CLINICAL RESEARCH

Association of Hydrogen Sulfide with Femoral Bone Mineral Density in Osteoporosis Patients: A Preliminary Study

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Accumulated evidence has suggested that hydrogen sulfide (H_2S) has a role in bone formation and bone tissue regeneration. However, it is unknown whether the H_2S content is associated with bone mineral density (BMD) in patients with osteopenia/osteoporosis.

Material/Methods: In the present study, we aimed to explore the changes of serum H_2S in osteopenia and osteoporosis patients. We analyzed femur expression of cystathionine β synthase (CBS), cystathionine γ lyase (CSE), and 3-mercapto-pyruvate sulfurtransferase (3-MST), which are key enzymes for generating H_2S.

Results: Sixteen (16%) patients had osteopenia, 9 (9%) had osteoporosis, and 75 (75%) had normal BMD. In comparison with patients with normal BMD (controls), the serum levels of H_2S were unexpectedly increased in patients with osteopenia and osteoporosis. This increase was much higher in patients with osteoporosis than in those with osteopenia. Serum H_2S levels were negatively correlated with femoral BMD, but not lumbar BMD. Interestingly, the expression of CBS and CSE were downregulated in femur tissues in patients with osteoporosis, whereas the expression of 3-MST remained unchanged. Serum phosphorus levels, alkaline phosphatase, hemoglobin, and triglycerides were found to be closely associated with CBS and CSE scores in femur tissues.

Conclusions: Serum H_2S levels and femur CBS and CSE expression may be involved in osteoporosis pathogenesis.

Keywords: Alkaline Phosphatase • Bone Diseases, Metabolic • Hydrogen Sulfide • Osteoporosis

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Background

Osteoporosis is a prevalent disease that is characterized by reduced bone mass, diminished bone integrity, and increased risk of fractures [1]. It is estimated that osteoporosis affects 200 million people worldwide [2]. Osteoporosis is a common disorder in women after menopause, and it may also develop in men or in people with hormonal disorders or conditions that require chronic glucocorticoid medications [3]. Age-related osteoporosis occurs in both women and man, with bone loss beginning from the age of 40 years and continuing for the remainder of adult life [4]. Early diagnosis and treatment of osteoporosis can protect patients with osteoporosis from experiencing bone fractures [5]. Advanced radiological technology and laboratory detection of bone turnover biomarkers are commonly used for the differential diagnosis of osteoporosis [6]. The measurement of bone mineral density (BMD) by dual-energy X-ray absorptiometry is frequently used to diagnose osteoporosis and to assess osteoporotic fracture risk [7]. However, the presence or absence of osteoporotic fractures varies in osteoporotic patients [8]. Recently, determination of plasma bone biomarkers such as procollagen type 1 amino-terminal propeptide (P1NP) and C-terminal telopeptide of type 1 collagen (CTX) has emerged as a novel strategy to diagnose osteoporosis [9]. Nevertheless, such biomarkers are clinically limited due to their irrelevance with regard to fracture risk and their low specificity or sensitivity for osteoporosis [10].

In spite of the high prevalence of osteoporosis, its prevention and treatment have been problematic. Under normal conditions of homeostasis, bone undergoes constant turnover throughout an individual's lifespan. This turnover is precisely controlled by bone-resorbing osteoclasts and osteocytes, as well as bone-forming osteoblasts [11,12]. Activation of osteoclasts and inactivation of osteoblasts synergistically contribute to the development and progression of various bone diseases, including osteoporosis [13,14]. Therefore, current treatment regimens for osteoporosis fall into 2 categories: antiresorptive drugs, such as estrogen-receptor analogs or bisphosphonates, that inhibit osteoclast function, and anabolic drugs, namely parathyroid hormone, that induce osteoblastic bone formation. However, clinical use of these drugs is limited because of their side effects. For example, prolonged use of estrogen replacement is associated with various complications, including breast cancer, uterine bleeding, and cardiovascular events, while bisphosphonates can lead to osteonecrosis of the jaw [15]. The prescription of parathyroid hormone is also restricted by the potential incidence of osteosarcoma [16]. Furthermore, some patients do not respond to these drugs, whether administered individually or in combination [17,18]. It should also be noted that bone loss is closely associated with primary as well as secondary forms of osteoporosis, and secondary osteoporosis needs to be treated according to the primary concern, which may be diabetes, uremia, or chronic glucocorticoid therapy, among others [19,20]. Although the etiopathology of primary osteoporosis has yet to be fully understood, differential therapies should be considered for distinct subgroups, such as juvenile, postmenopausal, and senile osteoporosis [19]. Therefore, it is necessary to identify novel strategies and/or targets for preventing and treating osteoporosis.

Hydrogen sulfide (H$_2$S) is a ubiquitous gaseous molecule that is endogenously produced by cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST) within the transsulfuration pathway [21-23]. Since it was first identified as a novel gasotransmitter, along with nitric oxide and carbon monoxide, ample evidence has shown the importance of H$_2$S in the regulation of multiple physiological functions [24-26]. In bone tissue, H$_2$S has been well documented to play a critical role in bone pathologies. Deficiency of H$_2$S is involved in the process of bone remodeling by impairing bone formation in ovariectomized mice, a classical model of osteoporosis [27]. The dysregulated biogenesis of H$_2$S disrupts osteogenic differentiation of stromal cells [28], and CBS-deficient mice display an osteopenic phenotype [28,29]. Also, a recent study has demonstrated that H$_2$S donor GYY4137 significantly relieves the inhibitory effects of dexamethasone on bone formation by activating Wnt signaling [30]. Accordingly, sodium hydrosulfide (NaHS), a donor for H$_2$S, has also been shown to attenuate dexamethasone-induced systemic damage of bone mass and osteoblast dysfunction [31]. Treatment with NaHS normalizes plasma H$_2$S and prevents osteoporotic bone loss in CBS-deficient mice by inhibiting inflammatory cytokines [32,33]. Exogenous H$_2$S ameliorates damage induced by high glucose or oxidative stress in osteoblasts [34-36], critical events involved in osteoporosis pathogenesis. Furthermore, NaHS administration inhibits the differentiation of osteoclast progenitor cells via a nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent mechanism [37]. These exciting findings suggest that H$_2$S could play a therapeutic role in systemic bone diseases, such as osteoporosis, by regulating the functions of osteoblast and osteoclast precursors [38,39]. However, the relationship between H$_2$S and osteopenia/osteoporosis has yet to be fully elucidated in humans. In addition, the association of H$_2$S-generating enzyme expression with bone metabolic factors is unclear. In light of the significance of H$_2$S in bone metabolism and reconstruction, our present study aimed to identify the relationship between H$_2$S with BMD in patients enrolled in a preliminary investigation.

Material and Methods

Patients

The preliminary clinical study was approved by the Ethics Committee of Kunshan People's Hospital, and all procedures

Table 1. Clinical characteristics of the study participants.

Variables	Control (n=75)	Osteopenia (n=16)	Osteoporosis (n=9)	P value
Age	50.2±1.1	55.6±1.5*	79.2±3.1*#	0.045
Male/Female	58/17	10/6	2/7	0.041
BMD (lumbar spine)	1.23±0.11	1.01±0.12*	0.76±0.04*#	0.012
BMD (femoral neck)	0.97±0.08	0.78±0.06*	0.54±0.02*#	0.011
T value (lumbar spine)	0.66±0.12	-1.59±0.10*	-2.6±0.32*#	0.008
T value (femoral neck)	0.64±0.12	-1.44±0.11*	-3.17±0.18*#	0.005

Values are expressed as mean±SD. * $P<0.05$ vs control, # $P<0.05$ vs osteopenia. The gender in three groups was analyzed using χ^2 test, other variables in three groups was analyzed one-way analysis of variance.

were in compliance with the Declaration of Helsinki. The informed consent form was signed by all participants. The exclusion criteria were as follows: (1) patients with multiple fractures, osteoporotic fractures, open fractures, or violent fractures (eg, traffic injuries, fall injuries); (2) patients with chronic liver and kidney diseases, metabolic diseases, tumors, or blood system diseases; (3) patients who had undergone ovariectomy; and (4) patients receiving long-term treatment with glucocorticoids, estrogen, calcitonin and bisphosphonates, or other treatments (including traditional Chinese medicine). A total of 100 patients in Jiangsu Kunshan People's Hospital between October 2017 and October 2019 were enrolled in this study. All blood specimens and BMD measurements were collected at Kunshan People's Hospital during the patients' visit. The clinical characteristics of the participants are listed in the **Table 1**. A total of 100 patients (70 men and 30 women) were enrolled, and their ages ranged from 25 to 91 years (average age, 53.7 years). Based on lumbar and femoral neck BMD assessments, the control, osteopenia, and osteoporosis groups consisted of 75 (58 men and 17 women), 16 (10 men and 6 women), and 9 subjects (2 men and 7 women), respectively (**Table 1**). Significant differences were found between the control, osteopenia, and osteoporosis groups with regard to sex, lumbar and femoral neck BMD, as well as the lumbar and femoral neck T-values (**Table 1**).

Blood Samples and Biochemical Analysis

Blood samples were collected by venipuncture from all participants after overnight fasting, and serum samples were obtained by centrifugation at 3000 rpm for 5 min. The sera were frozen and stored at -80°C until processing. Serum levels of alkaline phosphatase (ALP), calcium, hemoglobin, and phosphorus were measured by using an AU5800 automated chemistry analyzer (Beckman Coulter, Brea, CA, USA) in accordance with standard laboratory methods. Fasting blood glucose and triglycerides were measured with commercially available kits (Konelab, Finland), using a chemistry autoanalyzer.

Measurement of BMD

After blood sampling, BMD was assessed by dual-energy X-ray absorptiometry (Lunar, DPX-NP, GE Healthcare, Madison, WI, USA). Lumbar spine (L1-L4) and femoral (neck and total) scans were performed, and the T-score was defined as the number of standard deviations from the mean BMD of sex-matched young control subjects, while the Z-score reflected the standard deviations matched by sex, age, weight, and ethnicity. On the basis of World Health Organization criteria, osteoporosis was defined as a T-score of -2.5 or less and osteopenia as a T-score of greater than -2.5 to -1.0; a T-score of -1.0 or more was recognized as the normal control value.

Measurement of H₂S in Blood Samples

The serum levels of H₂S in all participants were measured by commercially available human H₂S kits (catalog number: HG14903, Trust Specialty Zeal, San Francisco, CA, USA) according to the manufacturer's instructions. In brief, the samples and standards were added to plates coated with purified antibody. After incubation, biotinylated anti-IgG was added and reacted with streptavidin to generate the antibody-antigen-enzyme-antibody complex. Next, the substrate 3,3',5,5'-tetramethylbenzidine was added and reacted with horseradish peroxidase, and the solution eventually turned yellow under the action of acid. The optical density in each sample was measured at 450 nm, and the H₂S content was transformed and calculated by a standard curve and expressed as micromoles per liter.

Femur Tissue Collection and Immunohistochemistry

Femur tissues were collected from a separate group of 19 patients with osteoarthritis who underwent hip replacement surgery. Their femur BMD was measured before surgery, and based on T-scores, 9 of the patients had osteoporosis and 10 had normal BMD. There was no significant difference in terms of age and sex between the 2 groups (age: 79.2±3.1 vs

77.5±5.1; men/women: 2/7 vs 3/8). A total of 19 femur tissue biopsies were fixed in 4% paraformaldehyde and embedded in paraffin. After slicing of the samples, histological sections (5 μm) underwent deparaffinization and rehydration through serial immersion in xylene followed by graded alcohol. Antigen retrieval was conducted by incubating sections with bone tissue-specific antigen-retrieval solutions (Showbio, Shanghai, China) for 1 h at room temperature. Normal goat serum was used to block nonspecific binding for 15 min at room temperature, and the sections were then probed with the primary antibodies against human CBS (ab140600, Abcam, Cambridge, UK), CSE (ab54573, Abcam, Cambridge, UK), and 3-MST (ab224043, Cambridge, UK) at 4°C overnight in a humidified container. After being rinsed with phosphate-buffered saline 3 times, the sections were incubated with the secondary antibodies (Sangon, Shanghai, China) for 1 h at room temperature, followed by incubation in 3,3'-diaminobenzidine tetrahydrochloride solutions (ZLI-9017, ZSGB-Bio, Beijing, China) for 30 s. The images were photographed using a microscope (Leica, Microsystems, Germany), and immunohistochemistry was evaluated by 2 blind observers by counting the number of positive cells (at least a total of 100 in randomly selected regions) for CBS, CSE, and 3-MST. The semiquantitative evaluation was carried out as previously described [40-42]. The extent of positively stained cells was estimated and classified on a 5-point scale as follows: grade 0 (the percentage of positive cells in selected fields <10%); grade 1 (the percentage of positive cells in selected fields ≥10% and ≤25%); grade 2 (the percentage of positive cells in selected fields >25% and ≤50%); grade 3 (the percentage of positive cells in selected fields >50% and ≤75%); and grade 4 (the percentage of positive cells in selected fields >75%). The intensity of the positive staining was categorized into 3 groups: weak (1, grade 0-1), moderate (2, grade 2), and strong (3, grade 4).

Statistical Analyses

All continuous variables are expressed as mean±standard deviation (SD), while nonnormally distributed data are expressed as medians and interquartile ranges. The statistical analyses were carried out using SPSS for Windows (version 17.0; SPSS Inc., Chicago, IL, USA). Normality testing was conducted for all continuous variables. Data distribution was evaluated using the Kolmogorov-Smirnov test. The differences were determined using the *t* test between the 2 groups. Those with skewed distributions were expressed as median (interquartile range), and differences between groups were assessed using the Mann-Whitney *U* test. Categorical data are expressed as the number of patients and were analyzed using the χ^2 test. The significance of differences between groups (control, osteopenia, and osteoporosis) was determined using 1-way analysis of variance for normally distributed data. Pearson or Spearman correlation analysis was used to determine the correlation

between H$_2$S level and BMD. Multiple linear regression analyses were conducted to evaluate the association between CBS/CSE scores and biochemical indicators. Significant differences were set as *P*<0.05.

Results

Association of Serum H$_2$S Level with Femoral BMD

Participants' serum H$_2$S levels were measured to assess their significance with regard to lumbar and femoral BMD. Pearson correlation analysis showed no significant correlation between serum H$_2$S level and lumbar BMD (**Figure 1A**). Interestingly, serum H$_2$S had a significant bivariate correlation with femoral BMD among these individuals (**Figure 1B**), suggesting a negative association between serum H$_2$S level and hip region BMD. Serum H$_2$S levels were obviously higher in osteopenia patients compared with controls (*P*=0.041 for control vs osteopenia), while patients with osteoporosis exhibited higher levels of H$_2$S (**Figure 1C**, *P*=0.022 for control vs osteoporosis; *P*=0.034 for osteoporosis vs osteopenia) in accordance with T-scores measured by dual-energy X-ray absorptiometry (**Figure 1D**).

Expression of H$_2$S-producing Enzymes in Femoral Tissues

Given that the close relationship between serum H$_2$S level and femoral BMD, we tested the expression of CBS, CSE, and 3-MST in the femoral tissues of 19 osteoarthritis patients who underwent hip replacement surgery. Among these 19 patients, 10 subjects had normal BMD and 9 had osteoporosis. In line with previous reports [43-47], blood phosphorus, hemoglobin, and triglyceride levels tended to be decreased in osteoporosis patients (**Figure 2**). By contrast, ALP was upregulated in osteoporosis patients compared with the control group (**Figure 2**). However, blood calcium and fasting blood glucose did not differ statistically between the 3 groups (**Figure 2**). To verify the correlation between H$_2$S-producing enzymes in femoral tissue and clinical parameters, the expression of CBS, CSE, and 3-MST was evaluated by immunohistochemistry. The results demonstrated that the expression of CBS and CSE was downregulated in femur tissues from patients with osteoporosis, whereas the expression of 3-MST was unchanged, relative to the control group (**Figure 3**). The correlation between H$_2$S-generating enzymes in femur tissue and the clinical biochemical indicators in serum was analyzed in 2 different groups of patients (**Figures 4, 5**). We found that the femoral CBS score was positively correlated with serum levels of phosphorus, hemoglobin, and triglycerides, as well as the femoral T-score (**Figure 4**). The univariate regression analysis showed that serum ALP was negatively associated with the femoral CBS value (**Figure 4**). Similar results were also found regarding the association of the femoral CSE value with serum phosphorus,
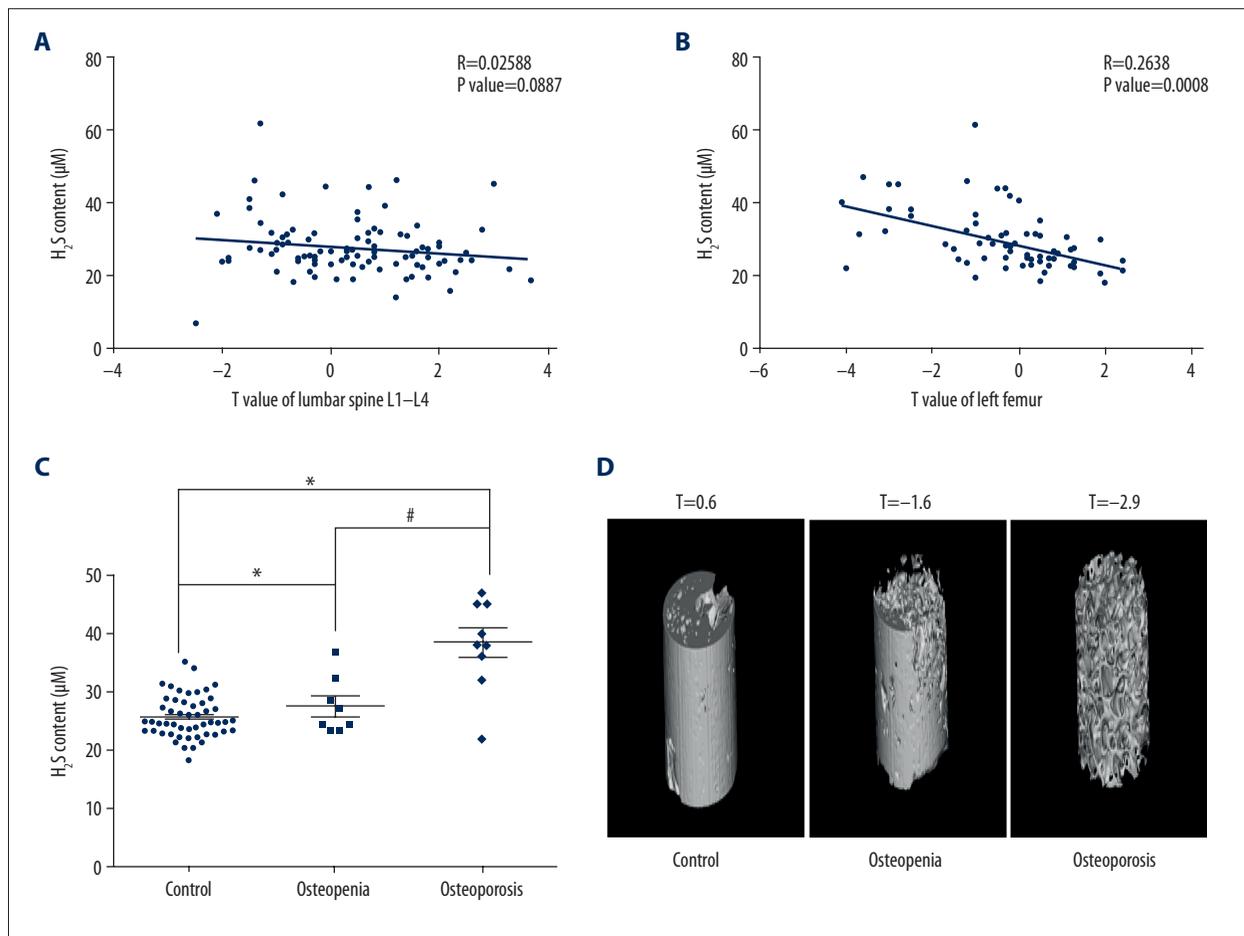
Figure 1. Association of serum H$_2$S level with bone mineral density (BMD). (**A**) Correlation between serum H$_2$S levels and lumbar BMD. (**B**) Correlation between serum H$_2$S levels and femoral BMD. (**C**) Serum H$_2$S levels in osteopenia/osteoporosis patients and control subjects. (**D**) Representative images of femoral scans from dual-energy X-ray absorptiometry. Pearson correlation analysis was used to determine the correlation between H$_2$S level and lumbar BMD. Pearson correlation analysis was used to determine the correlation between H$_2$S level and femoral BMD. One-way analysis of variance was used to compare serum H$_2$S level in osteopenia/osteoporosis patients and control subjects. Values are expressed as mean±SD. * *P*<0.05 vs control, # *P*<0.05 vs osteopenia.

hemoglobin, ALP, and triglycerides, as well as femoral T-score (**Figure 5**). Notably, both serum calcium and fasting blood glucose levels showed no correlation with femoral CBS/CSE values (**Figures 4, 5**).

Discussion

A previous study found that 2 H$_2$S-generating enzymes, CBS and CSE, were remarkably inhibited in dexamethasone-exposed osteoblastic MC3T3-E1 cells [48]. In addition, serum H$_2$S levels were shown to be downregulated in dexamethasone-induced osteoporosis in rats, with concomitant reductions in CBS and CSE expression in the bone marrow [30]. Consistent with this finding, serum H$_2$S levels and the bone marrow levels of 2 key H$_2$S-generating enzymes, CBS and CSE, were also diminished

in a model of postmenopausal bone loss induced by ovariectomy [27]. Behera et al [33] demonstrated that plasma levels of H$_2$S were decreased in a mouse model of hyperhomocysteinemia-induced bone loss and osteoporosis. Reduced plasma levels of H$_2$S have also been observed in spinal cord injury-induced sublesional bone loss and osteoporosis [49]. These experimental results clearly suggest that diminished production of H$_2$S is critically involved in the pathologies of osteoporosis, and H$_2$S might represent a novel therapy for boss loss and osteoporosis.

In the present study, we sought to determine the association of serum H$_2$S levels with lumbar and femoral BMD and to explore the relationship between femoral H$_2$S-generating enzyme expression and clinical biochemical indicators. We found that serum H$_2$S levels were significantly elevated in patients with osteopenia or osteoporosis compared with a control group with
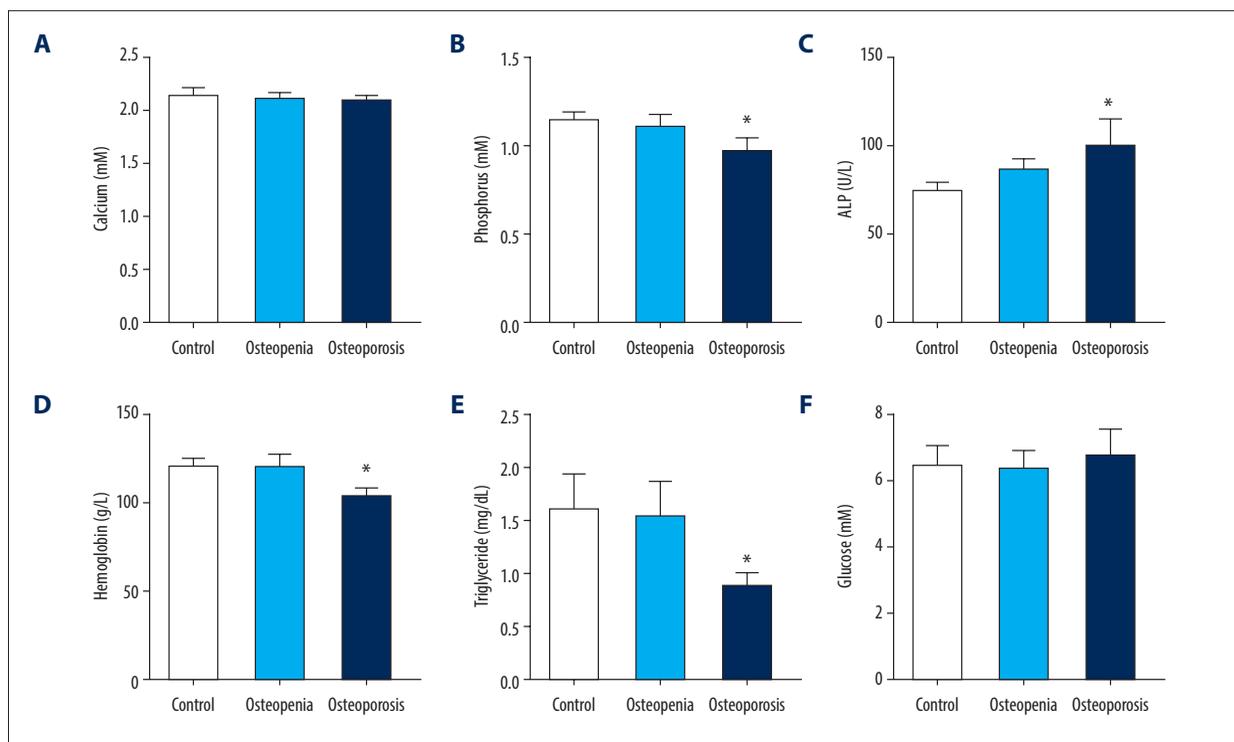
Figure 2. Clinical biochemical indicators in control subjects and osteopenia/osteoporosis patients: (**A**) serum calcium levels; (**B**) serum phosphorus levels; (**C**) serum alkaline phosphatase (ALP) levels; (**D**) serum hemoglobin levels; (**E**) serum triglyceride levels; and (**F**) fasting blood glucose levels. One-way analysis of variance was used to compare these clinical biochemical indicators in osteopenia/osteoporosis patients and control subjects. Values are expressed as mean±SD. * *P*<0.05 vs control.

normal BMD. Although the correlation of serum H$_2$S levels with femoral BMD was statistically different (*P*=0.0008), the correlation coefficient was low (R=0.2638), thus indicating a weak association. Moreover, other correlations between expression of femoral H$_2$S-generating enzymes and femoral T value were moderate (R=0.4). Therefore, a larger sample of clinical cases is needed to determine the biological significance of the correlation between serum H$_2$S and femoral BMD. Interestingly, serum H$_2$S levels were negatively associated with femoral BMD, but not lumbar BMD. This difference might be explained by the H$_2$S system possibly playing a more important role in stabilizing femoral BMD than lumbar BMD, which merits a comprehensive study. In addition, the decreased expression of CBS and CSE in femoral tissues were closely associated with serum levels of phosphorus, ALP, hemoglobin, and triglycerides. We anticipate that modulation of the H$_2$S system might have potentially therapeutic value for osteoporosis patients. It is particularly noteworthy that the opposite correlation of H$_2$S levels and CBS or CSE level with femoral BMD was observed in the present study. We speculated that an increase in circulating H$_2$S levels may be a compensatory mechanism to resist osteoporosis. In addition, we cannot rule out that peripheral H$_2$S levels might vary according to distinct stages of osteoporosis. By contrast, decreased H$_2$S levels in the femur tissues may induce disturbances in the bone metabolism system, causing

bone loss and osteoporosis. Certainly, further experimental results are needed to test these hypotheses.

Aging is categorized by a decline in tissue and organ functions with an increased risk of mortality [50]. In this study, we found that patients with osteoporosis were older and the proportion of women with osteoporosis was significantly higher among the 100 participants, which is consistent with the previous reports [43-45,51]. Evidence for a role of H$_2$S in bone tissue repair and regeneration is emerging as H$_2$S is being shown to regulate the migration and survival of cells involved in bone formation and breakdown [22]. Specifically, H$_2$S promotes the proliferation and survival of mesenchymal stem cells that give rise to the fracture callus [52]. Mechanistically, H$_2$S accelerates bone tissue regeneration through regulation of bone cell activities, oxidative stress, calcium intake by bone cells, and angiogenesis [22]. CBS and CSE, the H$_2$S-producing enzymes, are widely expressed in osteoblasts [53-55]. In particular, CSE is the predominant source of H$_2$S in osteoblasts [54]. H$_2$S has a well-established role in the protection of osteoblasts against homocysteine- or hydrogen peroxide-induced cell toxicity [56,57]. Impaired H$_2$S synthesis due to a deficiency of CBS is linked to impaired osteogenesis and bone loss in mice [28]. The WNT pathway [27,58], BMP pathway [59], and p38-MAPK and ERK signaling pathways [57,60], as well as S-sulfhydration of
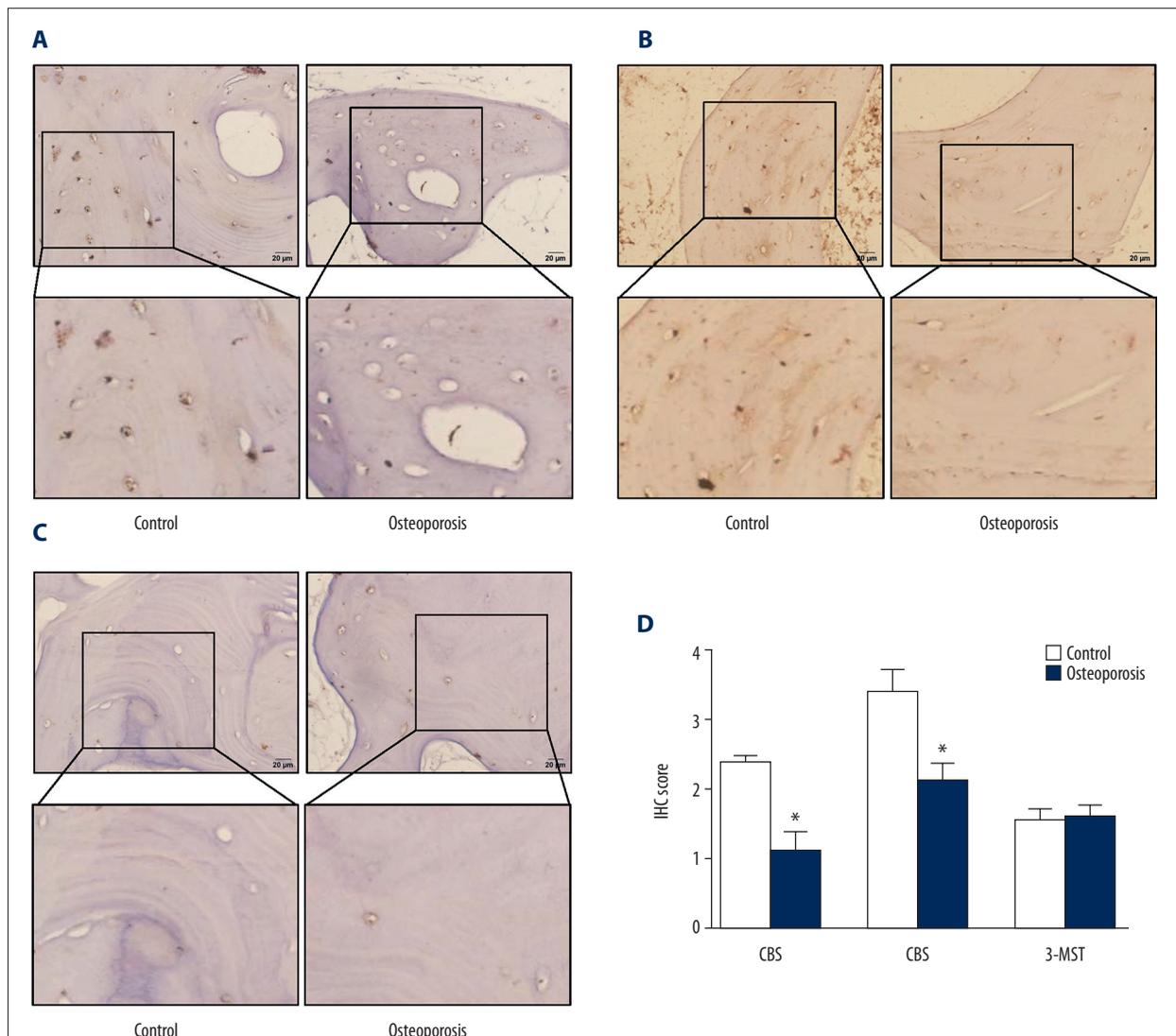
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Figure 3. Expression of H$_2$S-producing enzymes in femoral tissues. (**A**) Representative images of CBS immunohistochemistry staining. (**B**) Representative images of CSE immunohistochemistry staining. (**C**) Representative images of 3-MST immunohistochemistry staining. (**D**) Scores for CBS, CSE, and 3-MST. Independent *t* test was used to compare scores for CBS, CSE, and 3-MST in control and osteoporosis patients. Values are expressed as mean±SD. * *P*<0.05 vs control. CBS – cystathionine β synthase; CSE – cystathionine γ lyase; 3-MST – 3-mercaptopyruvate sulfurtransferase.

Runx-2 [54] and Ca^{2+} TRP channels [28], have been implicated in H$_2$S-dependent induction of osteogenesis. H$_2$S has been found to reverse nicotine- and lipopolysaccharide-induced osteoclastic differentiation [61]. In addition to these findings, exogenous administration of H$_2$S donors confers therapeutic potential against bone loss induced by modeled microgravity or distraction osteogenesis [49,61,62]. Overall, these data demonstrate that H$_2$S is an important player in osteogenesis and bone formation under both normal and pathological conditions.

In the present study, we recruited a cohort of 100 individuals and performed a systematic analysis of the relationship between BMD and serum H$_2$S level. Our results showed that serum H$_2$S levels were negatively associated with femoral BMD rather than lumbar BMD. We also divided our study cohort into control, osteopenia, and osteoporosis groups based on the T-scores of the individual participants. The serum H$_2$S levels were found to be obviously enhanced in patients with comorbid osteopenia and osteoporosis. Based on these results, we postulated that high serum H$_2$S levels may be a promising marker for early diagnosis of osteoporosis, which deserves further investigation. Further studies are indispensable to determine whether increased circulating H$_2$S levels may be a compensatory mechanism to protect against osteoporosis.
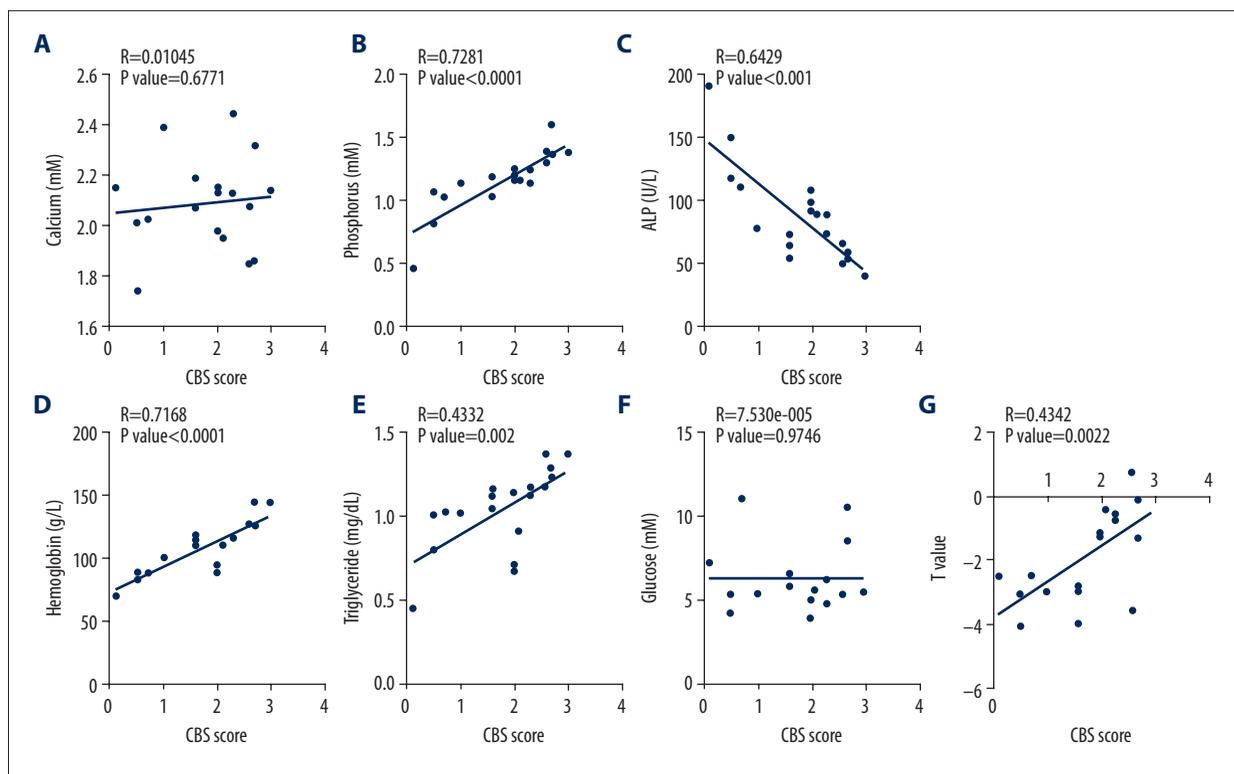
Figure 4. Association of femoral cystathionine β synthase (CBS) expression with clinical characteristics. (**A**) Correlation between femoral CBS expression and serum calcium levels. (**B**) Correlation between femoral CBS expression and serum phosphorus levels. (**C**) Correlation between femoral CBS expression and serum alkaline phosphatase (ALP) levels. (**D**) Correlation between femoral CBS expression and serum hemoglobin levels. (**E**) Correlation between femoral CBS expression and serum triglycerides levels. (**F**) Correlation between femoral CBS expression and fasting blood glucose levels. (**G**) Correlation between femoral CBS expression and femoral T value. Multivariate forward stepwise linear regression analysis was used to identify the correlation between femoral CBS expression and clinical characteristics.

In light of the importance of H$_2$S in femoral BMD, the expression of H$_2$S-producing enzymes in femoral tissues were determined in 19 osteoarthritic patients who underwent hip replacement surgery. Based on their femoral T-score, 9 osteoarthritis patients had osteoporosis, while 10 osteoarthritis patients had normal BMD. Immunohistochemistry results showed that femoral expression of CBS and CSE was downregulated, whereas 3-MST expression was unaltered in patients with osteoporosis, hinting that an impaired CBS/CSE system might be involved in the etiology of osteoporosis. Although we measured femur H$_2$S-producing enzymes in subjects with normal BMD or osteoporosis, these cases were complicated by osteoarthritis. As such, the clinical significance of H$_2$S-producing enzymes in the pathogenesis of osteoporosis warrants further study.

It is accepted that serum calcium levels are within a normal range in patients with osteoporosis, but serum phosphatase and ALP can be somewhat changed in patients with osteoporosis [63,64]. ALP is a marker of bone turnover and vascular calcification in patients with chronic kidney disease [65]. Hemoglobin is an important biomarker for fractures [66], and

normalization of hemoglobin levels is critical for the treatment of thalassemia-associated osteoporosis, a form of secondary osteoporosis [67]. However, the clinical significance of hemoglobin in primary osteoporosis remains to be investigated. A few clinical studies have suggested that high serum triglyceride levels might lower the risk of vertebral fractures [68,69], indicating that triglycerides may be important in bone metabolism homeostasis. In the current study, blood phosphorus, hemoglobin, and triglyceride levels were downregulated, while ALP was elevated in patients with osteoporosis, which was similar to previous reports [43-47]. We further examined the relationship between femoral CBS/CSE expression and femoral BMD as well as clinical biochemical indices. We found significant associations of femoral CBS/CSE expression with serum phosphorus levels, ALP, hemoglobin, and triglycerides. These findings suggest an important role of H$_2$S in femur-related osteoporosis. However, the precise roles of the endogenous H$_2$S system in the biological behaviors of osteoblasts and osteoclasts, fundamental events involved in osteoporosis pathophysiology, require further research. Here, it should be mentioned that the choice of hemoglobin, triglycerides, and glucose as markers of
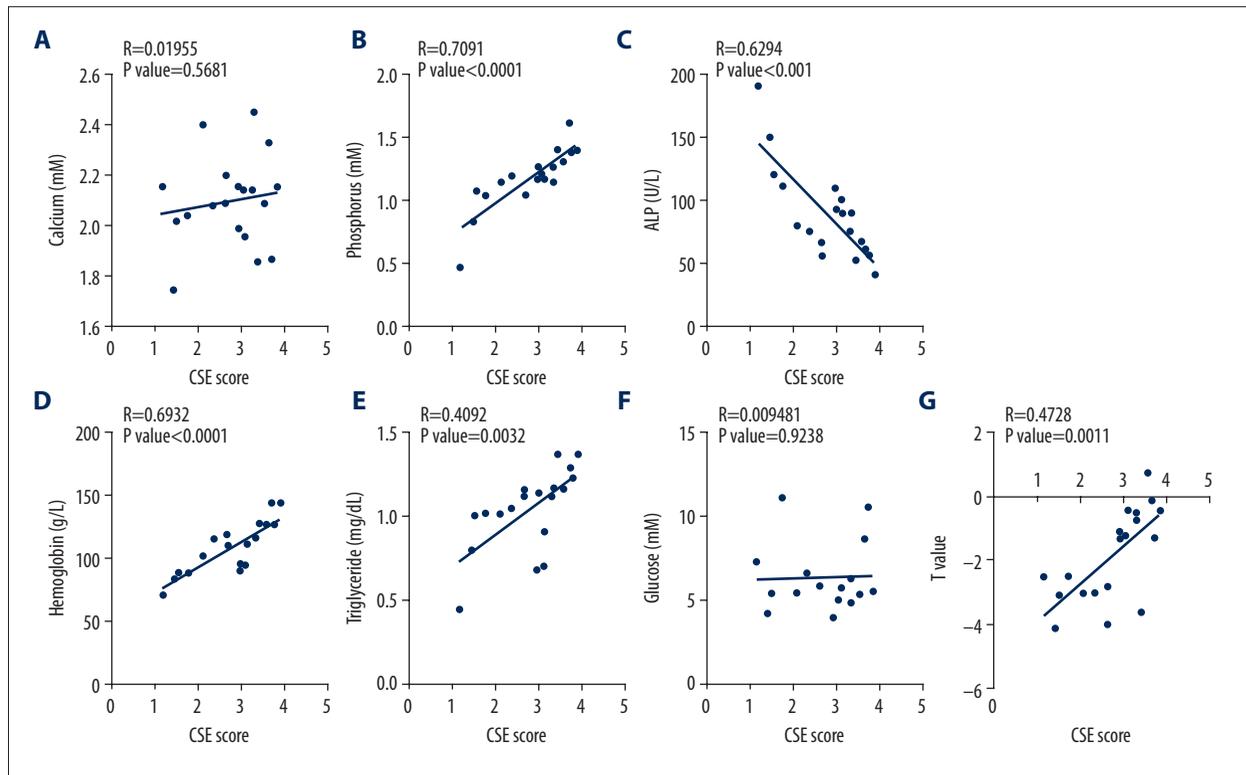
Figure 5. Association of femoral cystathionine γ lyase (CSE) expression with clinical characteristics. (**A**) Correlation between femoral CSE expression and serum calcium levels. (**B**) Correlation between femoral CSE expression and serum phosphorus levels. (**C**) Correlation between femoral CSE expression and serum alkaline phosphatase (ALP) levels. (**D**) Correlation between femoral CSE expression and serum hemoglobin levels. (**E**) Correlation between femoral CSE expression and serum triglycerides levels. (**F**) Correlation between femoral CSE expression and fasting blood glucose levels. (**G**) Correlation between femoral CSE expression and femoral T value. Multivariate forward stepwise linear regression analysis was used to identify the correlation between femoral CSE expression and clinical characteristics.

bone loss or bone metabolism might be insufficient and unspecific. Further studies are required to investigate the association of H₂S with serum levels of bone turnover biomarkers, including parathyroid hormone, P1NP, and CTX [6].

Limitations

Immunohistochemistry-related results are only semiquantitative experiments, which is a limitation in this study. As such, quantitative evaluations (such as quantification of mRNA or expression of CBS, CSE, and 3-MST by reverse-transcription polymerase chain reaction or western blotting, respectively) would be more appropriate to investigate the association of femoral expression of H₂S-producing enzymes with biochemical indicators. In line with this, the quantitative analysis of H₂S in femoral tissues is necessary to further confirm the importance of femoral H₂S concentrations in osteoporosis. Given that the distribution of H₂S-producing enzymes in bone cells is still unclear, immunofluorescence double-staining experiments are needed to verify which H₂S-producing enzymes are expressed by each type of bone cells, especially osteoblasts

and osteoclasts. The close relationship between H₂S and BMD shown in the current study is only the first step in determining the exact roles of H₂S in osteoporosis. It remains to be explored whether H₂S could serve as a biomarker for prediction of comorbidities and prognosis of osteoporosis, and future cohort studies or randomized control trials are required to confirm the nature of the causal relationship. Additionally, multicenter studies are necessary to strengthen the generalizability of our results. Since age and sex are factors that are closely related to osteoporosis, our analysis might not be representative for incidence, sex, and age. In addition, the groups of osteopenic and osteoporotic patients might be pooled with different etiologies. A more rigorous clinical experimental design is needed to verify the exact significance of H₂S in the pathogenesis of osteoporosis. Regardless, the newly developed therapeutic approaches for osteoporosis, particularly those designed to target bone H₂S, should be extensively studied in the near future. We speculate that genetic analyses of CBS/CSE gene polymorphisms might predict which patients are at higher or lower risk of osteoporosis and deserve further studies.

Conclusions

In conclusion, we reported the relationship between H$_2$S and BMD in an osteopenia/osteoporosis population. We found that serum H$_2$S levels were significantly negatively associated with femoral BMD, while femoral CBS/CSE expression was positively related with femoral BMD. These findings support the view that H$_2$S plays a central role in bone metabolism and osteoporosis. Considering that the etiologies of primary or secondary osteoporosis are quite different, the biological significance of H$_2$S may vary in different types of osteoporosis. Future research is needed to explore the potential roles and mechanisms of H$_2$S in primary and secondary osteoporosis.

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Acknowledgments

Conflict of Interest

None.

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