Reactive Sulfur and Selenium Species in the Regulation of Bone Homeostasis

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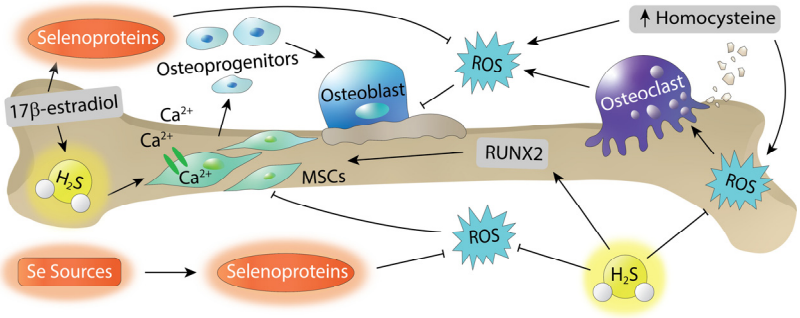
Abstract

Reactive oxygen species (ROS) are important modulators of physiological signaling and play important roles in bone tissue regulation. Both reactive sulfur species (RSS) and reactive selenium species (RSeS) are involved in ROS signaling, and recent work suggests RSS and RSeS involvement in the regulation of bone homeostasis. For example, RSS can promote osteogenic differentiation and decrease osteoclast activity and differentiation, and the antioxidant activity of RSeS play crucial roles in balancing bone remodeling. Here, we outline current research progress on the application of RSS and RSeS in bone disease and regeneration. Focusing on these investigations, we highlight different methods, tools, and sources of RSS and RSeS, and we also highlight future opportunities for delivery of RSS and RSeS in biological environments relating to bone.



Introduction

Reactive oxygen species (ROS) are often viewed as detrimental to health, but these reactive species also play important signaling roles in different systems. For example, ROS have established roles in the cardiovascular, muscular, and immune systems. In the cardiovascular system, multiple redox pathways are involved in angiogenesis and vasodilation.[1] The muscular system relies on redox signaling for skeletal muscle regeneration and muscle cell activity regulation.[1] Furthermore, chronic granulomatous disease (CGD) is caused by decreased levels of ROS that result from mutations in NADPH oxidase 2 (NOX2) and leads to hyperinflammation in patients.[2, 3] ROS are also critical for bone remodeling in processes associated with both bone strengthening and healing.[4] Looking farther down the chalcogens on the periodic table, both reactive sulfur species (RSS) and reactive selenium species (RSeS) are intertwined with ROS, and recent work suggests that all three of these chalcogens are involved in different components of bone regulation.

Many RSS are established to play important roles in biological processes, but hydrogen sulfide (H_2S) has garnered significant recognition in the past two decades. As a recent addition to the gasotransmitter family, H_2S is endogenously produced, membrane permeable, and active at physiologically relevant concentrations. Moreover, many of its biological effects can be mimicked by exogenously applied H_2S sources.[5] H_2S is produced by three main enzymes including cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST). These enzymes are localized throughout various organ systems and cell types with CBS primarily localized in the brain, CSE in the liver and cardiovascular system, and 3-MST in the mitochondria.[6] As evidenced by the diverse localization of these enzymes, H_2S plays a wide variety of important physiological roles throughout the body.[7, 8] For example, H_2S

provides neuroprotection and cardioprotection, promotes vasodilation and angiogenesis, and has recently been found to regulate bone homeostasis. H_2S promotes bone formation through persulfidation of Ca^{2+} channels, which stimulates the differentiation of mesenchymal stem cells (MSCs) to bone-forming osteoblasts.[9] CBS and CSE expression are also closely correlated with the transition of human MSCs (hMSCs) towards the osteoblastic phenotype with CSE potentially serving as a novel marker of osteogenic differentiation.[10] Additionally, irregular H_2S levels have been correlated with certain bone diseases including osteoporosis, which has brought forth efforts to better understand the therapeutic potential and role of H_2S and other RSS in the skeletal system.[11-14]

Reactive selenium species (RSeS) have also emerged as an important class of bioorganic molecules with broad roles in different nutrient pathways and biological functions. Selenomethionine and selenate salts are two main dietary sources of selenium that result in selenium incorporation into biomolecules through different metabolic pathways. Selenium-depleted diets in humans can result in Kashin-Beck disease, which is a debilitating cartilage disease characterized by chondrocyte necrosis in the growth plate cartilage and articular cartilage that can lead to growth retardation and osteoarthritis.[15, 16] Because selenium deficiencies are often manifested in cartilage, selenium levels are thought to impact bone health indirectly.[17] Low selenium levels have also been linked to reduced bone turnover rates and bone mineral density. Interestingly, at least nine selenoproteins are naturally expressed in osteoblasts, which suggests a critical role of selenium in the maintenance of bone homeostasis.[18] In particular, selenoprotein P is an essential selenium transporter to bones.[19] Mechanistic investigations into the direct effects of selenium on bone health suggest that the potent antioxidant effects of RSeS may play a critical role in cell proliferation and differentiation, as seen in studies with Chinese hamster ovary

(CHO) cells.[18] Many other selenoproteins are involved in antioxidant processes, which further supports their role in regulating the redox landscape of different osteogenic processes.

RSeS and RSS have synergistic activities against reactive oxygen species and oxidative stress, which is in part due to the different chemical properties of Se and S. For example, RSeS are more polarizable and more nucleophilic than their sulfur counterparts. RSeS are also generally more toxic than RSS. For example, the LD_{50} of selenocystine is 8.5 mg/kg[20] in rats, whereas the LD_{50} for cystine is> 2000 mg/kg.[21] In addition, selenols have a much lower pK_a and a superior redox lability compared to thiols. For example, selenocysteine (Sec) has a pK_a of 5.2 and redox potential of -383 mV (to the diselenide form) whereas cysteine (Cys) has a pK_a of 8.3 and a redox potential of -238 mV (to the disulfide form).[22, 23] These properties allow RSeS and RSS to play different roles in ROS protection and antioxidant activity. For example, glutathione (GSH) is the most abundant thiol in eukaryotic cells (0.5 to 10 mM) and plays an important role in mitigating general oxidative stress, and may act as a redox buffer to control thiol and disulfide redox equilibria. By contrast, Sec typically performs its antioxidant role as a key amino acid in different antioxidant and ROS-scavenging enzymes. For example, glutathione peroxidases (Gpx) contain a selenol moiety in the active site that is oxidized by peroxide to form a selenenic acid, which is subsequently reduced by two equivalents of GSH to regenerate the active selenol and release H_2O and GSSG (Figure 1b). When taken together, the redox buffering of GSH and high ROS scavenging rate constants for ROS-degrading enzymes provide significant capacity to rapidly scavenge and degrade different ROS.[24] Additionally, thioredoxin reductases (TrxR) are an additional class of selenoproteins that act as principal reductants in the thioredoxin (Trx) system (Figure 1c), in which a thiol and selenol functional group are oxidized to form a selenyl-sulfide bond that is subsequently reduced by NADPH to generate the reduced TrxR. The Trx system,

including peroxiredoxins that directly reduce ROS, is crucial in modulating oxidative stress.[25, 26] Further illustrating the connectivity between RSeS and RSS, the transsulfuration pathway is also important in Se metabolism because it breaks down selenomethionine for selenoprotein synthesis (Figure 1a).[27]

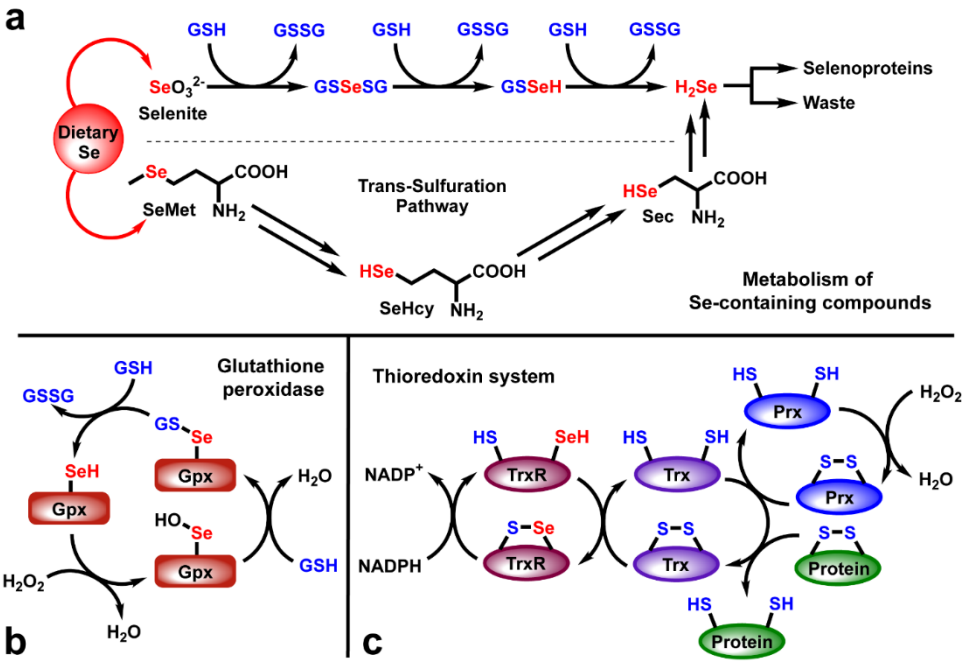


Figure 1. (a) Simplified metabolic pathways of selenium-containing compounds. (b) Selenium contribution to glutathione peroxidase function. (c) Selenium and sulfur interplay in the regulation of the thioredoxin system.

Based on the role of RSeS and RSS in oxidative stress and other processes involved in tissue regeneration, it is also likely that RSeS and RSS also play key roles in bone repair and homeostasis. Although the direct interplay between RSeS and RSS in bone-related processes has

not yet been investigated directly, prior work suggests that there are emerging opportunities in this area. This review summarizes recent work on the applications of Se and S related to bone regeneration and highlights different methods, tools, and common sources of chalcogenides used in different investigations (summarized in Tables 1 and 2). We also identify future opportunities for delivery of RSS and RSeS in biological environments relating to bone.

Role of RSeS and RSS in Bone

Osteoporosis

ROS play critical roles in bone regulation and remodeling. For example, ROS upregulate receptor activator of nuclear factor κβ ligand (RANKL), which in turn increases osteoclastogenesis (the formation of bone-resorbing osteoclast).[28, 29] Continuous remodeling by osteoclasts and osteoblasts is necessary for maintaining healthy and strong bone, and ROS serve a vital role in this process. Excessive ROS, and consequently excessive osteoclast activity, can disrupt bone homeostasis, resulting in bone loss diseases like osteoporosis.[30] Osteoporosis is a prevalent bone disease primarily occurring in postmenopausal women and elderly men. The disease is characterized by low bone mineral density, which leads to increased risk of fracture. Common therapeutic strategies to treat osteoporosis include the use of amino-bisphosphonates. Bisphosphonates have a high affinity for bone, but the acidic pH caused by osteoclast resorption causes the dissociation of bisphosphonates from bone and subsequent internalization by osteoclasts. Once internalized, amino-bisphosphonates induce cell death in osteoclasts by inhibiting the mevalonate pathway.[31] Despite the success of bisphosphonates in decreasing bone resorption activity, extended use can suppress osteoblast activity, which has led to the decline of

bisphosphonate therapies. Developing alternative strategies to restore balance in ROS-dependent pathways that regulate bone mineral density remains an unmet need.

H_2S has been shown to decrease osteoclast activity and differentiation and reduce bone resorption by decreasing ROS production (Figure 2). For example, Gambari et al. demonstrated that administration of sodium hydrosulfide (NaSH) as an exogenous H_2S source to CD11b+ human monocytes prevented osteoclast differentiation and function in a dose-dependent manner. The concentration range of NaSH (100 – 200 μM) that prevented osteoclastogenesis was not cytotoxic to cells, but rather inhibited RANKL-induced ROS production by upregulating NRF2 protein expression.[32] Excess ROS in osteoporotic bone additionally impacts osteoblast proliferation and differentiation. Xu et al. showed that pretreatment of MC3T3-E1 osteoblastic cells with NaSH (100 μM) protected against H_2O_2 (400 μM)-induced cell injury through a MAPK-dependent mechanism.[33] Taken together, the ability of H_2S to inhibit osteoclast activity and differentiation, promote osteogenic differentiation, and protect osteoblasts against oxidative stress make it a potential target for future treatment for osteoporosis.[34]

Early work aimed at combining current therapeutic strategies for osteoporosis with H_2S delivery included the development of the amino-bisphosphonate H_2S donor DM-22.[31] DM-22 combines alendronate, an established osteoporosis therapeutic, with an aryl isothiocyanate that releases H_2S in the presence of excess cysteine. Although DM-22 showed relatively low H_2S release efficiency (4% of total DM-22, measured using an H_2S electrode), it enhanced the mineralization of hMSCs under osteogenic stimulation after 21 days as evidenced by the increased Alizarin Red-S staining when compared to the vehicle experiments. In contrast, alendronate alone inhibited mineralization compared to the vehicle treatment and demonstrated dose-dependent cytotoxicity to hMSCs over 72 h. Despite enhancing the mineralization of hMSCs, DM-22

treatment resulted in a high variability of gene expression of osteogenic biomarkers, making it difficult to confirm the effects of DM-22 in promoting osteogenic differentiation in hMSCs. One of the main goals of combining H_2S delivery with currently used bisphosphonate osteoporosis therapeutics is to achieve both anabolic activity and anti-catabolic activity. Although the bone-forming activity of DM-22 is still unclear, DM-22 was able to retain the anti-resorptive activity of alendronate as observed through osteoclast activity assays (TRAP and PIT) at DM-22 levels that were previously determined as not cytotoxic to hMSCs (33 μM). Collectively, the enhanced mineralization of hMSCs in the presence of DM-22 paired with the anti-resorptive effects of DM-22 demonstrate therapeutic potential for incorporation of H_2S delivery in current osteoporosis therapeutics. We anticipate H_2S donors with increased H_2S release efficiency could be more effective at promoting osteogenic differentiation at lower doses.

Selenium in the form of selenite (SeO_3^{2-}) has also been shown to decrease osteoclast differentiation by suppressing RANKL-mediated ROS generation.[35] Notably, dietary selenium status has been directly linked to bone mineral density and osteoporosis in a variety of clinical studies. For example, Wang et al. demonstrated an inverse dose-dependent relationship between dietary selenium intake and osteoporosis, where consumption of <50 μg/day showed an approximate 70% increase in osteoporosis incidence.[36] An earlier 2012 study found that dietary selenium supplementation has a positive effect on bone mineral density in postmenopausal women, but only when calcium levels were also reduced.[37] These observations in supplementation studies highlight the opportunity to investigate more targeted approaches for selenium delivery in osteoporosis, such as selenium-containing prodrugs or nanoparticles. In recent work, Yu et al. reported that selenium-containing polysaccharide-protein nanoparticles can enhance osteoblast

activity in fish (medaka) and increase mineralization and bone formation through BMP-2/Smad-mediated pathways.[38]

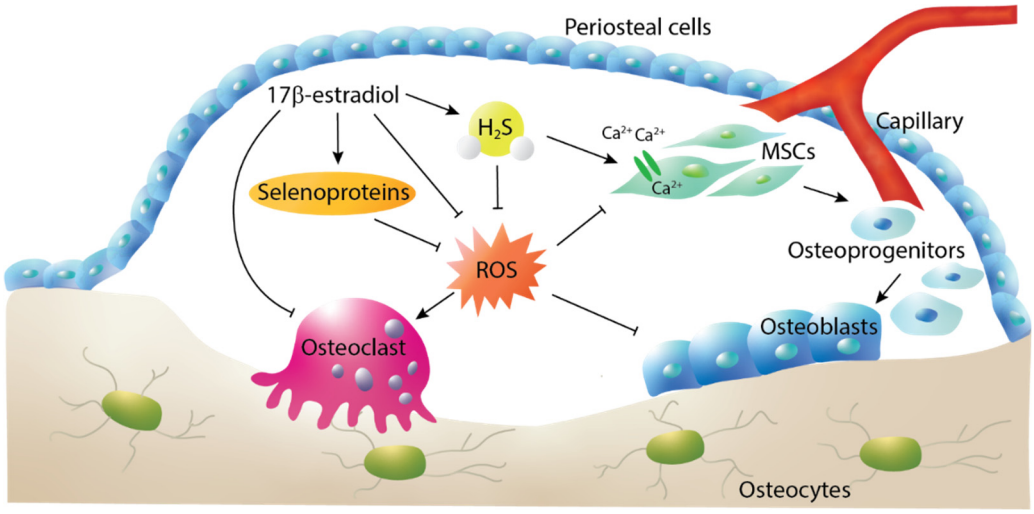


Figure 2. H_2S and selenoprotein regulation of osteoclast and osteoblasts in a trabecular bone remodeling cavity. H_2S activates Ca^{2+} channels to increase osteoblast production. Both H_2S and selenoproteins protect hMSCs and osteoblasts against oxidative stress and decrease osteoclast generation and activity through ROS-dependent pathways. Estrogen levels have been associated with both H_2S and selenium levels, suggesting that RSS and RSeS may be important in pathways associated with estrogen-mediated regulation of bone.

Ovariectomy-induced bone loss

Osteoporosis is predominately observed in postmenopausal women because of the natural decline of estrogen production from the ovaries. Estrogen plays an important role in bone health in both women and men. For example, estrogen inhibits bone remodeling activation by osteocytes

(cells embedded into formed bone), inhibits bone resorption by osteoclasts, and protects osteoblasts from apoptosis and oxidative stress (Figure 2).[39] Since ovaries are a major source of estrogen, ovariectomy (ovx) osteoporosis models are useful to replicate postmenopausal osteoporosis.

To investigate the effects of estrogen deficiency on endogenous H_2S, Grassi et al. used an ovx-postmenopausal bone loss model in mice. Compared to sham-operated mice, ovx mice had lower serum H_2S levels by 65% and significantly reduced bone marrow levels of CBS and CSE.[40] Administration of the small molecule H_2S donor, GYY4137 (1 mg/mouse/day), normalized serum H_2S levels and completely prevented bone loss induced by ovariectomy. MSCs harvested from ovx and sham-operated mice showed that GYY4137 treatment promotes bone formation through activating Wnt signaling in MSCs. Further investigating the relationship between estrogen and H_2S, Grassi et al. treated human bone marrow stromal cells with 17β-estradiol (an estrogen steroid hormone) and showed upregulated levels of CBS and CSE. This work suggests that estrogen regulation of H_2S levels may be one mechanism by which estrogen stimulates bone formation, and consequently the restoration of H_2S levels could be potential approach for combating postmenopausal osteoporosis. In addition, H_2S has also been found to help treat glucocorticoid-induced secondary osteoporosis in rats where intraperitoneal injection of GYY4137 (1 mg/rat every other day) significantly decreased the inhibitory effect of dexamethasone (5 mg/kg body weight/day) on bone formation.[41]

As discussed above, an increase in ROS production leads to increased osteoclastogenesis and decreased osteoblast activity, which can culminate in osteoporosis. Ovariectomized rats have increased ROS levels in bone[42] in addition to decreased selenium levels and Gpx activity.[43] Treatment of ovariectomized rats with 17β-estradiol resulted in recovery of selenium levels and

Gpx activity, which suggests a potential underlying link between estrogen and selenium-derived antioxidant function in bone remodeling.[44] In a study investigating the effects of selenium pretreatment on bone repair in ovariectomized rats exposed to radiation, Freitas et al. demonstrated that treatment with sodium selenite (0.8 mg/kg injection, 40 days post-ovx, 2 days post bone damage, pre-irradiation event) leads to increased bone regeneration over the non-treated, irradiated specimens as measured by bone density analyses during the repair process.[45] This increase in bone regeneration as a result of selenite pretreatment was only observed in irradiated animals, with the pretreated and untreated control animals ultimately showing no differences. These results suggest that although RSeS application protects bones against radiation-induced impairment of regeneration processes, such treatment does not directly impact ROS-derived issues related to osteoporosis in estrogen-deficient rats. Although these findings highlight the benefits of selenium supplementation to combat ROS-related pathologies, the lack of clear mechanisms of selenium activitiy or clear delineation of the active selenium metabolites complicate the development of more targeted selenium-based delivery approaches.

Hyperhomocysteinemia

Hyperhomocysteinemia (HHcy) is a condition characterized by elevated levels of homocysteine (>15 μM) and can arise from the dysfunction of enzymes associated with homocysteine (Hcy) metabolism.[46] Although CBS deficiency is the most prevalent cause of HHcy, HHcy can also result from other genetic deficiencies, excess methionine intake, certain diseases, and certain drugs.[46] CBS deficiency in murine models has been shown to result in decreased levels of plasma H_2S since CBS generates H_2S from Hcy and cysteine condensation.[9] In contrast, clinical studies on CBS deficient patients have shown that patients compensate for the

decreased CBS-derived H_2S production by activating other H_2S-producing enzymes.[47] As a whole, these studies highlight the potential for species-specific or tissue-specific effects of differential enzyme expression and metabolite levels in *in vivo* models of HHcy. A variety of different disease states and physiological conditions are associated with HHcy including lens dislocation, cardiovascular disease, Alzheimer's disease, schizophrenia, and osteoporosis.[48] HHcy-induced bone loss can be attributed to increased levels of intracellular ROS, which enhances osteoclast differentiation and activity while decreasing osteoblast activity.[49] Highlighting the role of the transsulfuration pathway in HHcy (Figure 3), both Hcy buildup and depleted H_2S levels can lead to increased oxidative stress and excessive bone resorption through RANKL upregulation. Understanding the role of H_2S and other RSS in HHcy-induced bone loss may provide future opportunities for useful research models or potential therapeutic interventions.

A number of investigations have already highlighted the roles of H_2S in HHcy-induced bone loss. For example, Behera et al. fed mice a methionine enriched diet (1.2% methionine) to elevate Hcy levels and treated the HHcy mice with NaSH (10 mg/kg/day) daily through intraperitoneal injection.[50] In the absence of NaSH treatment, the HHcy mice showed decreased mRNA expression of CBS and CSE, decreased CBS enzymatic activity, and lower H_2S levels than mice on a normal diet. Furthermore, mice induced with HHcy epigenetically upregulated RANKL and downregulated osteoprotegerin (a RANKL inhibitor), which increased osteoclastogenesis and osteoclast activity and led to osteoporotic phenotypes. Pre-treatment of mice with NaSH increased H_2S levels, resulted in upregulated CBS expression and activity, and prevented the HHcy-induced bone loss by enhancing osteogenesis and inhibiting osteoclastogenesis. In a follow-up study, heterozygous CBS knockout ($CBS^{+/-}$) mice were fed a high methionine diet to investigate whether H_2S alleviates HHcy-induced epigenetic changes that lead to bone loss.[51] HHcy-induced $CBS^{+/-}$

mice showed inhibited histone deacetylase 3 (HDAC3) activity, which causes inflammatory cytokine transcriptional activation in MSCs and increases osteoclastogenesis. The HHcy-induced $CBS^{+/-}$ mice also showed accelerated bone resorption, significantly lower bone mineral density, and reduced trabecular bone volume fraction compared to wild-type mice. H_2S supplementation by intraperitoneal NaSH injection (10 mg/kg/day) provided protection against these epigenetic alterations and inflammatory NF-κB signaling to prevent bone loss. In addition, NaSH administration led to persulfidation of runt-related transcription factor 2 (RUNX2), which is a key transcription factor for osteoblast differentiation, and highlights the role of S-based posttranslational modifications in signaling pathways associated with bone regeneration.

Osteoblast function is also disrupted by elevated levels of Hcy, which results in mitochondrial oxidative damage and further exacerbates bone loss in HHcy. For example, Zhai et al. demonstrated that treatment of MC3T3-E1 osteoblastic cells with Hcy (50-300 μM) decreased osteoblast activity, resulted in a dose-dependent increase in ROS, and increased mitochondrial dysfunction.[52] Furthermore, treatment of MC3T3-E1 cells with 300 μM Hcy decreased H_2S levels and reduced CBS and CSE expression. Pretreatment with NaSH (30 μM) attenuated Hcy-induced mitochondrial toxicity in the MC3T3-E1 cells and reduced Hcy-induced apoptosis. Across these various models of HHcy, supplementation with NaSH successfully reversed osteoblast dysfunction and bone loss, suggesting that H_2S or related RSS may play an important role in mitigating HHcy effects.

Multiple sources have shown that selenium-depleted diets in murine and avian models result in reduced Hcy levels, potentially through transsulfuration pathway impairment as evidenced by the high levels of plasma glutathione.[53-55] These results suggest that reducing selenium intake could be used as a strategy to lower Hcy levels in HHcy-related conditions.

Selenium supplementation in humans, however, does not appreciably influence Hcy levels, which highlights the complexity of the relationship between selenium and Hcy status.[56, 57] Clues to this interaction can be seen in certain physiological conditions that exhibit both increased Hcy levels and decreased selenoprotein expression, such as treatment-resistant schizophrenia.[58] Individuals with schizophrenia have low Gpx levels, which can lead to oxidative stress and epigenetic changes that contribute to the disease.[58] Elevated levels of Hcy have been previously shown to decrease Gpx protein expression in cells (MSCs, endothelial cells, fibroblast cells), and this relationship is also observed in higher-ordered organisms.[59] Further investigation into the Hcy/selenium connection is needed to discern whether modulation of selenium levels has subsequent and predictable effects on HHcy and related conditions.

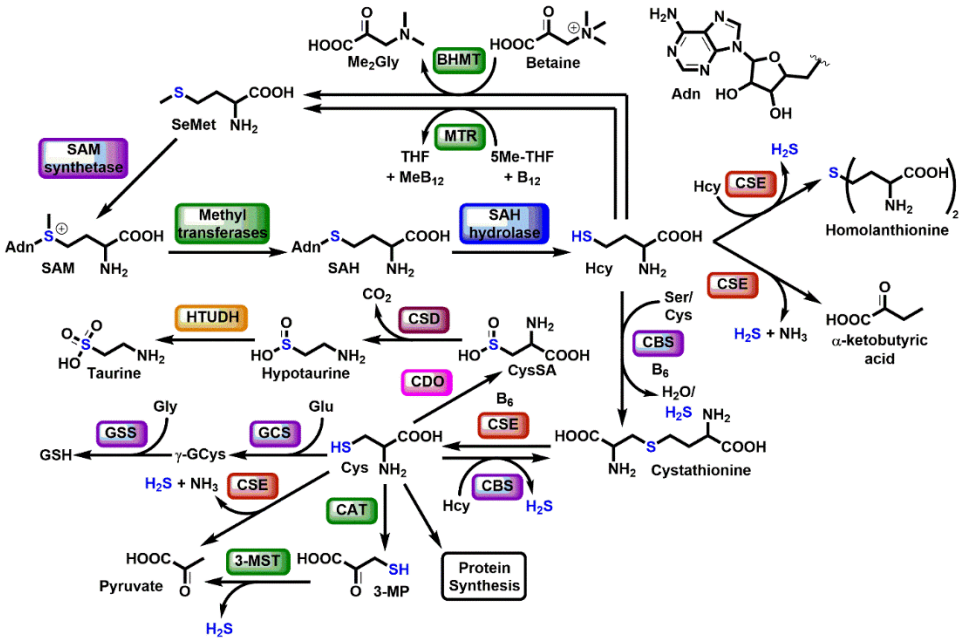


Figure 3. The transsulfuration pathway. Metabolites: SAM – *S*-adenosylmethionine, SAH – *S*-adenosylhomocysteine, CysSA – cysteine sulfinic acid, γ-GCys – γ-glutamylcysteine, 3-MP – 3-mercaptopyruvate. Enzymes: BHMT – betaine homocysteine *S*-methyltransferase, MTR – methionine synthase, CDO – cysteine dioxygenase, CSD – cysteine sulfinate decarboxylase,

HTUDH – hypotaurine dehydrogenase, GCS – γ-glutamylcysteine synthetase, GSS – glutathione synthetase, CAT – cysteine aminotransferase.

Bone Cancer

There are three types of bone cancer that make up over 80% of all cases: osteosarcoma, chondrosarcoma, and Ewing sarcoma.[60] In each of these conditions, tumor growth is a primary indicator of cancer development; however, tumors resulting from the metastasis of other cancers are not considered to be true bone cancers. Bone tumors often require surgical removal, which can leave patients with moderate cases in severe pain and patients with severe cases physically debilitated. It is difficult to mitigate the pain associated with bone cancer and tumors, and additional challenges are presented in patients that undergo bone-cancer-related surgeries. Research in the last decade shows that bone cancer pain can be reduced in murine models by inhaling gaseous H_2S at 40 ppm in air for 8 hours per day over 7 days; this treatment deactivates microglia and inhibits inflammation in the spinal cord, preventing neuropathic pain from bone cancer.[61] Similar antinociceptive properties after administration of H_2S has also been demonstrated for gastrointestinal pain in murine models.[61-63] These approaches to managing pain with H_2S treatment deliver positive outlooks possible future avenues of non-opioid pain management strategies for cancer patients, who are at high risk for opioid dependence.[64]

Generally, cancer research seeks to develop treatments that are more effective in eliminating cancer while simultaneously being less harmful to patients, and different selenium-containing compounds are now being examined under this lens. The innate toxicity of RSeS has sparked investigations into whether selenium-containing species can be tuned to have greater

cytotoxicity toward cancerous cell lines than non-cancerous cell lines. For example, the abundance of reduced thiols in cancerous cells, creating a reducing environment, makes them more susceptible to selenite-mediated oxidation, which in turn inhibits the ability of cancer cells to grow and replicate.[65]

Alternative approaches to the delivery of RSeS, such as nanoparticle (NP) technology, have been developed to incorporate selenite into hydroxyapatite nanoparticles (HANs) to form selenite-doped nanoparticles (Se-HANs).[66] HANs were designed to help fill voids in bones created by surgical tumor removal and can provide a viable scaffold for bone healing and regeneration because hydroxyapatite (HAp) is the primary inorganic mineral in vertebrate bones.[66] Additionally, selenite metabolism results in ROS generation, which activates caspase-based apoptotic pathways in cancerous cells but can also be toxic at higher concentrations in non-cancerous cells.[67] The use of HANs in combination with selenite to combat bone cancer has been studied extensively in the last decade, beginning with the initial Se-HAN synthesis and characterization as well as demonstrated anti-cancer activity (40% decrease in cell viability) against MG63 osteosarcoma cells at 200 μg/mL.[66-71]

Recent efforts to improve the efficacy of the Se-HANs have included modified NPs that contain catechins (CC), which improved NP anti-cancer activity (85% lower cancer cell viability) compared to non-CC analogs.[72] CC inclusion allows for greater treatment efficacy at lower NP concentrations due to an observed selectivity for cancer cells, which reduces toxicity toward healthy bone marrow cells. This selectivity is due to CC-induced ROS production that reduces the mitochondrial membrane potential in cancerous cells but not healthy cells.[72, 73] In 2019, Khan et al. showed that CC-doped Se-HANs are highly toxic (<10% viability) to MNNG/HOS cells (human osteosarcoma TE85 cells treated with 0.01 μg/mL *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine)

at 100 μg/mL treatment over 48 hours with only minimal observed toxicity toward bone marrow MSCs.[72] Se-HANs have further been tested in animal models of osteosarcoma, which primarily affects adolescents and young adults.[60] BALB/c mouse models of osteosarcoma treated every 3 days with 100 μg/mL Se-HANs showed a significant reduction in tumor weight (>60%) and volume (>70%) over 30 days when compared to the untreated mice.[67] These and other recent advances in NP technology may provide efficacious approaches for Se-based therapeutics for cancer treatment.

Fracture healing

Bone fractures are common injuries (~6 million occur annually in the United States) that often fully heal without significant scarring.[74] This efficient healing is due to the fracture repair model that can be broken down into four main phases.[75] In the first phase, a hematoma forms from bleeding within the fracture site. Inflammatory cells within the hematoma secrete cytokines and growth factors that recruit stem cells to the injury site and differentiate cells towards osteogenic fates. A soft fibrocartilage callus is formed by chondrocytes and fibroblasts in the second stage to provide mechanical support to the fracture and offer a scaffold for vascularization. The soft callus is gradually replaced with a hard callus in the third stage. The hard callus stage is where osteogenesis primarily occurs, and osteoblasts form a mineralized bone matrix that contains mineralized extracellular matrix tissue.[75] The final stage includes the remodeling of the hard callus into cortical and/or trabecular bone through osteoclast resorption and osteoblast anabolism, resulting in restored bone homeostasis. Although the fracture repair model is effective for healing

most fractures, there can be complications due to factors such as age, underlying conditions, severity of injury that result in defect bone sites called nonunion.

According to the FDA, a nonunion is a fracture that persists for a minimum of nine months without signs of healing for three months. It is estimated that up to 10% of all fractures proceed to nonunion status.[76] Nonunions are classified into four categories: hypertrophic, atrophic, oligotrophic, or septic nonunion.[76] Each category differs in the extent of callus formation, blood supply, stability, and infection, which provides information on whether biological stimulation, fixation, or antibiotics are necessary. If biological stimulation is required, treatment strategies include the use of a bone graft or the delivery of FDA-approved recombinant human bone morphogenic protein 2 (BMP-2).

BMP-2 is naturally secreted by cells during fracture healing to recruit osteoprogenitor cells and induce osteogenic differentiation. The clinical use of BMP-2 can result in numerous complications. Typically, BMP-2 is loaded up in a porous scaffold which is embedded in the defect site. Large doses of BMP-2 are required to produce a therapeutic effect; however, a surplus of BMP-2 can also lead to inflammation and abnormal bone growth into soft tissues surrounding the defect region.[77] Rapid clearance of protein therapeutics from the tissue requires the use of large quantities of BMP-2, which significantly increases the cost and decreases the safety profile of treatment.[78] Controlling the release of BMP-2 from these scaffolds has recently emerged as effective strategy to decrease ectopic bone growth and reduce the amount of BMP-2 required to promote defect healing.[79]

Analogous to BMP-2, physiologically sufficient levels of H_2S have also been shown to be important for bone formation and fracture healing. Endogenous H_2S production in osteoblasts is primarily attributed to CSE. To investigate the role of H_2S in osteoblast function and bone

formation, Zheng et al. overexpressed CSE by transfection of recombinant adenovirus in pre-osteoblasts and implanted the adenovirus in a rat femur defect.[80] CSE overexpression in pre-osteoblasts increased osteoblast differentiation and maturation, resulting in upregulated BMP-2 and osteopontin expression, increased ALP activity, and increased calcium nodule formation. CSE overexpression additionally increased RUNX2 nuclear accumulation, DNA binding activity, and transcription of its target genes. Using NaSH as an exogenous source of H_2S, Zheng et al. found that RUNX2 was persulfidated, and that persulfidated RUNX2 had increased binding to the osteocalcin promoter. Mutations at the two cysteine residue sites, C123 and C132, prevented persulfidation and DNA binding activity, suggesting that persulfidation of RUNX2 promotes its transactivation. Implantation of a gelatin sponge containing the CSE adenovirus in a rat bone fracture resulted in repair of the fracture lesion after 2 weeks. Less inflammatory cell infiltration and increased collagen secretion was observed after only 1 week when compared to the vehicle treatment. Collectively, this work suggests that H_2S promotes osteoblast differentiation and maturation through persulfidation of RUNX2 to enhance bone healing. Furthermore, the anti-inflammatory effects observed after 1 week of fracture healing suggest that H_2S may provide a variety of important regenerative effects throughout the healing process.

The dynamic therapeutic potential of H_2S in fracture healing has been recently highlighted by Gambari et al. who hypothesized that exogenous H_2S delivery through both early and late stages of bone healing could be beneficial (Figure 4).[12] In early stages, H_2S could aid in regulating inflammation and restoring vasculature in the injury site. In later stages, H_2S promotion of osteoblast differentiation and decrease of osteoclast differentiation and activity could also aid defect bone healing.[12] The effects of exogenous H_2S delivery in H_2S deficient mice has been investigated in the healing of mandibular defects.[81] CSE knockout ($CSE^{-/-}$) inhibited osteogenic

differentiation of bone marrow MSCs and suppressed defect healing, whereas intraperitoneal injection of GYY4137 (1 mg/kg) reversed these effects by regulating expression and activity of ALP and RUNX2. Administration of GYY4137 has also been shown to promote bone formation in unfavorable osteogenic environments such as distraction osteogenesis (bone lengthening) in rabbits (intravenous injection of 50 mg/kg twice per day) and in microgravity (intraperitoneal injection of 25 mg/kg/day).[82, 83] These examples highlight the opportunity for treatment of bone defects in more nontraditional bone fracture cases.

Although the effects of selenium in fracture healing are still largely uninvestigated, the antioxidant activity of selenium may have the potential to protect MSCs and osteoblasts against oxidative stress and decrease osteoclast activity, akin to the function of selenoproteins. Protective effects of selenium have been demonstrated in murine models of bone loss from cadmium exposure where sodium selenite treatment (3.5 mg/kg/day for 4 weeks, then every other day for 8 weeks) rescued antioxidant enzyme activity in bones and recovered bone mass.[84] Similarly, sodium selenite treatment (1.0 mg/kg/day for 4 weeks) in murine models of diabetes-induced structural defects in the mandible and long bones resulted in reduced bone alterations when compared to the control animals.[85]

To control the delivery of potential therapeutic agents in bone, researchers have developed a number of scaffolds that can be embedded into the defect site and serve as a vehicle for chemical compounds while simultaneously breaking down as the bone heals. Both H_2S and RSeS sources have been included in such bone healing scaffolds. For example, the common H_2S donor GYY4137 has been embedded in a silk fibroin scaffold which, when applied to hMSCs, resulted in enhanced osteogenic differentiation observed through increased mineralization and upregulation of osteogenic biomarkers.[86, 87] As discussed above, different selenium

nanoparticle scaffolds have been developed to treat bone defects caused by tumor removal. Similarly, selenium and silver substituted (5% Se) hydroxyapatite-based bone grafts have shown potent antibacterial activity (*Escherichia coli, Staphylococcus aereus*) without impeding the growth of healthy SaOS-2 bone cells.[88] Collectively, H_2S and RSeS delivery could provide advantageous osteogenic and protective effects in implants for bone defects, particularly resulting from osteosarcomas.

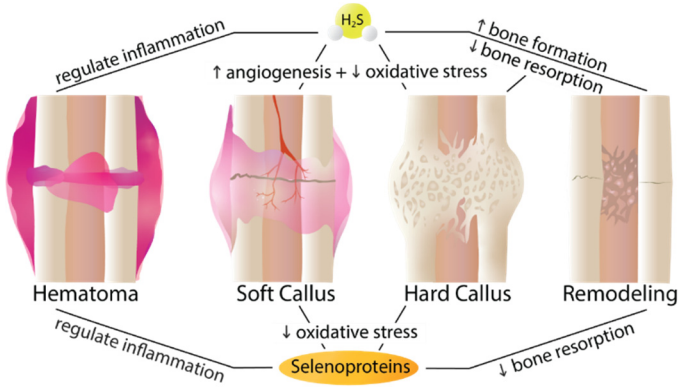


Figure 4. Potential therapeutic roles of H_2S throughout the four stages of the bone healing process highlighting selenoprotein modulation of oxidative stress.

Future Outlook

Sources of RSS and RSeS

The investigations of RSS and RSeS highlighted above set the groundwork for future investigations into the importance of RSS and RSeS in bone. As this area of research continues to expand, new chemical tools for investigating RSS and RSeS are needed. For example, most prior work on H_2S in bone, which is summarized in Table 1, has utilized NaSH or GYY4137 as H_2S sources. NaSH is an exogenous H_2S source that rapidly releases a large dose of H_2S upon

administration. Much of the produced H_2S is immediately volatilized or oxidized, which makes it difficult to determine actual H_2S concentrations, especially over longer time periods, and adds uncertainty to the proposed therapeutic levels of H_2S. As an alternative to NaSH administration, other researchers have used GYY4137, which is one of the first and most widely used controlled-releasing molecular H_2S donors to date. H_2S release from GYY4137 relies on hydrolysis and is relatively inefficient. In addition, GYY4137 is often sold as a dichloromethane complex. Dichloromethane is metabolized to form carbon monoxide, which is another important gasotransmitter with similar biological effects as H_2S, and could potentially contribute to the observed effects of GYY4137.[89] DM-22, a bisphosphonate isothiocyanate, was developed as an H_2S donor with a high affinity to bone; however, isothiocyanates are also inefficient H_2S sources that require cysteine to release H_2S.[31] Similarly, broccoli-derived sulforaphane as a dietary source of isothiocyanate was recently shown to reduce cancer-induced bone pain in rats,[90] suppress osteoclastogenesis and breast cancer-induced osteolytic bone resorption,[90-92] promote osteoblastic differentiation, and increase bone volume in mice.[93] Although it is unclear whether these results are from sulforaphane-derived H_2S, these investigations highlight the significant impacts of dietary-derived sources of RSS on bone health. Looking to other sources of RSS, Gambari et al. also recently demonstrated an increase in mineralization and increase in expression of certain osteogenic biomarkers after treatment of hMSCs with sulfurous thermal water.[94] Despite these findings, it remains unclear which specific contents of the thermal water were contributing to the observed results.

The palette of tools for H_2S research has greatly expanded since the development of GYY4137 in 2008. Small molecular H_2S donors activated in response to hydrolysis, endogenous species, photoactivation, and biorthogonal chemistry have been developed.[95, 96] With new

available technologies, there is now a significant opportunity to tune H_2S release to respond to biological stimuli specific to bone or even within specific stages of the bone healing process. Donors that release H_2S in response to ROS and mildly acidic conditions (both of which are naturally occurring in bone healing environments)[97] have already been developed.[98-100] Applying such donors in therapeutic scaffolds and developing other H_2S donors that can target specific stages of bone healing could lead to more efficient therapeutic strategies for treating bone defects and diseases.

When compared with available chemical tools for RSS delivery and investigation, the palette of tools for RSeS investigations remains in its infancy. Most biological studies have used selenite, selenomethionine, or selenium doped nanomaterials as precursors for RSeS (Table 2). To better understand the roles of selenium in bone regulation as well as other systems, it is imperative to develop exogenous and bioavailable sources of RSeS. Complementing the commonly-used inorganic sources of selenium, the organoselenium compound Ebselen has been studied as an exogenous source of selenium.[101] Ebselen is a potent antioxidant, essentially functioning as a Gpx mimic, and has been examined in a wide array of conditions from Alzheimer's to Zika Virus, but further investigation are needed to advance the utility of this RSeS.[102] Drawing parallels to the early development of chemical tools for H_2S and RSS delivery, related tools are now emerging for H_2Se delivery and include phthalic selenoanhydride, TDN1042 and its cyclic analogs, selenoamides, and selenocyclopropenes.[103-106] As this field continues to grow, investigations into the biological implications of elevated/diminished selenium levels in the body will be facilitated, and opportunities to pursue selenium-based therapeutic strategies will become easier to identify. Therefore, there is tremendous opportunity for developing selenium-containing

compounds like prodrugs to release discrete selenium species, tune release kinetics, and temporally control selenium delivery and localization.

Table 1. Sources of H_2S used to study H_2S in bone regeneration and their observed effects.

Exogenous source of H_2S	Observed effects	Model	Year
$H_2S_{(g)}$	Prevents bone cancer pain in rats[61]	in vivo	2018
Dietary	Sulforaphane promotes osteoblastic differentiation and inhibits osteoclastogenesis, resulting in a net bone volume increase in mice[90, 93]	in vitro/vivo	2016, 2021
	Sulforaphane suppresses breast cancer-induced osteolytic bone resorption[91]	in vitro/vivo	2020
	Sulforaphane helps alleviate bone cancer pain in rats[90]	in vivo	2021
NaSH	Decreases osteoclast differentiation[32]	in vitro	2014
	Alleviates HHcy-induced bone loss[50, 51]	in vitro/vivo	2018
	Attenuates HHcy-induced mitochondrial oxidative damage in osteoblasts[52]	in vitro	2019
NaSH and GYY4137	Protects osteoblasts against oxidative damage[33, 107]	in vitro	2011, 2017
	Activates Ca^{2+} channels to promote osteogenic differentiation[9]	in vitro/vivo	2014
	Increases osteoblast activity to promote bone fracture healing[80]	in vitro/vivo	2017
GYY4137	Exhibits anti-cancer effects towards an osteosarcoma cell line[108]	in vitro	2011
	Accelerates bone formation in mandibular distraction osteogenesis[82]	in vitro/vivo	2015
	Rescues bone loss induced by estrogen deficiency[40]	in vitro/vivo	2016
	Promotes osteoclastogenesis in mechanical loading model[109]	in vitro/vivo	2018
	H_2S-releasing scaffold promotes osteogenic differentiation of hMSCs[87]	in vitro	2019
	Rescues bone loss induced by glucocorticoids[41]	in vitro/vivo	2019
	Decreases bone loss induced by modeled microgravity[83]	in vitro/vivo	2019
	Promotes mandibular defect healing[81]	in vitro/vivo	2020
Other	H_2S-releasing bisphosphonate (DM-22) decreases osteoclast function and stimulates mineralization with hMSCs undergoing osteogenic differentiation[31]	in vitro	2017
	Sulfurous thermal water increases H_2S levels in hMSCs and increases mineralization and expression of some osteogenic biomarkers[94]	in vitro	2020

Table 2. Sources of selenium used in studies on bone regeneration and their observed effects.

Exogenous source of Se	Observed effects	Model	Year
Dietary	Increased serum Se levels correlates with lower Hcy levels[57]	clinical	2004
	Se deficiency impairs bone and cartilage growth[16]	in vivo	2007
	Se status inversely related to bone turnover and positively correlated to bone mineral density[110]	clinical	2012
	Lower Se intake results in higher prevalence of osteoporosis[36]	clinical	2019
Selenomethionine	Se supplementation does not increase Hcy levels[56]	clinical	2003
Sodium selenite	Protects against diabetes-induced mandible structural alterations[85]	in vivo	2002
	Elevated Se intake negatively effects bone but only with low calcium intake[37]	clinical	2012
	Inhibits osteoclast differentiation by decreasing ROS generation[35]	in vitro	2012
	Provides radioprotective effect on bone repair in ovariectomized rats[45]	in vivo	2012
	Protects bone against cadmium-induced oxidative stress in rats[84]	in vivo	2014
Selenium nanoparticles	Se-doped bone mineral nanoparticles demonstrate antitumor activity towards osteosarcomas[67, 72]	in vitro/vivo	2016, 2019
	Polysaccharide-protein complex-coated selenium nanoparticles enhance bone formation[38]	in vitro/vivo	2018
	Nanoscale selenium protects against Hcy-induced oxidative damage[111]	in vitro/vivo	2020

Conclusion

The investigation of RSS, and especially RSeS, in bone is still an emerging field, but significant research contributions that demonstrate important roles for RSS and RSeS in regulating bone homeostasis have already been reported. Building from this recent progress, we expect that this area is poised for the development and application of chemical tools specifically designed to control the delivery of RSS and RSeS in environments relevant to bone homeostasis and regeneration. These future investigations will lay the foundation for expanding our knowledge and

therapeutic potential of the intertwined roles of RSS and RSeS in this important research and clinical area.

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