Laboratory Investigations

Partial Prevention of Long-Term Femoral Bone Loss in Aged Ovariectomized Rats Supplemented with Choline-Stabilized Orthosilicic Acid

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Abstract. Silicon (Si) deficiency in animals results in bone defects. Choline-stabilized orthosilicic acid (ch-OSA) was found to have a high bioavailability compared to other Si supplements. The effect of ch-OSA supplementation was investigated on bone loss in aged ovariectomized (OVX) rats. Female Wistar rats ($n = 58$, age 9 months) were randomized in three groups. One group was sham-operated (sham, $n = 21$), and bilateral OVX was performed in the other two groups. OVX rats were supplemented orally with ch-OSA over 30 weeks (OVX1, $n = 20$; 1 mg Si/kg body weight daily) or used as controls (OVX0, $n = 17$). The serum Si concentration and the 24-hour urinary Si excretion of supplemented OVX rats was significantly higher compared to sham and OVX controls. Supplementation with ch-OSA significantly but partially reversed the decrease in Ca excretion, which was observed after OVX. The increase in bone turnover in OVX rats tended to be reduced by ch-OSA supplementation. ch-OSA supplementation increased significantly the femoral bone mineral content (BMC) in the distal region and total femoral BMC in OVX rats, whereas lumbar BMC was marginally increased. Femoral BMD was significantly increased at two sites in the distal region in OVX rats supplemented with ch-OSA compared to OVX controls. Total lumbar bone mineral density was marginally increased by ch-OSA supplementation. In conclusion, ch-OSA supplementation partially prevents femoral bone loss in the aged OVX rat model.

Key words: Choline-stabilized orthosilicic acid — Silicon — Bone — Bone mineral density — Ovariectomy

Age-related diseases such as osteoporosis have emerged as major public health problems. Although the etiology of osteoporosis is multifactorial [1], the most common type of osteoporosis is the bone loss associated with ovarian hormone deficiency at menopause. Drugs such as calcium (Ca) and vitamin D supplements, bisphosphonates, and estrogen and estrogen receptor modulators [2] are extensively used to try to slow or reverse osteoporosis. Osteoporosis results from an imbalance between bone resorption by osteoclasts and bone formation by osteoblasts [3]. Estrogens and bisphosphonates decrease bone resorption. Only a few drugs, such as strontium ranelate [4] and daily injections of parathyroid hormone [5], can increase osteoblast activity and enhance bone formation. There has also been interest in bone minerals (magnesium and fluoride [6]) and nutritional trace elements (zinc, copper, and manganese). Zinc, copper, and manganese are essential cofactors of enzymes involved in the synthesis of the constituents of bone matrix [7]. Dietary intake of these trace elements is positively associated with bone mass, while deficiency has been correlated with reduced bone mass or slow healing of fractures [8].

Silicon (Si) may be useful as a preventive or therapeutic agent against osteoporosis in combination with estrogen, Ca, and vitamin D, considering its suggested role in bone mineralization. Previous studies with Si-deficient diets in rats and chicks have described signs of aberrant connective tissue and bone metabolization (thinner cortex, less calcified bone matrix). Carlisle [9] found a close relationship between Si concentration and the degree of mineralization of young bone. Si deficiency was also associated with a reduction of the number of osteoblasts in bone matrix of chickens [10]. The formation of glyco-

saminoglycans and the synthesis of collagen in bone and cartilage were decreased by nutritional Si deficiency [11]. More recently, Seaborn and Nielsen [12, 13] showed that Si deprivation in rats results in altered bone mineral composition and decreased activity of bone enzymes such as femur alkaline phosphatase (ALP). Keeting et al. [14] and Schütze et al. [15] reported that the Si-containing compound zeolite (sodium zeolite A) stimulates DNA synthesis in osteoblasts and inhibits osteoclast-mediated bone resorption *in vitro*. In a recent study, a positive correlation was shown between dietary Si intake and bone mineral density (BMD) at the hip in men and premenopausal women, suggesting that higher Si intake may have a beneficial effect on cortical bone health [16].

Orthosilicic acid (OSA), also known as soluble silica, is present in low concentrations ($< 10^{-4}$ M) in beverages and water. Dietary silicates undergo hydrolysis, forming OSA, which is readily absorbed in the gastrointestinal tract [17]. Physiological concentrations of OSA were recently found to stimulate collagen type I synthesis and osteoblastic differentiation in human osteoblast-like cells *in vitro* [18]. A stabilized form of OSA called choline-stabilized OSA (ch-OSA) was found to have a high bioavailability compared to other Si supplements, which contain polymerized species of OSA [19]. Supplementation of young animals with ch-OSA resulted in a higher collagen concentration in the skin [20] and increased femoral bone density [21]. Oral intake of ch-OSA in humans, over 20 weeks, resulted in a significant positive effect on skin surface and skin mechanical properties [22]. The improvement in skin parameters after ch-OSA supplementation suggested regeneration or *de novo* synthesis of collagen fibers.

In the present study, we used aged ovariectomized (OVX) rats to evaluate the effect of ch-OSA on bone turnover in an animal model for postmenopausal bone loss.

Materials and Methods

Animals and Diet

Nine-month-old female Wistar rats ($n = 58$; Elevage Janvier, Le Genest Saint Isle, France) with an average weight of 250 g were randomly assigned to three groups. The rats were individually housed in cages at 25°C with a 12-hour/12-hour light/dark cycle. One group was sham-operated ($n = 21$), and bilateral OVX was performed in the other two groups. OVX rats were supplemented with ch-OSA (OVX1, $n = 20$; 1 mg Si/kg body weight daily; Bio Minerals n.v., Destelbergen, Belgium) over 30 weeks or used as controls (OVX0, $n = 17$).

The rats were weighed weekly. A casein-based diet (C1000: 0.9% Ca, 0.7% P, vitamin D_3 500 IU/kg, vitamin K_3 10 mg/kg, choline chloride 1,012 mg/kg; Altromin, Lage, Germany) was available *ad libitum* in the sham group, while OVX rats were pair-fed. The drinking water contained 0.60 ± 0.05 mg Si/L. The mean analyzed Si concentration in two batches of this diet was 322 ± 47 μg Si/g. ch-OSA supplementation was started immediately after OVX and continued for 30 weeks. ch-OSA was given daily in a freshly prepared 0.1% citric acid solution

(5 mL) in demineralized water. During administration of ch-OSA, rats had no access to drinking water. Rats without ch-OSA supplementation were administered 5 mL of a 0.1% citric acid solution as a placebo.

The study protocol was approved by the local ethical committee (University of Antwerp).

OVX

OVX was performed under anesthesia with ether, using a dorsal approach with a small single midline skin incision. Control animals were subjected to sham surgery, exposing but not removing the ovaries. The success of OVX was confirmed at necroscopy for failure to detect ovarian tissue and atrophy of the uterine horns.

Urine and Serum Analysis

Urine was collected in metabolic cages after 20 weeks of supplementation and stored at –70°C. The rats were anesthetized after 30 weeks of supplementation (or 7.5 months postsurgery) with ether and killed by cardiac puncture. Blood was collected in Si-free polypropylene labware (Sarstedt, Nümbrecht, Germany). Following centrifugation of clotted blood, serum samples were stored in polypropylene tubes at –70°C (Sarstedt). Si concentration in serum and urine was analyzed in one batch by electrothermal atomic absorption spectrometry with inverse longitudinal Zeeman background correction (AAnalyst 800; Perkin Elmer, Bodenseewerk, Germany). Pyrolytic-coated graphite tubes were used. The hollow cathode lamp settings were, respectively, 30 mA lamp current, 251.6 nm spectral line, and 0.2 nm bandwidth. The injected sample volume was 20 μL, and signals were measured in the peak-area mode. Serum samples were measured in duplicate by standard addition. Standards and serum dilutions were prepared in matrix modifier solution containing 72 mg/L $CaCl_2$ (Aldrich, Bornem, Belgium), 1.508 g/L $NH_4H_2PO_4$ (Merck, Overijse, Belgium), and 0.5 g/L Na_4EDTA (Aldrich) in ultrapure water (conductance ≤ 0.08 μS). Sensitivity, determined as the amount of Si yielding a 0.0044 Abs.s signal, was 90 pg. A pool of serum obtained from fasting healthy subjects was analyzed on several days to determine the interassay coefficient of variation, which was 8.7% for a mean Si concentration of 109.09 μg/L ($n = 16$).

Total urinary Ca and phosphorus concentrations were determined by spectrophotometry by means of a Vitros 250 instrument (Johnson & Johnson, Ortho Clinical Diagnostics, Beerse, Belgium). The Vitros Ca slide method is based on formation of a colored complex of Ca with Arsenzo III dye. Coefficients of variation are 1.50% for a mean Ca concentration of 8.0 mg/dL and 1.02% for a mean concentration of 11.7 mg/dL.

The phosphorus slide method is based on the reaction of inorganic phosphate with ammonium molybdate to form an ammonium phosphomolybdate complex, which is reduced by *p*-methylaminophenol sulfate to form a stable heteropolymolybdenum blue chromophore. Coefficients of variation are 1.63% for a mean phosphorus concentration of 51.4 mg/dL and 1.77% for a mean concentration of 24.9 mg/dL.

ALP activity in serum was determined by spectrophotometry, measuring the release of *p*-nitrophenol from *p*-nitrophenol phosphate. The enzyme activity was determined as the change of absorbance at 405 nm over a 5-minute period as acknowledged by the International Federation of Clinical Chemistry. Coefficients of variation were 4.26% for a mean ALP activity of 68.0 U/L and 1.55% for a mean activity of 422 U/L.

Osteocalcin (OC) was measured using the rat OC immunoradiometric assay kit (Immunotopics, San Clemente, CA). Coefficients of variation of 3.8% for a mean OC concentration of 186.83 ng/mL and 4.41% for a mean concentration of 15.6 ng/mL.
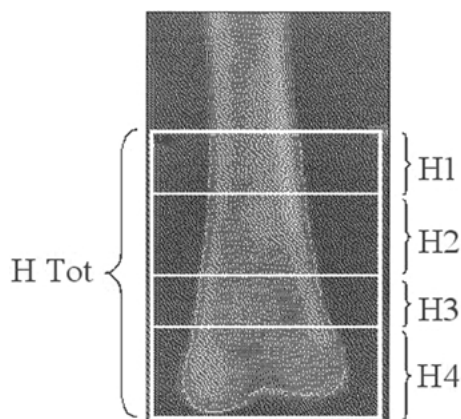
Fig. 1. Scanned areas of interest in the femur were located in the midshaft (H1) and the distal metaphysis region (H2, H3, H4).

Pyridinoline and deoxypyridinoline in urine were measured by means of high-performance liquid chromatography (HPLC) on a Bio-Rad (Munich, Germany) apparatus, with reagents also from Bio-Rad. Internal standard (25 μL) was added to 175 μL urine and 200 μL HCl 3% and incubated overnight at 100°C. Three hundred microliters of the hydrolized sample was run through conditioned cellulose columns on a vacuum box, according to the instructions of the manufacturer. Fifty microliters of the eluate was injected into the HPLC apparatus. The analytical column was a reversed-phase 100×4.0 mm Bio-Rad column, and the fluorescence detector was from Shimadzu (Kyoto, Japan; RF-551, excitation 295 nm, emission 400 nm). A flow cell of 25 μL was used, flow was 0.8 mL/min, temperature was 50°C, and run time was 15-17 minutes. Coefficients of variation for pyridinoline were 6.66% for a mean concentration of 290 pmol/L and 6.67% for a mean concentration of 1,330 pmol/L; coefficients of variation for deoxypyridinoline were 3.32 for a mean concentration of 280 pmol/L and 6.71% for a mean concentration of 70 pmol/L.

Bone Mineral Measurements

The lumbar spine (vertebrae L1-L4) and the femur were dissected, carefully freed of soft tissue, and stored at −20°C until examination. Bone mineral content (BMC) and BMD were analyzed by dual-energy X-ray absorptiometry (QDR1000; Hologic, Waltham, MA) according to a previously described protocol [23]. Scans were recorded for total femur, four regions of interest in the femur (H1, midshaft; H2, H3, H4, distal metaphysis; see Fig. 1), and lumbar vertebrae (L1-L4). The coefficient of variation was <1%.

Statistical Analysis

Results are expressed as means ± standard error (SE). Statistical evaluation of the data was performed using SPSS (Chicago, IL) software (version 13.0). OVX rats supplemented with ch-OSA were compared with both sham and OVX controls, and differences between groups were evaluated with the Mann-Whitney U-test. $P < 0.05$ was considered significant.

Results

Despite pair feeding, the body weight of OVX rats was higher compared to control rats (sham) at all times (Fig. 2). The final body weights of OVX0, OVX1, and sham rats at 29 weeks were, respectively, 343.30 ± 38.62, 343.75 ± 18.27, and 297.15 ± 19.49 g. Rats supplemented with 1 mg Si/kg body weight increased their Si intake by 5.5%.

OVX marginally increased the serum Si concentration, whereas urinary Si excretion tended to be lower after OVX. Supplementation with ch-OSA significantly increased both the serum Si concentration and urinary Si excretion in OVX rats compared to unsupplemented controls (Table 1) and sham-operated rats.

OVX rats differed from intact rats by, respectively, decreased urinary excretion of Ca and P and increased levels of serum OC and ALP activity. The urinary excretion of deoxypyridinoline was also significantly increased after OVX. Supplementation with ch-OSA significantly but partially reversed the decrease in Ca excretion. OC and ALP levels in OVX rats tended to be lower after ch-OSA supplementation compared to controls. The amount of deoxypyridinoline excreted in the urine after OVX was about twice that of intact controls and was not changed by ch-OSA supplementation. Urinary excretion of pyridinoline was similar in OVX controls and sham-operated rats but significantly increased after ch-OSA supplementation.

The mean diameter and length of the femur were not statistically different between the groups (data not shown). BMC was significantly decreased by OVX in the femur (−15%, sham versus OVX0, $P = 0.0001$) and spine (−17%, sham versus OVX0, $P < 0.0001$). ch-OSA supplementation of OVX rats increased significantly the femoral BMC in the distal region (H3 +9.4%, OVX1 versus OVX 0, $P = 0.01$) and total femoral BMC (+6.4%, OVX1 versus OVX 0, $P = 0.025$) compared to OVX controls. Lumbar BMC was marginally increased by ch-OSA supplementation (+3.5% versus OVX0, $P =$ not significant) (data not shown).

OVX significantly decreased BMD in the femur (−15%, sham versus OVX 0, $P < 0.0001$) and spine (−14%, sham versus OVX 0, $P = 0.0005$). ch-OSA supplementation increased significantly the femoral BMD in OVX rats at two sites in the distal region (H2 +4.2%, H3 +7.2%, OVX1 versus OVX0, $P < 0.05$) compared to controls (Fig. 3). Total lumbar BMD was marginally increased by ch-OSA supplementation in all vertebrae (Fig. 4).

Discussion

Until now, three studies have investigated the effect of Si on bone in OVX rats. Hott et al. [24] demonstrated that supplementation with an organic Si compound (1 mg Si/kg body weight) significantly reduced bone loss in OVX rats compared to intact controls. The supplementation period was very short, and the effect on BMD was not investigated. Rico et al. [25] showed that
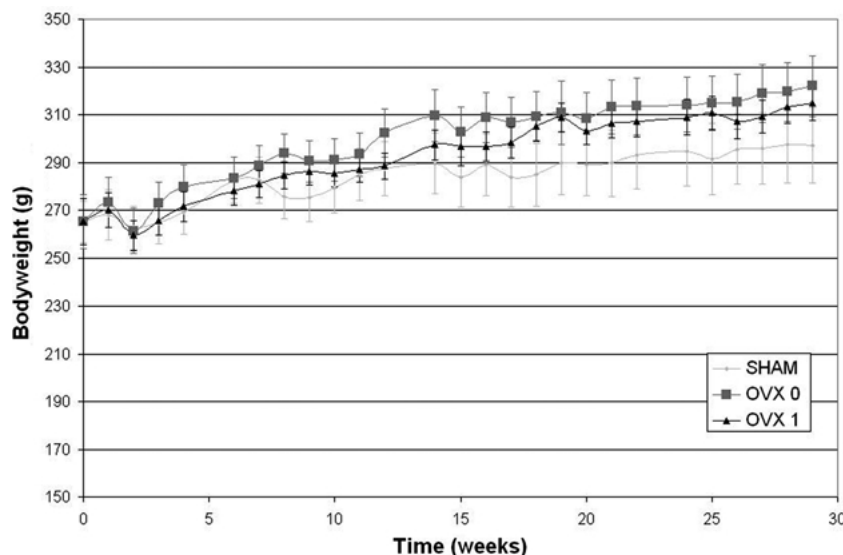
Fig. 2. Evolution of the mean body weight (mean $\pm$ SE) of each treatment group during the experimental period of 30 weeks. Sham, intact rats; OVX0, nonsupplemented OVX controls; OVX1, supplemented OVX rats (1 mg Si/kg body weight).

Si supplementation with sodium silicate (500 mg Si/kg feed) inhibits the loss of bone mass in OVX rats and promotes the longitudinal growth of long bones – in this case, the femur. In both the above studies, young animals (age 3 months) were used and supplementation was limited to 4 weeks. Previously, it was shown in a study using 4.5-month-old rats that there is rapid periosteal new bone apposition through a modeling process as well as rapid bone mineral deposition in the femoral cortical bone until the age of 7.5 months [23]. Since we wanted to evaluate the effect of ch-OSA on both cortical and trabecular bone, we choose rats aged 7.5 months at baseline. In a recent study [26], a low dietary Si intake was used in premature (56 g body weight at baseline) intact and OVX rats. It was found that the Si status affected the response to estrogen deficiency; i.e., OVX increased plasma ALP in Si-supplemented, but not in Si-low, rats. Furthermore, the authors suggested that Si has a biochemical role that affects bone growth processes by affecting bone collagen turnover and sialic acid-containing extracellular matrix proteins such as osteopontin. We emphasize that in the present study a normal and not an Si-low purified diet was used. In fact, compared to the basal purified diet in the study by Nielsen and Poellot [26], the Si content of the diet in our study was > 150-fold higher. The administered Si dose in the form of ch-OSA was only 5.5% of the dietary Si intake.

ch-OSA is a stabilized form of OSA in which choline chloride is used as a stabilizing agent. Comparative studies have shown that ch-OSA has a high bioavailability compared to other Si compounds such as colloidal silicic acid and phytolytic silicon [19, 27]. This is confirmed by the significant increase in both the serum Si concentration and the urinary Si excretion in OVX rats supplemented with ch-OSA compared to controls.

Interestingly, OVX marginally influenced Si status as the serum Si concentration tended to be higher and urinary Si tended to be lower compared to intact rats.

Previously, we showed that supplementation of young animals with ch-OSA resulted in a higher collagen concentration in the skin [20] and increased femoral bone density [21]. In both these experiments, an Si-adequate diet was used and the dietary Si intake was increased < 5% in the form of ch-OSA. Furthermore, oral intake of ch-OSA in women with photoaged skin resulted in a significant improvement in skin roughness and mechanical skin anisotropy, which was suggested to be the result of a potential regeneration or *de novo* synthesis of collagen fibers [22].

As shown by others [28], OVX increased bone turnover markers and decreased both 24-hour urinary Ca excretion and bone mass. These changes observed after OVX are similar to the changes seen in women after menopause due to decreased Ca absorption from the gut.

Reffitt et al. [18] showed that physiological concentrations of OSA stimulate collagen type I synthesis and osteoblastic differentiation in human osteoblast-like cells in vitro. We found in a speciation study that in both serum and urine Si is present as OSA after administration of ch-OSA (unpublished results). Consequently, we suggest that ch-OSA supplementation is likely to result in stimulation of collagen synthesis. Recently, we demonstrated in women with osteopenia that 12 months of combined therapy of Ca/vitamin D plus ch-OSA has a beneficial effect on bone turnover, especially on procollagen type 1 N-terminal propeptide, which was significantly less reduced compared to therapy with Ca/vitamin D only [29].

Urinary Ca excretion was partially normalized after ch-OSA supplementation in OVX rats, which may sug-

Table 1. Serum and urine parameters (mean ± SE) measured in OVX rats supplemented with ch-OSA (OVX1) compared to nonsupplemented intact (sham) rats and OVX controls (OVX0)

| Group	Silicon Serum µg/L	Silicon Urine µg/24h	Calcium Urine mg/24h	Phosphorus Urine mg/24h	OC Serum ng/ml	ALP Serum U/L	PYR Urine nmol/24h	DYP Urine nmol/24h
SHAM	113.00 ± 24.93	119.82 ± 8.84	1.18 ± 0.11	13.08 ± 1.04	9.12 ± 0.53	29.49 ± 4.12	5.23 ± 0.39	1.60 ± 0.17
OVXO	147.35 ± 25.55	94.09 ± 9.37	0.65 ± 0.09[a]	9.23 ± 1.42[a]	21.80 ± 3.30[a]	45.73 ± 3.64[a]	5.18 ± 0.22	3.05 ± 0.18[a]
OVX1	253.35 ± 20.12[ab]	164.86 ± 10.03[a,b]	0.88 ± 0.08[ab]	8.77 ± 1.27[a]	15.97 ± 1.34[a]	38.10 ± 2.26[a]	5.66 ± 0.29[a]	3.20 ± 0.23[a]

[a] $P < 0.05$ vs. sham (Mann-Whitney U-test)
[b] $P < 0.05$ vs. OVX controls (Mann-Whitney U-test)
PYR, pyridinoline; DYP, deoxypyridinoline
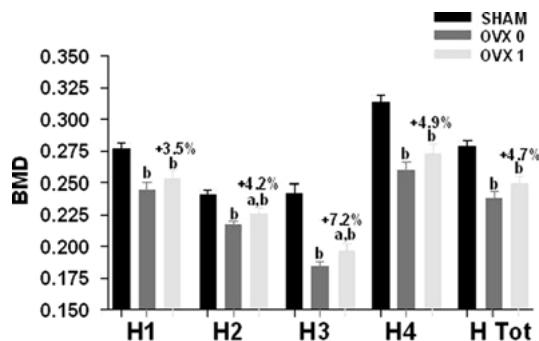
Fig. 3. Femoral BMD (mg/cm^2, mean ± SE) of OVX rats supplemented with ch-OSA (OVX1) compared to nonsupplemented intact rats (sham) and OVX controls (OVX0). [a]$P <$ 0.05 vs. OVX0 controls (Mann-Whitney U-test). [b]$P < 0.05$ vs. sham (Mann-Whitney U-test). The percent increase in BMD of OVX1 versus controls is indicated.
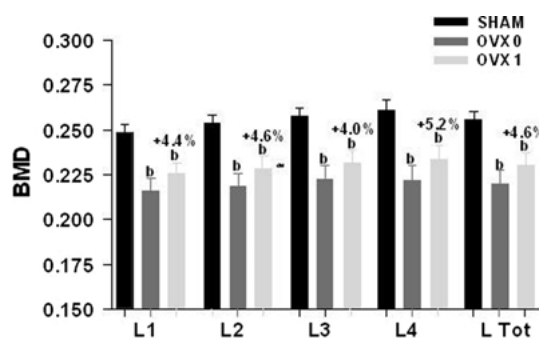
Fig. 4. Lumbar BMD (mg/cm^2, mean ± SE) of OVX rats supplemented with ch-OSA (OVX1) compared to nonsupplemented intact rats (sham) and OVX controls (OVX0). [b]$P <$ 0.05 vs. sham (Mann-Whitney U-test). The percent increase in BMD of OVX1 versus controls is indicated.

gest that declined intestinal Ca absorption is to some extent restored by ch-OSA. In a previous animal study [20], we demonstrated that serum Si and Ca concentrations are positively correlated, suggesting also a possible role for Si in Ca metabolism. Interestingly, vitamin D_3, which is important for the rate of Ca entry from the intestinal lumen into the enterocyte [30], was also reported to influence the Si concentration in serum [31]. Similar to estradiol treatment, increased OC and ALP levels tended to be lower after ch-OSA supplementation in OVX rats, which points to a decrease in bone turnover compared to nonsupplemented OVX controls.

Decreased femoral bone mass was partially normalized after ch-OSA supplementation in OVX rats. The effect on the lumbar spine was of the same order of magnitude, although not statistically significant, most likely due to higher standard deviations.

In conclusion, long-term preventive treatment with ch-OSA prevents partial femoral bone loss in the aged OVX rat model. The present results confirm earlier

studies in humans and other animals suggesting that OSA is involved in bone metabolism.

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