the luciferase activity of the cell lysates. **B.** Sodium selenite (3.2 µM) was pretreated to MC3T3 E-1 cells for 24 hours followed by H_2O_2 (100 µM) treatment for 12 and 24 hours. After 12 and 24 hours, protein lysates were collected, and western blotting was performed. To normalize the densitometry of western blot bands, β-actin was used as a loading control. Data are shown as the mean ± SD of three independent experiments. *; P≤0.05 compared to the control, **, ##; P≤0.01 compared to the H2O2-stimulated osteoblasts, and hr; Hours.

Discussion

Selenium is a vital trace element and is an essential constituent of selenocysteine (SeCys) residues. It has been known to modulate the functioning of various selenocysteine-containing intracellular selenoproteins (26). Selenium is an essential constituent of several antioxidant enzymes which plays a vital role in scavenging the free radicals released during normal oxygen metabolism (27). Lately, a case-control study on the elderly population showed a decreased risk of osteoporotic hip fracture in people with high selenium intake. However, it was also largely dependent on smoking status (28). Moreover, a bone phenotype showing poorly developed cortical and trabecular mineralization in rodents was due to low dietary selenium intake (22). Also, selenium as sodium selenite has been shown to regulate OCN expression in osteoblasts, required for the formation of a mineralized matrix of bone (29). Thus, we tried to observe the effect of selenium on the osteogenic differentiation process of osteoblasts. Our results showed that in MC3T3-E1 cells sodium selenite stimulated the ALP activity. Additionally, induction in the expression levels of mRNAs of osteogenic transcriptional factors (*OSX* and *Runx-2*), osteogenic markers *(Col1α* and *OCN*) and increased collagen synthesis further confirmed the stimulatory ability of sodium selenite on the osteogenic activity of MC3T3-E1 cells.

Varied environmental conditions or agents or even the normal cellular metabolism may produce ROS, which has been found responsible for the pathogenesis of various pathologies, including osteoporosis (30). H_2O_2, being a member of the ROS family has the ability to diffuse across biological membranes and cause varied kinds of biological activities. It has been reported that exogenous treatment of H_2O_2 inhibits osteoblastic differentiation in MC3T3-E1 and bone marrow stromal cells (MSCs) cells (31-34). Previously, sodium selenite has been shown to protect bone MSCs against H_2O_2-induced inhibition of osteoblastic differentiation. The study observed that the effect of sodium selenite was associated with oxidative stress inhibition and the Mitogen-activated protein kinase (MAPK) signaling pathway (17). Hence, in MC3T3-E1 cells, we tried to confirm the effect of H_2O_2 and, at the same time, observed the rescue effect of sodium selenite, if any, on the osteogenic activity (ALP activity). It was observed that the treatment of sodium selenite (3.2 µM) significantly rescued the adverse effect of H_2O_2 on the osteogenic differentiation marker (ALP activity) in pre-osteoblast cells. This rescued effect of sodium selenite on H_2O_2 suppressed ALP activity in MC3T3-E1 cells was due to rescuing the repressed expression of osteogenic transcription factors (*OSX* and *Runx-2*). Lately, it has been observed that in postmenopausal women with osteoporosis, oxidative stress is negatively associated with BMD of total femora (35). Moreover, in ovariectomized rats, H_2O_2 and the levels of lipid peroxidation were increased, while enzymatic antioxidants like glutathione S transferase (GST), superoxide dismutase (SOD),

glutathione peroxidase (GPx) were reduced in femora tissue homogenates (36). In osteoblasts and bone MSCs, various types of selenoproteins or selenoenzymes, including thioredoxin reductases, GPx, selenoprotein P, and types 2 iodothyronine deiodinases are known to be expressed. In bone MSCs, the expression of the antioxidant enzyme GPx is required to protect against the oxidative damage induced by H_2O_2 (37). Liu et al. (17) also observed that the pretreatment of sodium selenite to bone MSCs effectively suppressed H_2O_2-induced oxidative stress by increasing the total antioxidant capacity (TAOC) and decreased glutathione (GSH) levels and suppressing intracellular ROS levels and lipid peroxidation. Thus, it might be expected that pretreatment of sodium selenite was able to rescue the inhibitory role of the H_2O_2 on the osteoblastic differentiation process by enhancing the expression of antioxidant enzymes like GPx, as observed by the previous researchers.

The WNT/β-catenin signaling pathway is essential for bone formation by regulating the various processes of osteoblastogenesis like proliferation, differentiation, and mineralization (12). Since sodium selenite was able to attenuate the decrease in osteogenic markers and ALP activity in MC3T3-E1 cells treated with H_2O_2, we next tried to examine the role of the WNT/β-catenin signaling pathway in the rescue effect mediated by sodium selenite. Treatment of H_2O_2 decreased the *Axin-2* reporter activity after 12 and 24 hours. A similar decrease in β-catenin stability was observed in H_2O_2 treated MC3T3-E1 cells. Co-treatment of sodium selenite along with H_2O_2 to MC3T3-E1 cells significantly recovered the suppressed *Axin-2* reporter activity after 24 hour of treatment. This was further established by increased stability of β-catenin after the co-treatment of sodium selenite with H_2O_2 to MC3T3-E1 cells. An increase in β-catenin stability and *Axin-2* reporter activity after sodium selenite treatment implicates the involvement of the WNT signaling pathway in osteoblasts. The stimulatory effect of sodium selenite on the WNT signaling pathway might explain the rescue effect of sodium selenite on the osteogenic activity of H_2O_2 stimulated MC3T3-E1 cells. MAPKs play an essential role in bone formation. Studies have shown that the ERK1/2 signaling pathway is responsible for the inhibitory effect of H_2O_2 on osteoblastic differentiation (31, 32). Though other MAPKs like (JNK and p38) have also been implicated in the physiological processes mediated by oxidative stress (38) but their role in H_2O_2-induced inhibition of osteoblastic differentiation is not clearly understood (31, 32). Lately, it was observed that H_2O_2-mediated adverse effect on osteoblastic differentiation of bone MSCs was inhibited by selenium. The effect was due to a decrease in oxidative stress and partly stimulation of the ERK signaling pathway (17). Previous studies have highlighted a crosstalk between MAPKs and WNT signaling pathways during the osteoblast differentiation process, largely decided by the kind of stimuli (25, 39). Thus, future studies are essential to understand any physical crosstalk of MAPKs with the WNT signaling pathway under the influence of selenium in the induction of osteogenic activity in osteoblasts.

Conclusion

Our study demonstrates the osteogenic stimulatory ability of selenium in osteoblasts. Sodium selenite was able to exert induction in the differentiation of osteoblasts as represented by elevation in ALP activity, increased mRNA levels of *OSX*, *Runx2*, *Col1α*, and *OCN*, and enhanced collagen synthesis. Moreover, sodium selenite was able to rescue the H_2O_2-mediated suppression of osteoblastic differentiation in osteoblasts. Sodium selenite was able to achieve this by activating WNT signaling pathway in osteoblasts. With these findings, it may be concluded that the role of selenoproteins in bone formation has just been recognized, and further detailed studies may validate them as potential therapeutic interventions for osteoporosis.

Acknowledgments

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Authors' Contributions

A.R.S., G.S.; Designed the experiments, performed the experiments, generated data, and wrote the manuscript. Y.-H.L.; Performed the experiments, generated data, and wrote the manuscript. C.C.; Helped with results, discussion and interpretations. S.-S.L., E.-M.S.; Provided the facilities and helped drafting the manuscript. All authors have read and approved the final manuscript.

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