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ORIGINAL ARTICLE

Positive association between serum silicon levels and bone mineral density in female rats following oral silicon supplementation with monomethylsilanetriol

R. Jugdaohsingh · A. I. E. Watson · P. Bhattacharya · G. H. van Lenthe · J. J. Powell

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Abstract
Summary Observational (epidemiological) studies suggest the positive association between dietary silicon intake and bone mineral density may be mediated by circulating estradiol level. Here, we report the results of a silicon supplementation study in rats that strongly support these observations and suggest an interaction between silicon and estradiol.
Introduction Epidemiological studies report strong positive associations between dietary silicon (Si) intake and bone mineral density (BMD) in premenopausal women and indicate that the association may be mediated by estradiol. We have tested this possibility in a mixed-gender rodent intervention study.
Methods Tissue samples were obtained from three groups of 20-week-old Sprague Dawley rats (five males and five females per group) that had been supplemented ad libitum for 90 days in their drinking water with (i) <0.1 mg Si/L (vehicle control), (ii) 115 mg Si/L (moderate dose) or (iii) 575 mg Si/L (high dose). All rats received conventional laboratory feed, whilst supplemental Si was in the form of monomethylsilanetriol, increasing dietary Si intakes by 18 and 99 %, for the moderate- and high-dose groups, respectively.
Results Fasting serum and tissue Si concentrations were increased with Si supplementation ($p<0.05$), regardless of gender. However, only for female rats was there (i) a trend for a dose-responsive increase in serum osteocalcin concentration with Si intervention and (ii) strong significant associations between serum Si concentrations and measures of bone quality ($p<0.01$). Correlations were weaker or insignificant for tibia Si levels and absent for other serum or tibia elemental concentrations and bone quality measures.
Conclusions Our findings support the epidemiological observations that dietary Si positively impacts BMD in younger females, and this may be due to a Si-estradiol interaction. Moreover, these data suggest that the Si effect is mediated systemically, rather than through its incorporation into bone.

Keywords Animal study · Bone μCT · Estradiol · Matrix mineralisation · Nutrition · Silicon

Introduction

Silicon, a major component of the mammalian diet via the consumption of plant-based foods, is present in all mammalian tissues and especially the connective tissues [1, 2]. However, it is not clear whether it has a biological/biochemical role in higher animals, including mammals. Evidence amassed over the past 40 years suggests that Si may be important for normal bone and connective tissue health [1]. We have previously reported, in the Framingham Offspring cohort, that higher intakes of dietary Si are associated with higher bone mineral density (BMD) at the hip sites in premenopausal women and to some extent in men but not at

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R. Jugdaohsingh (✉) · A. I. E. Watson · J. J. Powell
Elsie Widdowson Laboratory, MRC Human Nutrition Research, Fulbourn Road, Cambridge CB1 9NL, UK
e-mail: ravin.jugdaohsingh@mrc-hnr.cam.ac.uk

A. I. E. Watson
School of Sport and Exercise Health Sciences, Loughborough University, Loughborough LE11 3TU, UK

P. Bhattacharya · G. H. van Lenthe
Department of Mechanical Engineering, KU Leuven, Heverlee, Belgium

all in postmenopausal women [3]. These findings suggested that there may be an interaction between Si intake and estrogen status, and this was investigated further in a female-only cohort (the Aberdeen Prospective Osteoporosis Screening Study) where postmenopausal use of hormone replacement therapy (HRT) was documented in detail. We confirmed the Si–BMD relationship in premenopausal women and the lack of association in postmenopausal women who had never taken HRT [4]. However, for postmenopausal women, the Si–BMD relationship was regained in past users of HRT and especially so in current users of HRT [4]. These findings, from observation studies, imply a possible interaction between Si intake and estrogen status.

Others have also suggested a possible interaction between silicon and estrogen. Charnot and Peres [5, 6] reported that endogenous sex and endocrine hormones affect the absorption and metabolism of Si in rats, whilst Nielsen and Poellot [7] reported that dietary Si (or Si status) affects the response to a change in estrogen status (i.e. ovariectomy/estrogen deficiency). Here, we have taken advantage of rat tissue samples that were collected from a 12-week (90 days) oral intervention study with the Si supplement 'monomethylsilanetriol' (MMST, $CH_3Si(OH)_3$) to directly investigate the interaction between Si intake and estrogen status with respect to bone health. The effect of Si supplementation on body Si pools (Si status) was investigated by measuring fasting Si levels in serum, ear (non-calcified collagenous tissue and potential Si pool) and bone (calcified collagenous tissue and Si pool). The study was carried out by a commercial clinical research organisation for separate, regulatory purposes (i.e. a safety study), but it provided an opportunity for us to investigate the effects of 3-month Si supplementation on bone quality (bone microarchitecture and bone mineralisation) in male and female rats, where there is natural separation of circulating estradiol levels [8, 9]. Silicon supplementation was given on a normal dietary Si background; i.e. this was not a deficiency study, the rats received a maintenance diet with its normal high Si content.

Previous human studies have shown, over a 1-month intervention period, that MMST ($CH_3Si(OH)_3$) is a safe Si supplement and that it undergoes metabolism to orthosilicic acid (OSA, $Si(OH)_4$) which is considered the bioactive form of Si [10, 11]. Unlike OSA, however, this MMST precursor form has the advantage of remaining soluble and bioavailable at the supplemental levels used in this study [10–13]. The overall purpose of this study was to investigate the effect of MMST (Si) supplementation on connective tissue Si concentrations and bone quality measures. The data, however, also allowed us to investigate the interaction between Si and estrogen status.

Methods

Animal study and tissue collection

Rat tissue samples were collected at the end of a 90-day supplementation study with MMST, which was performed at a Good Laboratory Practice-accredited commercial clinical research organisation (CRO; Charles River Laboratories Pre-Clinical Services, Ireland). The study consisted of three groups each of ten rats: group 1 = vehicle control (reverse osmosis-treated drinking water with <0.1 mg Si/L), group 2 = 115 mg Si/L drinking water ('moderate Si dose') and group 3 = 575 mg Si/L drinking water ('high Si dose'). The drinking waters were prepared and provided by LLR-G5 Ltd (Castlebar, Ireland), with Si supplemented in the form of MMST ($CH_3Si(OH)_3$), and Si contents were confirmed in our laboratory by ICP-OES analysis. The supplemental dosing undertaken in this study was, primarily, for regulatory safety assessment purposes, and therefore, the doses were high. Each group consisted of five male (8 weeks old at start) and five female (8 weeks old at start and nulliparous and non-pregnant) Sprague Dawley rats (Charles River Laboratories, Margate, UK). All rats were individually housed in polypropylene cages with stainless steel lids (with dust-free sawdust as bedding) at 22 °C with a 12/12-h light/dark cycle. The drinking water, with and without the Si supplementation, was provided ad libitum in individual (dedicated) plastic drinking units. All rats also received ad libitum the same maintenance feed (2018 Teklad 18 % Protein Rodent diet).

Body weight and the consumption of the drinking waters were monitored on a daily basis, but feed intakes were not monitored. On day 89, the animals were fasted except for de-ionised water with no added Si. Fifteen to 16 h later, on day 90, animals were anesthetised, terminal blood samples were collected, animals euthanised and necropsy performed. Fasting blood, sera, plasma and tissue samples were generated for clinical biochemistry analysis, haematological analysis and histopathology at the CRO and were not available to the authors for the below analyses. In addition, sera (obtained without use of anticoagulants), ears and tibias were also collected and stored at −80 °C prior to being couriered frozen on dry ice to the authors' laboratory for analysis. This study was approved by the animal ethics committee of the CRO.

Analyses

A brief summary of the analyses is given below with more details in Online Supplementary Materials. All samples were analysed in a blinded fashion.

Serum 17β-estradiol

Fasting serum samples were analysed for 17β-estradiol using a commercially available high-sensitivity ELISA kit (Enzo Life Sciences UK Ltd, Exeter, UK) to confirm the higher circulating serum levels in female rats compared to male rats and to investigate potential changes in circulating levels with Si treatment.

Serum osteocalcin

Aliquots of fasting serum samples were also analysed for osteocalcin, a marker of bone formation/bone turnover, using the commercially available Rat N-Mid Osteocalcin kit (MyBioSource Inc, Sand Diego, USA).

Total elemental analysis

Total elemental analyses of the rodent feed, fasting serum samples and one of the pairs of ear and tibia samples were carried out by inductively coupled plasma optical emission spectrometry (ICP-OES), Jobin Yvon 2000-2 (Instrument SA, Longjumeau, France), using peak profiles [12, 14] and sample-based standards for Si and other elements. Prior to analyses, tissue samples (ear, tibia) and rodent feed were digested by microwave-assisted (nitric) acid digestion, whilst the serum samples were diluted in 0.2 % nitric acid (see Online Supporting Materials for further details). Serum iron and phosphorus were not measured, the latter due to possible haemolysis [15].

Bone quality measurements

The second of the pair of tibias collected from each animal at necropsy was cleaned as previously described [16] at the authors' laboratory and couriered, frozen on dry ice, from the authors' laboratory to the Laboratory of Pathophysiology, University of Antwerp, Belgium, for micro-CT analysis (Skyscan 1076 in vivo X-ray micro-CT scanner, Aartselaar, Belgium). The following bone quality parameters (measures) were obtained: trabecular BMD (tBMD), tissue volume (TV), bone volume (BV), bone volume fraction (BV/TV), total surface (TS), bone surface (BS), bone surface/volume ratio (BS/BV), bone surface density (BS/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N) and total porosity (Po(T)).

Biomechanical testing

Following bone quality measures, the tibias were subjected to three-point bending test at room temperature in a custom-made loading device, integrated in a materials testing machine (Bose ElectroForce Test Bench LM1, Bose Corp, USA). The following parameters were obtained from the data collected: stiffness (k; N/mm), yield strength (F_y; N) and fracture load (F_{max}; N).

X-ray diffraction

One tibia from each of the three groups of the female rats that had undergone micro-CT analysis underwent powder X-ray diffraction (XRD) analysis at the Institute for Materials Research, University of Leeds (UK) to determine changes in the mineral phase with Si supplementation. Prior to analysis, the bones were processed [17] to remove organic components from the bone matrix that could interfere with the XRD analysis.

Statistical analyses

Results are reported as means±SD unless otherwise stated. Grubbs' test for outliers was carried out (in GraphPad Prism 6; GraphPad Software Inc, La Jolla, USA) on all the datasets collected. One of the serum Si values (1120 μg/L), in a female rat from the 'high' Si dose group, was identified as a significant outlier (at $p<0.05$) and is omitted from the data shown. Test for linearity was used to test for a dose-responsive increase in bone, serum and ear Si concentrations and serum osteocalcin concentration with Si supplementation and significance was taken as $p\leq0.05$. In the absence of a significant trend, individual group differences between Si treatments and diluent control were then assessed by independent (unpaired) samples two-tailed t test and, because there was comparison for two groups (moderate and high Si dosing), a Bonferroni correction was applied to the p value (i.e. p/n), and significance was taken as $p\leq0.025$. Pearson correlation (with two-tailed t test) was used to test for correlations between fasting serum and tibia element concentrations with bone quality measures. All statistical analysis was conducted in IBM SPSS version 21 (IBM Corporation).

Results

Serum 17β-estradiol concentrations

Fasting serum levels of 17β-estradiol, in the samples collected at necropsy, were, on average, 1.7-fold higher in female rats compared to male rats (94.8±20.6 vs. 57.9±12.6 pg/mL; $p=0.0002$; Supplementary Figure 1).

Silicon intakes and body weights

The feed (chow pellets) consumed by all groups contained, on average, 610 μg Si/g feed (Supplementary Table 1), and

assuming typical average feed intakes of 23 g feed/day in adult female rats and 32 g feed/day in adult male rats of the same strain [18], this would contribute ~14 and 19 mg Si to daily Si intakes, respectively. Mean daily intake of Si from drinking waters (supplemented with and without Si) is shown in Table 1, separately for male and female rats, and did not differ between genders: overall mean Si intakes from the moderate and high Si-supplemented drinking waters added a further 18 and 99 % Si to that from the feed.

Weight gain of the animals was unaffected by Si supplementation (Supplemental Figure 2; $p>0.2$ for female rats and $p>0.4$ for male rats). There was also no association between serum Si and body weight or body weight gain ($r=-0.2$, $p>0.5$). Moreover, no adverse effects (clinically, biochemically or pathologically) were associated with 3 months' MMST supplementation at either the moderate or high doses investigated (data not shown), consistent with previous findings in a lower-dose, 4-week human supplementation study [10].

Tissue silicon measurements

A dose-responsive increase in Si concentrations of the serum and collagen-rich ear tissue was apparent with Si supplementation ($p<0.05$, test for linearity; Fig. 1a, b). However, for the tibia, there was not a dose response: whilst the moderate dose of supplemental Si led to a significant increase in bone Si levels ($p=0.03$), the high dose had no effect (Fig. 1c). Similar patterns were observed for male and female rats (data not shown).

Bone-associated elements

Serum Cu and Zn concentrations were significantly increased in female rats on moderate dose Si supplementation and a similar, but non-significant, trend was seen for tibia Cu and Zn levels (Supplemental Tables 2 & 3). Male rats showed a similar trend for serum and tibia Cu levels (Supplemental Tables 2 & 3). Moreover, in the female rats, tibia Ca and tibia Ca/P ratios were increased with the high dose of Si compared to controls ($p=0.025$ and 0.016, respectively; Fig. 2), whilst in male rats, no statistically significant differences in tibia concentrations of Ca, P or Ca/P were found with Si supplementation ($p>0.1$ compared to controls; data not shown).

Serum osteocalcin

Serum osteocalcin concentrations were similar in female and male rats ($p=0.45$), but in female rats, there was a trend for a dose-dependent increase in osteocalcin concentration with Si supplementation (Fig. 3). Moreover, although serum osteocalcin concentrations showed no correlation with serum Si concentrations in female rats ($p=0.3$), strong *negative* correlation was seen in male rats ($r=-0.72$, $p=0.008$).

Bone quality measures

Silicon supplementation had no effect on tBMD in male rats (Fig. 2d and Supplemental Table 4), and although mean tBMD increased for female rats, it was not significant either (Fig. 2d and Supplemental Table 4). Nonetheless, given the relatively low group numbers and the variance around serum and bone Si levels, we considered that an association between Si levels and tBMD could have been masked by categorical analysis. To this end, direct correlations showed a strong relationship between fasting serum Si levels (a recognised proxy for Si status (10)) and tBMD in female rats but not in male rats (Fig. 4a vs. b). Bone Si levels also correlated with tBMD for female rats only (Fig. 4c vs. d), albeit not as strongly as between serum Si and BMD and perhaps explained by the association between serum Si and bone Si levels (Fig. 4e, f).

Moreover, fasting serum Si concentrations were found to correlate strongly with other bone quality measures and, again, only for the female rats. Associations were positive

Table 1 Mean daily silicon intakes from drinking waters

	Female rats			Male rats		
	Group 1: control ($n=5$)	Group 2: 115 mg Si/L ($n=5$)	Group 3: 575 mg Si/L ($n=5$)	Group 1: control ($n=5$)	Group 2: 115 mg Si/L ($n=5$)	Group 3: 575 mg Si/L ($n=5$)
Drinking water (mL/day)[a]	21.5 (2.2)	22.9 (3.9)	26.5 (3.1)[c]	29.9 (3.0)	29.0 (5.4)	29.4 (4.7)
Si intake from water (mg/day)[b]	< 0.002	2.64 (0.45)	15.21 (1.77)	< 0.002	3.33 (0.62)	16.86 (2.71)

Means (±SD) calculated from daily measurements between days 40 and 50. Feed intake was not measured but was estimated to be 23 g/day in female rats and 32 g/day in male rats, contributing ~14 and 19 mg Si/day, respectively, in female and male rats

[a] A vehicle control (reverse osmosis-treated drinking water with <0.1 mg Si/L) or Si-supplemented drinking water (115 or 575 mg Si/L) was consumed by the animals ad libitum as a substitute for normal drinking water

[b] There was no significant difference in Si intake between female and male rats

[c] Intake was significantly greater than control ($p=0.02$, unpaired t test)

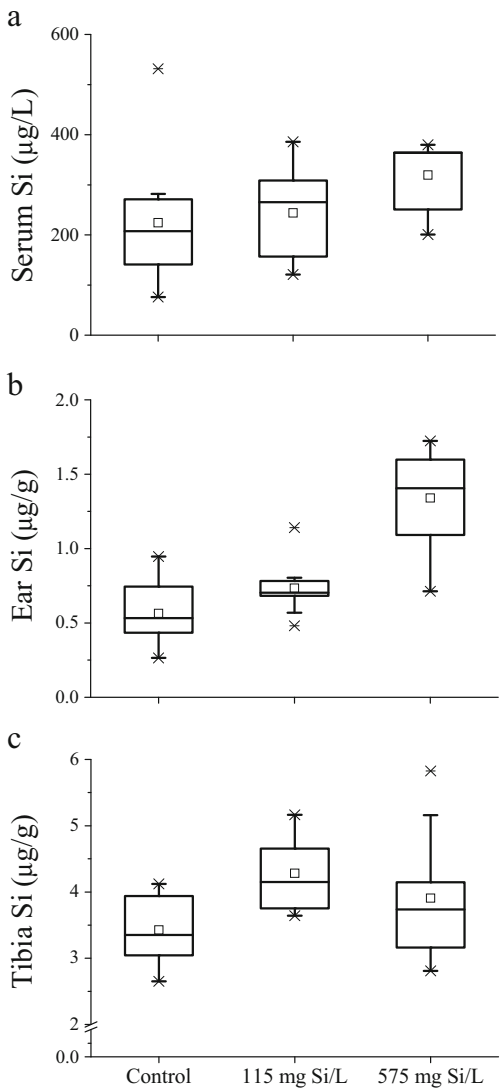

Fig. 1 **a**–**c** Fasting silicon concentrations in the tissues of rats (n=10 per treatment: 5 males and 5 females), in the control and Si-supplemented groups, collected at necropsy (i.e. after 12-week intervention). Data is shown as box plots where the *horizontal lines* indicate the 5th, 25th, 50th (i.e. median), 75th and 95th percentiles, the *open square* shows the mean and the *crosses* the minimum and maximum values. Test for linearity was significant for serum Si (p<0.05) and ear Si (p<0.0001) concentrations, but not tibia Si concentration (p=0.17). By subsequent t test of the tibia, p=0.003 for 115 mg Si/L versus control and was not significant for 575 mg Si/L versus control

with BV/TV, BS/TV and with Tb.N and negative with Tb.Sp and Po(T) (Fig. 5a–e). Strong positive correlations were also obtained for fasting serum Si levels and, individually, BV (r=0.79, p=0.0008) and BS (r=0.72, p=0.004) but not TV (r=−0.06, p=0.835) or TS (r=0.02, p=0.934). So, serum Si in female rats was associated with the amount of bone, but not the size of bone. A weaker (positive) correlation was obtained with Tb.Th (r=0.64, p=0.014).

The above correlations were also generally found with tibia Si levels but, again, were not as significant as for the fasting serum Si levels (data not shown). In the single indicator tibia samples taken from each group for ex vivo X-ray diffraction analyses, there was no suggestion that Si supplementation altered bone mineral phase (Supplemental Figure 3). Si intervention did not significantly alter bone strength based upon categorical analysis (Supplemental Table 5) whilst, unlike for tBMD, the positive correlation with serum Si was not significant (Fig. 5f–h).

Finally, to address the specificity of Si's association with bone quality, we next assessed correlations of other serum (n=5) and tibia (n=8) elemental concentrations with tBMD (see 'Methods' section). Of these, only serum Mg yielded a (weak) correlation with tBMD (r=0.64, p=0.015 in female rats and r=0.61, p=0.016 in male rats), but this did not carry through with any other bone quality measures (data not shown).

It should be noted that in female rats, serum estradiol showed no correlations with serum Si, tibia Si, serum osteocalcin or tBMD (Supplemental Table 6). A significant correlation was obtained with TbTh (r=−0.67, p=0.05; data not shown), but this was in the opposing direction to serum Si. In male rats, serum estradiol showed no association with bone quality measures, although significant association with serum osteocalcin (r=0.71, p=0.033) and Fy (r=−0.88, p=0.004) was obtained (Supplemental Table 6).

Discussion

As noted above, we have previously reported, in human epidemiological studies, a strong positive association between dietary Si intake and BMD in premenopausal women [3, 4], whilst the lack of association in postmenopausal women was restored for those taking hormone replacement therapy [4]. We thus proposed that the dietary Si-BMD effect is estradiol mediated [3]. Assuming that Si has some active beneficial role in human and other mammalian connective tissues, then these prior studies [3, 4], and other data [1], indicate that the chemical species responsible is almost certainly orthosilicic acid ($Si(OH)_4$). Dietary Si appears to be absorbed only in monomeric form from the gastrointestinal tract [12, 19], either directly so from fluids such as drinking water or following digestion of plant-based foods. For these reasons, the CRO-based 3-month supplementation study that is described herein provided an excellent opportunity to test the hypothesis that dietary Si positively impacts BMD in estradiol-replete mammals. Firstly, unlike orthosilicic acid which starts to form insoluble silicates much above 56 mg Si/L (2 mM Si), MMST ($CH_3Si(OH)_3$) may be added to drinking water at up to 588 mg Si/L (21 mM Si) without irreversible polymerisation and precipitation. Moreover, MMST appears entirely non-toxic, again as confirmed herein, and is metabolised to orthosilicic acid in vivo [10]. Secondly, in murine models, a 3-month time period should be sufficient time to see the impact

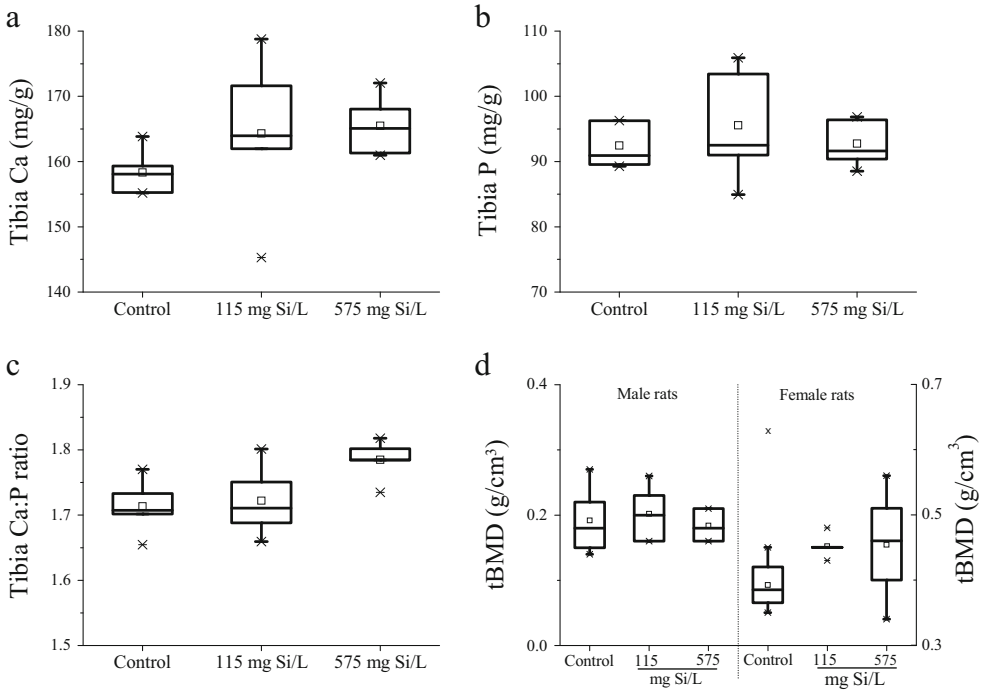

Fig. 2 Calcium (**a**), phosphorus (**b**) and Ca/P (**c**) concentrations of the tibias of female rats (n=5 per treatment), in the control and Si-supplemented groups, at necropsy after 12-week supplementation. (**d**) Trabecular bone mineral density (tBMD) of the tibia of male and female rats at necropsy. Data is shown as box plots where the *horizontal lines* indicate the 5th, 25th, 50th (i.e. median), 75th and 95th percentiles, the *open square* shows the mean and the *crosses* the minimum and maximum values. Tibia calcium concentration and Ca/P ratio were higher in the higher dose Si group, compared to the diluent control (p= 0.025, for the 575 mg Si/L vs. control; unpaired t test)

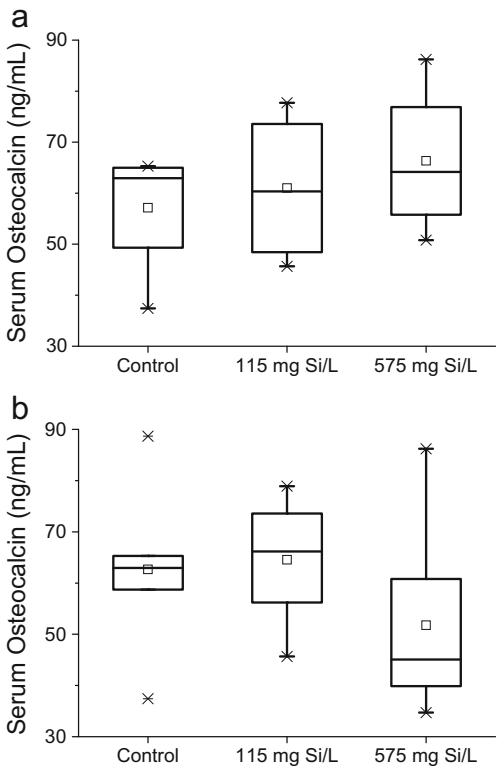

Fig. 3 N-mid osteocalcin concentrations of the fasting serum, collected at necropsy, of the female (**a**; n=4 per treatment) and male (**b**; n=4–5 per treatment) rats, in the control and Si-supplemented groups after 12-week supplementation. Data is shown as box plots where the *horizontal lines* indicate the 5th, 25th, 50th (or median), 75th and 95th percentiles, the *open square* shows the mean and the *crosses* the minimum and maximum values

on BMD of effective intervention [20]. Thirdly, male and female rats differ in their circulating estradiol levels by 1.7-fold in this study and by even greater amounts in prior studies [8, 9].

Fasting serum concentrations of Si provide the best known measure of Si status because recently ingested and absorbed Si is rapidly cleared from the circulation, and hence, fasting levels provide a steady state measure of Si that is presumed to be in equilibrium with body stores [10]. The finding that, following intervention, fasting serum Si levels were strongly positively correlated with trabecular BMD in female rats but not male rats supports the hypothesis that estradiol is required for the in vivo beneficial utilisation of Si. Of course other hormonal differences between male and female rats (i.e. other than estradiol) may explain or contribute to these findings. However, a previous study that looked at the effects/contribution of the different sex and endocrine hormones on the absorption of Si and tissue Si levels in adult rats found that estrogen deficiency in female and male rats produced the most pronounced effects [5], suggesting that estradiol may be the main or most potent mediator of Si metabolism. Similarly, with regard to bone, estrogen deficiency has the most marked effect on bone growth in male and female rats [21]. Nielsen and Poellot [7] reported that the effect of Si on bone growth/turnover depended on estrogen status, since the effect of Si was only seen in intact (non-ovariectomised) rats, but reduced/eliminated in ovariectomised rats. Replication of our findings in intact (sham-operated) and estradiol-supplemented ovariectomised rats but not in ovariectomised rats would provide the best proof for this, because, as mentioned above,

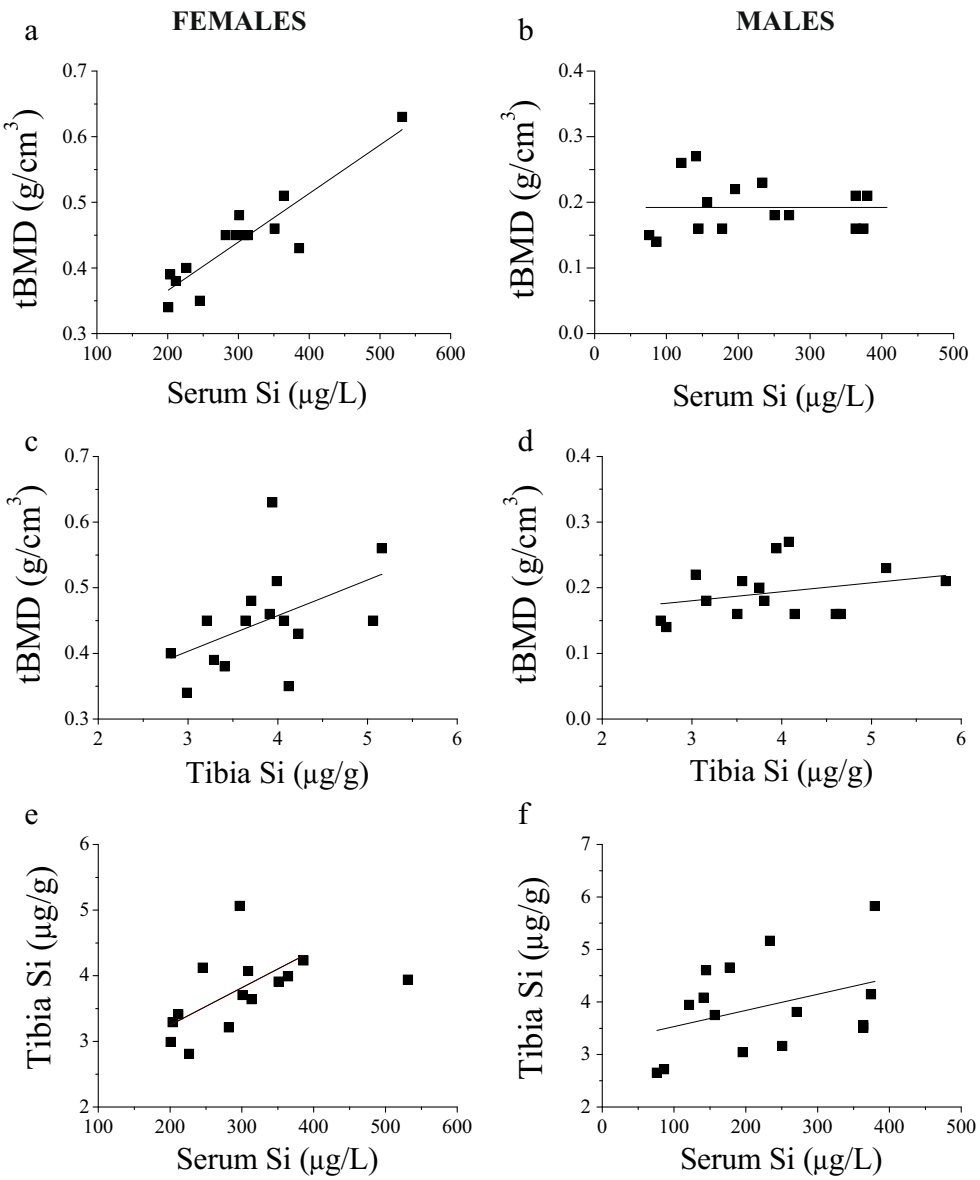

Fig. 4 **a**, **b** Associations between fasting serum silicon concentrations and trabecular bone mineral density (tBMD) of the tibias of female (**a**) and male (**b**) rats at necropsy (12-week intervention) (r=0.90, p<0.0001 for the female rats; Pearson correlation with two-tailed t test). **c**, **d** Associations between tibia Si concentrations and tBMD in female (**c**) and male (**d**) rats at necropsy (r=0.47, p=0.074 for the female rats; Pearson correlation with two-tailed t test). **e**, **f** Associations between fasting serum Si concentrations and tibia Si contents of the female (**e**) and male (**f**) rats at necropsy (r=0.47 and p=0.093 for the female rats; Pearson correlation with two-tailed t test). Note, the correlation reported in **a** was not dependent upon the serum Si value at 532 μg/L as its removal from the dataset only marginally affected the correlation reported, i.e. was still highly correlated (r=0.8, p=0.002)

in our previous observational study, the Si-BMD relationship was regained in postmenopausal women who were taking HRT [4].

Whether there is a small effect of oral Si on tBMD in male rats, as is observed for dietary Si–BMD associations in male humans [3], would probably require greater study numbers for intervention than we had access to in this work. It is also possible that in male rats, Si supplementation affects a different bone compartment, i.e. cortical bone rather than (or more than) trabecular bone. Cortical bone thickness was not measured in this study, and biomechanical data (which mainly evaluates cortical bone properties; see below) was incomplete for male rats (Supplemental Table 5). To our knowledge, the effects of Si supplementation on cortical and trabecular bone compartments have not been directly evaluated in the literature even though our previous epidemiological study showed similar Si–BMD associations at the different hip sites and the lumbar spine in men [3], implying that Si may affect both bone compartments equally.

How dietary Si could promote BMD in 'estradiol-replete' mammals is presently unclear, although additional observations herein may provide some clues. For example, tibia Si levels showed some correlation with tBMD and other bone quality measures but these were either not significant or weak compared to the serum Si correlations with BMD. This suggests that the Si effect is not due to and/or sensed from direct incorporation of Si into bone but, rather, is a peripherally generated signal as previously argued [16]. Indeed, although only three bone samples were analysed by XRD, there was certainly no obvious change to bone mineral with Si

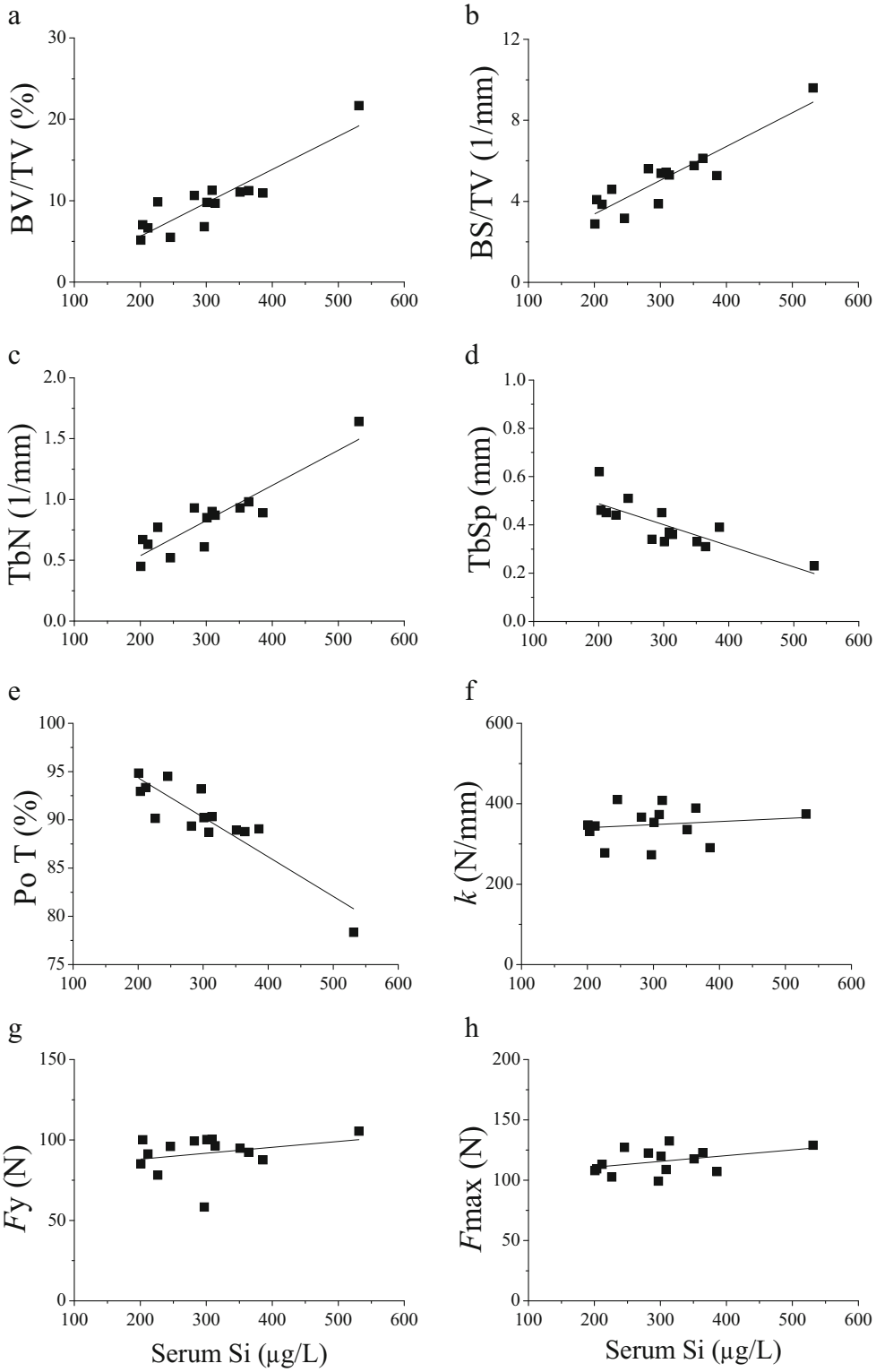

Fig. 5 Associations between fasting serum silicon concentrations and bone microarchitecture/quality of female rat tibias collected at necropsy (12-week intervention). Positive correlations were obtained for **a** bone volume fraction BV/TV (r=0.90, p<0.0001; Pearson correlation with two-tailed t test), **b** bone surface density BS/TV (r=0.91, p<0.0001; Pearson correlation with two-tailed t test), **c** trabecular number Tb.N (r=0.90, p<0.0001; Pearson correlation with two-tailed t test), whilst negative correlations were with **d** trabecular separation Tb.Sp (r=−0.80, p=0.001; Pearson correlation with two-tailed t test) and **e** total porosity Po(T) (r=−0.90, p<0.0001; Pearson correlation with two-tailed t test). Correlations between fasting serum Si concentrations and bone stiffness (**f**), yield strength (**g**) and fracture load (**h**) were not significant; r=0.15, 0.27 and 0.41 and p=0.6, 0.4 and 0.1, respectively. The correlations reported are not dependent upon the serum Si value at 532 μg/L, as its removal from the dataset only marginally affected the correlations reported; i.e. all were still highly correlated (r=0.8, p<0.004)

supplementation. This is not surprising, as the highest increase in bone Si content, with Si supplementation, was <0.01 atomic mole percent and thus unlikely to directly affect the mineral phase or its properties. In fact, tibia Si levels did not increase linearly with Si supplementation (Fig. 1). This was not a result of the higher dose being less bioavailable, as indicated by the increase in fasting serum and ear tissue Si levels compared to the moderate Si dose groups. It is more likely that it indicates a safety mechanism: a negative feedback to protect against marked changes in bone composition and/or over

mineralisation, which could affect bone quality and bone strength, as suggested by Reffitt et al. [22] and consistent with our more recent data [16, 23]. Together, these findings suggest that different tissues could have differing Si tolerances/requirements and, in bone, this may have been surpassed with the high Si dose albeit not for the collagenous ear tissue. However, to confirm this, additional doses of Si/MMST should be tested.

The specific strong correlations between serum Si concentrations and bone quality measures, and the lack of similar correlations between serum estradiol with either bone quality measures or with serum Si concentrations, suggest that the effect of Si is not directly through estradiol or changes in estradiol concentrations. As such, the findings suggest that estradiol mediates the effect of Si rather than vice versa.

The positive association between fasting serum Si concentration and tBMD in female rats was backed up by strong correlations with other bone quality measures (Fig. 5) and the trend for a dose-dependent increase in serum osteocalcin concentration. Overall serum Si in the female rats correlated positively with the amount of bone tissue (BV, BS, BV/TV, BS/TV, Tb.Th and Tb.N) and negatively with the amount of non-bone tissue/space (Tb.Sp and Po(T)), i.e. suggesting that Si supplementation is associated with increased bone tissue within the volume measured. These findings did not, however, proceed to a correspondingly significant increase in bone strength. There are two possible explanations. Firstly, it is possible for BMD to be increased without an increase in bone strength/bone stiffness. For example, the addition of bone to the endocortical surface of female rats does not lead to an increase in bone strength [21]. Female rats have higher BMD compared to male rats, but this is not associated with higher bone stiffness/bone strength and is in fact associated with lower bone stiffness/bone strength than male rats ([21]; Supplementary Tables 4 & 5). The second possibility is that Si affects trabecular bone (and therefore tBMD) but not cortical bone in female rats. The three-point bending test evaluates the shaft of the bone, i.e. cortical bone properties (e.g. cortical thickness and cross-sectional area). Hence, it is possible that Si could change bone microarchitecture without effects on bone stiffness as assessed by three-point bending. The lack of correlation between tBMD and bone strength measures here supports this statement (data not shown). Furthermore, Nielsen and Poellot [7] also reported no effect of Si on long bone bending test measures, despite increases in bone thickness with Si.

Finally, these data also show specificity in the association with tBMD to Si as the other serum and tibia elements investigated (including Cu, Zn, Mg, Ca and Ca/P ratio), either showed no correlation with bone quality measures or were markedly weaker (data not presented), regardless of gender. In female rats, the weak correlations observed for serum Mg concentrations with tBMD (r=0.64, p=0.015), BS/TV (r=0.53, p=0.051), TbN (r=0.53, p=0.053) and Po(T) (r=−0.55, p=0.044) are most likely driven by its association with serum Si concentrations (r=0.63, p=0.016). Silicon supplementation increased serum and tibia Cu concentrations in both male and female rats and serum and tibia Zn concentrations in female rats. Similar findings have previously been reported. Emerick & Kayongo-Male [24] reported that Si supplementation increased the Cu status (plasma Cu concentrations) of both Cu-deplete and Cu-replete rats, whilst, more recently, Seaborn and Nielsen [25] reported that Si deprivation reduced femoral and vertebral rat bone Cu and Zn concentrations. Emerick and Kayongo-Male [24] went further to suggest that some of the reported effects of Si (on connective tissues) may be attributed to an increase in Cu utilisation. However, as noted above, we did not find any correlations between serum or tibia Cu concentrations with bone quality measures, but we did with serum Si, suggesting that, at least here, the Si effect on bone quality was not driven by the increase in Cu utilisation.

Previous studies have shown that when the bone steady-state (equilibrium) is challenged, such as with ovariectomy, osteopenia or reproduction, oral or intravenous Si intervention can help maintain BMD [26–31] (see also reviews by Jugdaohsingh [1] and Price et al. [32]). In the work presented here, however, the rats were healthy. Moreover, the rats were not Si deficient so the effects seen are not the correction of a state of stress but, rather, are offering insights into ‘optimal nutrition’. The supplemental dosing undertaken in this study was, primarily, for regulatory safety assessment purposes, and therefore, the doses were high. In the ‘moderate’ dose group, 115 mg Si/L (4.1 mM MMST) was the sole source of fluid. In adult human supplementation, it would be just 90 mL/day out of, typically, 2 L total fluid intake per day [10]. The ‘high’ dose group was the same except the Si concentration was 575 mg Si/L (20.5 mM MMST) instead of 115 mg/L. Translating these findings to human intakes of Si is not easy. On the one hand, as noted above, supplementation in the rats was disproportionately high compared to human dosing. On the other hand, nutrient intakes are always disproportionately high for rats versus humans [33] and the Si supplementation of this study only increased the rats’ naturally high dietary Si intake by 18 and 99 % with moderate and high dosing, respectively. Interestingly, by analogy, the correlation between dietary Si intake and BMD in premenopausal women of the Framingham cohort [3] shows no tail-off in the relationship at the upper quintile of Si intake (30–63 mg/day), so perhaps optimal dietary Si intakes in premenopausal women could indeed be higher.

Conclusion

In conclusion, this paper reports that Si supplementation increases fasting serum and connective tissue Si concentrations

in rats. In female rats, concentrations of serum Si, but not other bone or serum elements, correlated strongly with trabecular BMD and other bone quality measures. These relationships were not seen in male rats and were not seen with measures of soft tissue quality for either gender, supporting the hypothesis that estradiol is required for the optimal utilisation of dietary Si in bone/connective tissues. However, additional animal models (e.g. estrogen receptor knockouts (ER-null) or ovariectomy with and without estradiol) are required to confirm this. The effect appears to be related to systemic signalling, governed by steady state circulating Si levels, rather than direct incorporation of Si into bone. Further work should also aim to identify the mechanism.

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Conflicts of interest JJP has consulted to companies involved with silicon supplementation including LLR-G5 Ltd. RJ, AIEW, PB and GHL declare that they have no conflict of interest.

Ethical approval All applicable institutional and/or national guidelines for the care and use of animals were followed.

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