Role of osteocytes in mediating bone mineralization during hyperhomocysteinemia

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Abstract

Hyperhomocysteinemia (HHCY) is a risk factor for osteoporosis but whether HHCY affects bone mineralization or not is still ambiguous. Herein we evaluated whether homocysteine affects tissue mineral density (TMD) of cortical bone and if so the role of osteocytes. CD1 mice administered with homocysteine (5 mg/100 g body weight, i.p.) for 7, 15 and 30 days showed temporal changes in TMD and osteocyte lacunar density in femoral cortices. Short-term administration of homocysteine (day 7) increased osteocyte lacunar density and reduced TMD evidenced by microCT50 while prolonged administration of homocysteine (day 30) reinstated TMD and lacunar density to baseline values. Major differences were decreased number of nucleated osteocyte lacunae, increased number of empty lacunae and cleaved caspase 3-positive osteocyte lacunae in day 30 HHCY bone evidenced by H&E staining and immunohistochemistry. Other differences were induction in mineralization genes like *Dmp1*, *Phex* and *Sost* in cortical bone by real-time PCR and increased number of Dmp1- and Sost-positive osteocyte lacunae in day 30 HHCY bone evidenced by immunohistochemistry. Both HHCY day 7 and day 30 samples showed reduced Young's modulus demonstrating that biomechanical property of bone was lost during early HHCY itself, which did not improve with recovery of TMD. Our results thus demonstrate occurrence of two phases in cortical bone upon HHCY: the early phase that involved loss of TMD and increase in osteocyte numbers and a late phase that involved osteocyte reprogramming, apoptosis and mineralization, which reinstated TMD but compromised biomechanical property. To conclude, osteocytes have a potential role in arbitrating bone pathogenesis during HHCY.

Key Words

- ▶ homocysteine
- ▶ osteocyte
- ▶ Sost
- ▶ E11
- ▶ Dmp1
- ▶ caspase 3
- ▶ lacunae

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Introduction

Throughout life bone is constantly modeled and remodeled via coordinated activities of two types of bone cells: the bone-forming osteoblasts and the bone-resorbing osteoclasts (Hadjidakis & Androulakis 2006). These cells are regulated by a third cell type called 'osteocytes' that originate from mesenchymal stem cells through osteoblast lineage differentiation by a process

called 'osteocytogenesis'. Interestingly, only 10–20% of osteoblasts differentiate into osteocytes (Aubin & Turksen 1996). During osteocytogenesis, the expression of genes that are characteristic of osteoblasts get changed and the induction of genes that impart osteocytic phenotype are promoted. For example, Pdpn (E11) and Dmp1 expressed by developing osteocytes enable formation of osteocytic dendrites, regulation of crystal (mineral) size and proper matrix mineralization while expression of sclerostin

imparts cell maturation and mineralization (van Bezooijen *et al.* 2005, Franz-Odenaal *et al.* 2006, Bonewald & Johnson 2008, Beniash *et al.* 2011, Wijenayaka *et al.* 2011). Palumbo and coworkers (Palumbo *et al.* 1990) demonstrated that the developing osteoblast transpire via three segments – the ‘osteoblast–osteocyte’, ‘osteoid–osteocyte’ and ‘pre–osteocyte’ before forming the mature osteocyte. Microscopic evaluation of the bone has shown that mature osteocytes reside in cavities called ‘osteocyte lacunae’. These lacunae are interconnected by tiny canals or canaliculi and form an extensive network called the lacunar–canalicular pore system or LCS (Lai *et al.* 2015). LCS buried within the mineralized matrix positions osteocytes to obtain nutrients from the blood supply, sense external mechanical signals, regulate structural reorganization after bone remodeling and communicate among itself and with other cells on bone surfaces (Dallas *et al.* 2013, Webster *et al.* 2013).

An interesting feature of osteocyte is its ability to live a remarkably long life of 10–20 years. Osteocytes therefore constitute 95% of the cellular component of adult bone tissue (Bonewald 2008). Earlier it was thought that osteocytes were passive and metabolically inactive cells. Nonetheless, today these are considered as an advanced cell type with endocrine functions (Dallas *et al.* 2013). In response to cellular stimuli, osteocytes secrete substances such as RANK ligand, OPG, fibroblast growth factor 23 (FGF23), prostanoids, nitric oxide, nucleotides, cytokines and growth factors that modulate bone remodeling and many physiological functions in the body (Schaffler & Kennedy 2012, Dallas *et al.* 2013). For example, FGF23 produced by osteocytes regulate serum phosphate level while sclerostin and DKK1 regulate bone formation (Bonewald & Johnson 2008, Feng *et al.* 2009).

Bone remodeling events are intricate processes influenced by diet, intrinsic hormonal milieu, physical stimulus, genetic factors and metabolism (Raisz 1999). A known metabolic factor that hampers bone remodeling process is homocysteine (Robert *et al.* 2005, Vacek *et al.* 2013). Homocysteine is essentially a metabolite of methionine metabolism that exists at a critical biochemical point in the methionine cycle from where it is used to synthesize cysteine and glutathione. Owing to enzyme (cystathionine B-synthase) and co-factor deficiency (folate, vitamin B₆ and vitamin B₁₂), the re-methylation and transulfuration pathways in methionine cycle fail to metabolize homocysteine causing the amino acid to accumulate in blood. This condition referred to as hyperhomocysteinemia (HHCY, >15 µmol/L) is an independent risk factor for heart-, kidney-, brain- and

bone-related diseases (Ganguly & Alam 2015). Clinically, HHcy is categorized as mild (16–30 µmol/L) and moderate to severe HHcy (≥31 µmol/L), and its prevalence varies with geography, age, sex and ethnicity with the lowest concentration seen in Asian Americans, intermediate in Hispanic Americans and highest in Caucasians (Yang *et al.* 2015). Many causative factors for HHcy have been identified, which include genetic variations, lifestyle habits, environmental factors, hormonal changes, drugs and disorders like cardiovascular disease, cancer and type 2 diabetes (Han *et al.* 2016).

HHcy has been associated with bone fragility, but the underlying basis for this association still remains ambiguous. Although experimental studies demonstrate that hyperhomocysteinemic rodent models suffer femoral neck and lumbar spine fragility (Herrmann *et al.* 2009), clinical data demonstrate inverse, mixed and even no association between HHcy and bone mineral density (van Meurs *et al.* 2004, Gjesdal *et al.* 2006, Bucciarelli *et al.* 2010). These reports accentuate the need for further research into the understanding of the pathophysiological processes that modulate bone mineral density during HHcy. At the same time, literature cites that bone cells such as osteoblasts and osteoclasts respond incongruously toward homocysteine. To our knowledge, homocysteine does not inhibit osteoblast activity but shifts osteoblast machinery to increase RANKL:OPG ratio favoring an osteoclastogenesis condition (Vijayan *et al.* 2013). There are also reports that homocysteine promotes bone matrix formation by osteoblasts and stimulates osteoclastogenesis (Herrmann *et al.* 2008, Vaes *et al.* 2009). As osteoblasts and osteoclasts are regulated by osteocytes, we were interested to know how osteocytes responded toward HHcy. All the more, existing reports demonstrate dysregulation of osteocyte as a cause for abnormal bone mineralization (Feng *et al.* 2013). Herein, our prime objective was to investigate whether HHcy affects bone mineralization and if so, the role of osteocytes.

Materials and methods

Animals

Animal experimentations were approved by animal ethics committee (IAEC No. 272/11). Female mice were housed in polypropylene cages at the animal house of NII under ambient room conditions: temperature (22 ± 4°C), humidity ~60–65% and light (08:00–20:00) with access to normal chow and water *ad libitum*.

CD1 mice were used for this study. For experimentation, mice aged eight weeks weighing 19 ± 5 g were acquired from the animal house facility and randomly divided into two groups. For one week, the mice were left to habituate to new cage environment (Bundgaard *et al.* 2012). Though young, the mice at week 10 showed calcified borders as evidenced by MicroCT50 (Fig. 1A).

Cages were then categorized as the following:

- Vehicle group that received intraperitoneal injections of $1 \times$ phosphate-buffered saline or PBS (pH 7.4).
- HHCY group that received intraperitoneal injections of homocysteine at a dose of 5 mg/100 g body weight. Briefly, homocysteine was dissolved in $1 \times$

PBS (pH 7.4) and administered to mice daily between 15:00 h and 16:00 h. One hour after injection of homocysteine, blood was collected by retro orbital bleeding. Analysis of homocysteine in serum by ELISA (CUSABIO Lifescience, Hubei, China) showed mice to have $49 \pm 1.9 \mu\text{mol/L}$ of homocysteine in circulation demonstrating moderate HHcy.

- For the time-dependent study, mice were administered homocysteine for 7, 15 and 30 days and killed on 8th day, 16th day and 31st day after overnight fasting along with age-matched vehicle control mice. Killing was executed by overdose of ketamine/xylazine mixture. Blood was collected by cardiac puncture.

Figure 1

Changes in femoral cortical bone geometry upon HHcy. (A) Gray scale segmentation of bone cylinder. Scale = 100 μm . (B and C) MicroCT50 data showing changes in relative bone volume (B) and cortical thickness (C) in cortical bones isolated at days 7, 15 and 30 after homocysteine induction (5 mg/100 g bwt i.p. for 30 days). (D) 3D rendering of proximal femoral cylinders by MicroCT50 (scale = 100 μm). (E) MicroCT50 data showing changes in closed pores of sizes (200–800 μm^3) per unit volume of cortical bone. (F) Changes in total cortical porosity (%) by microCT analysis. (G) Changes in cortical tissue mineral density by MicroCT50. (H) Changes in mineral content after total ashing. (I) Changes in Ca and P levels in ashed bone. (J) Alizarin staining of cortical bone. Values are mean $\pm$ S.E.M.; $n = 4$. *Statistical significance with vehicle control; **statistical significance with HHcy day 7 bone. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-16-0562>.

Histopathological analysis

Femoral bones after harvest were cleaned off bone marrow by flushing with ~1 mL of PBS (pH 7.4) several times using a 26-gauge needle. Briefly, samples were fixed in 10% neutral buffered formalin for 24 h, decalcified using 20% EDTA–0.1 M Tris solution (pH 7.2–7.3) for 5 days, dehydrated in ethanol and embedded in paraffin wax. Bone sections of 5 μ m thickness were cut on a rotary microtome, mounted onto clean slides, de-paraffinized using xylene and rehydrated through a series of decreasing ethanol solutions and finally water. The slides were either stained with hematoxylin-eosin (H&E) or Alizarin S (1 w/v%), washed, covered with a glass coverslip and imaged using USB 2.0 Camera Viewer (Leadoptics Microscope, England, UK).

Microcomputed tomographic analysis⁵⁰ (microCT⁵⁰)

Isolated femurs fixed in 10% neutral buffered formalin for 24 h were washed in 1 $\times$ PBS (pH 7.4) and stored in 70% ethanol until scanning. All samples were cut at diaphysis region ~3 mm below the epiphysis (from distal region) into cylinders to fit the sample holder (with an inner diameter of 2.5 mm) of microCT⁵⁰ (SCANCO Medical AG, Brüttisellen, Switzerland). All scans were performed using X-ray voltage of 70 kVp at an intensity of 57 μ A, using a 0.5 mm aluminum filter and a voxel size resolution of 0.8 μ m. 500 slices were analyzed covering a total of 0.4 mm. A CCD-based detector detected the photons, and projection data were computer reconstructed into a 3400 $\times$ 3400 image matrix. As osteocytes cannot be visualized, the spaces in which these cells reside viz. osteocyte lacunae were used as proxy to determine the osteocyte density. Closed pores of sizes of 200–300 μ m³ that corresponds to osteocyte lacunae were considered for analysis (Lai *et al.* 2015).

Immunohistochemical analysis

For immunostaining, the sections were de-waxed with fresh xylene and rehydrated through a series of ethanol dilutions (100–50%) and finally water. These sections were boiled in antigen retrieval citrate buffer pH 6.0 (Abcam) at 90°C for 15 min, digested with 0.05% trypsin for 10 min at 37°C and quenched with 3% hydrogen peroxide solution in methanol for 10 min at room temperature to nullify the endogenous peroxidase activity. Sections were then washed in 1 $\times$ PBS (pH 7.4),

permeabilized in 0.025% Triton X-100 and blocked using 10% normal serum (of species in which secondary antibody was raised) containing 1% bovine serum albumin. Thereafter, slides were incubated with primary antibodies. Antibodies used for this assay were (i) #9661 polyclonal antibody (1:300 dilution) raised in rabbit against cleaved caspase 3 (Asp175) from Cell Signaling Technology; (ii) ab103203 polyclonal antibody (1:300 dilution) raised in rabbit against Dmp1 from Abcam; (iii) ab63097 polyclonal antibody (1:20 dilution) raised in rabbit against sclerostin from Abcam. After overnight incubation at 4°C, the slides were washed with 1 $\times$ PBS (pH 7.4) and incubated with goat polyclonal HRP-conjugated secondary antibody against rabbit IgG (ab6721) for 45 min. The sections were then developed with anti-rabbit horse radish peroxidase/3,3'-diamino benzidine (DAB) kit (Abcam) followed by counter staining with hematoxylin, dehydration and coverslip mounting. Slides were visualized and photographed using on Eclipse 80i (Nikon) microscope. 'No primary antibody controls' were obtained by replacing primary antibody with primary antibody diluent.

Mineral content (% ash) analysis

Bone samples, with all bone marrow thoroughly removed (as previously described), were defatted in chloroform under vacuum constantly stirred for 16 days. The samples were then dried to a constant weight (4 days) and ashed in a furnace at 600°C for 24 h. Mineral content (% ash) was expressed by dividing the weight of the ashed bone (W_{AB}) by the weight of the dried defatted bone (W_{DB}) and multiplying this ratio by 100 ($(W_{AB}/W_{DB}) \times 100$).

Calcium and phosphorus estimation

Bone cortices were digested with nitric acid:perchloric acid (5:1), dried in an oven at 70°C and ashed in a muffle furnace at 600°C. Samples were dissolved individually using 3 N nitric acid and serially diluted to fit a standard curve of 1–5 ppm calcium carbonate standard solution. The total concentration of calcium in samples was then measured using flame photometry (Elico Ltd., India). For estimation of inorganic phosphate, weighed ash was allowed to react with 2.5% ammonium molybdate in 5 N sulphuric acid and 0.25% 1-amino-2-naphthanol-4-sulphuric acid in 20% sodium sulphite for 5 min and the complex formed was measured at 680 nm in a double-beam UV-2450 spectrophotometer (Shimadzu, Japan).

Scanning electron microscopy

Bone cortices after digestion with 6% Terg-a-zyne solution pH 8.0 (Sigma-Aldrich) for 3 weeks were dehydrated (50% ethanol, 2 h; 70% ethanol, 2 h; 100% ethanol, overnight) and then air dried. Samples were then pressure fractured to expose the inner surface. The endosteum surface of cortical bones was considered for analysis. These fragments were diced under a microscope, affixed onto carbon-taped metal subs, sputter coated in gold and viewed under a Zeiss EVO LS10 scanning electron microscope (Carl Zeiss AG). The number and size of vascular channels were determined by ImageJ, NIH.

Quantitative polymerase chain reaction

RNA was isolated from bone cortices using RNA lipid tissue mini kit (Qiagen). Genomic DNA elimination was performed using kit from Sigma-Aldrich. cDNA synthesis was done using cDNA synthesis kit (Qiagen). To perform qPCR, 10 ng of cDNA (per well) was amplified using the Light Cycler 480 Syber Green I Master reagent (Roche Diagnostics) and primers in the Light Cycler 480 (Roche Diagnostics) under following cycling conditions: 3 min at 95°C, 15 s at 95°C, 20 s at 60°C, 25 s at 72°C for 40 cycles. After amplification, fold changes in gene expression vs β -actin (reference) analysis were determined using the $2^{-\Delta\Delta CT}$ (Livak) method. The primers used for the reaction were commercially acquired from Qiagen. The catalog numbers of the RT² qPCR mouse primers are as follows: *Sost* (sclerostin) PPM36047A; *Pdpr* (E11) PPM24891B; *Dmp1* (dentine matrix protein-1) PPM04690F; *Bglap* (bone gamma carboxy glutamate protein or osteocalcin) PPM04465F; *Phex* (phosphate-regulating endopeptidase) PPM41830F; *Ahsg* (alpha-2HS glycoprotein) PPM32919B; *Actb* (β -actin) PPH00073G; *Col1a1* (collagen, type I, alpha1) PPM03845F; *Tnfrsf11* (tumor necrosis factor superfamily member 11) PPM03047F; *Runx2* (runt-related transcription factor 2) PPM04449C.

Osteocalcin and sclerostin assays

The level of carboxylated osteocalcin in mouse serum was measured employing a highly sensitive EIA kit from TaKaRa Bio (Cat #MK127). The kit uses a plate-bound solid-phase rat capture antibody that recognizes the C-terminal region of mouse osteocalcin. It is paired with a labeled monoclonal antibody that detects specifically osteocalcin with Gla residues.

The manufacturer's protocol was followed as described, and assays were measured in duplicate. A standard curve was generated using standards provided with the kit, and concentrations of the protein in serum were extrapolated from this curve.

Sclerostin was analyzed in serum using ELISA kit (Cat. MSST00) from R&D Systems according to the manufacturer's information. Absorbance was read at 450 nm (Tecan Microplate reader, Switzerland with Magellan software).

Mechanical testing of mouse bone

The mid-diaphysis portion of the femoral bones was cut and the ends were polished. Samples after moistening with 1× PBS (pH 7.4) were analyzed using Instron 3345, Instron Corporation (Canton, USA). Compression tests were performed with an elongation rate of 0.1 mm/s using a load cell of 5 kN. Specimens were loaded along the principal stress direction of the bone till it broke. The stress σ strain ϵ values were obtained. Stress is the force acting per unit area by the load i.e. the ability of the bone to withstand the load. Strain is the deformation per unit length to the load. Young's modulus is a ratio of stress to strain and is a measure of the stiffness of the bone.

Statistical analysis

The results were analyzed using a statistical program SPSS/PC+, version 11.0 (SPSS). For comparison among groups, one-way ANOVA followed by Duncan *post hoc* test was performed. The statistical significance was set at P value <0.05.

Results

Changes in cortical bone geometry with administration of homocysteine

This study used high-resolution microCT50 to examine how homocysteine affected cortical bone structure. Figure 1A is a 3D microCT reconstruction of cortical bone samples harvested from vehicle- and homocysteine-treated mice. We did not observe any change in % BV/TV even after 30 days of homocysteine administration (Fig. 1B). Administration of homocysteine however affected cortical bone thickness. We found a reduction in cortical bone thickness as early as day 7 (Fig. 1C).

On day 15, cortical bone thickness increased, but the change was not statistically significant. By day 30, HH CY bone samples showed an increase that was statistically significant ($P=0.012$) with respect to HH CY day 7 bone samples. Figure 1D shows distribution of closed pores in cortical bone. Figure 1E shows changes in the volume of closed pores in the given volume of bone. The density of closed pores (pores excluding all vascular channels) was increased up to 17% in HH CY day 7 bone. On day 15 a decline in closed pore density was observed, however, a statistically significant difference ($P=0.014$) was found only on day 30 (HH CY day 30 vs HH CY day 7 samples). Total cortical porosity represents all the space in the cortical bone not filled by the mineral. An increase in total cortical porosity was observed in HH CY day 7 bone samples, but the effect was not statistically significant (Fig. 1F). Total cortical porosity was not affected on day 15 and significant difference ($P=0.04$) in cortical porosity was observed in HH CY day 30 bones and vehicle control (Fig. 1F).

Figure 1G shows changes in cortical tissue mineral density (TMD) by microCT50 among groups studied. The cortical TMD of HH CY day 7 bone samples was significantly lower ($P=0.04$) compared to that of vehicle control. Cortical TMD was observed to re-establish with time. There was a statistical increase in cortical TMD in HH CY day 30 bone compared to that in day 7 bones. No significant difference in TMD existed between HH CY day 30 and vehicle-treated mouse cortical bones. Changes in mineral density were also assayed by evaluating the % ash content of whole femurs (Fig. 1H). By weight, the ash content was lower ($P=0.012$) in HH CY day 7 bone sample compared to bones from vehicle-treated mice (Fig. 1H). In HH CY day 30 samples, an increase in ash content was observed with respect to HH CY day 7 group. No significant difference in ash content was seen between bones from HH CY day 30 group and vehicle control. Ca and P levels in these bones were measured separately after digestion (Fig. 1I). The levels of Ca and P in bone ash were significantly lower ($P=0.013$) in HH CY day 7 bone samples as compared to those in vehicle control. HH CY day 30 bone samples showed significant higher ($P=0.012$) level of Ca and P as compared to HH CY day 7 bone samples (Fig. 1I). The mineral content of bone samples were also evaluated by Alizarin red staining (Fig. 1J). Alizarin S staining of HH CY day 7 bone samples showed a weak staining as compared to vehicle-treated mouse bones (Fig. 1J). HH CY day 30 samples showed higher staining as compared to HH CY day 7 bones, and intense staining was observed in few areas of bone cortex.

Changes in osteocyte numbers in cortical bone with administration of homocysteine

H&E staining of cortical bone sections showed a significant increase ($P=0.01$) in the average number of nucleated osteocyte lacunae (Av.Ot.N) in HH CY day 7 bone as compared to vehicle-treated bone samples (Fig. 2A). In HH CY day 30 bone, Av.Ot.N was significantly lower ($P=0.01$) as compared to vehicle control.

The average number of empty lacunae (Av.Ot.Lac) was lower in HH CY day 7 cortical bone ($P=0.01$) as compared to vehicle control. In HH CY day 30 bone samples, there was a significant rise in Av.Ot.Lac as compared to HH CY day 7 bone samples and vehicle control (Fig. 2A).

Osteocyte lacunar density is established as a common proxy for determining osteocyte density in techniques such as microCT50. Results showed that HH CY day 7 bone samples possessed higher density of osteocyte lacunar pores of sizes between 200 and 300 μm^3 as compared to vehicle control (Fig. 2B). HH CY day 30 cortical bone samples showed lacunar density similar to that of vehicle-treated mouse cortical bones. Bone samples retrieved at day 15 demonstrated a gradation indicating that osteocyte lacunar pores indeed reverted toward baseline values (Fig. 2B).

Changes in vascular pores in cortical bone with administration of homocysteine

Vascular pores on the cortical bones were visualized by S.E.M. Low power S.E.M. analysis showed that though the number of vascular pores on endosteal surface of the cortical bone did not change with administration of homocysteine on day 7 after homocysteine administration (Fig. 2Ca and d), the size of vascular pores in these cortical bones were significantly larger ($P=0.011$) with respect to vehicle control group (Fig. 2Cb and c). The area of vascular pores in HH CY day 30 bone samples did not vary with vehicle control bone samples. There was a significant difference ($P=0.012$) in the number of vascular channels between HH CY day 30 and vehicle control group (Fig. 2Cd).

Homocysteine mediated changes in osteocyte expressing genes in cortical bone

The mRNA levels of genes like *Pdpr*, *Dmp1*, *Bglap*, *Phex*, *Ahsg*, *Sost*, *Runx2* and *Tnfsf11* in vehicle and homocysteine-treated bones analyzed by qPCR and represented as fold

Figure 2

Changes in osteocyte population, osteocyte lacunar density and open pores in cortical bones during HHcy. (A) H&E-stained cortical bone sections showing changes in osteocyte numbers, scale=200 μm. Av.Ot.N, average number of nucleated osteocytes; Av.Ot.Lac, average number of empty lacunae. Quantitative analysis of these parameters were done based on $n=100$ unit measurements per sample. (B) MicroCT50 analysis showing changes in osteocyte lacunae pores (200–300 μm³). (C) Scanning electron microscopy microphotographs showing changes in vascular open pores, scale=2 μm. (a) Changes in vascular pores at lower s.e.m. magnification. (b) Changes in vascular pores at higher s.e.m. magnification. (c) Changes in size of open blood vessels in cortical bone between vehicle and HHcy groups. Quantitative analysis of this parameter was done based on $n=20$ unit measurements per sample. (d) Changes in the average number of open blood vessels in cortical bone between vehicle and HHcy groups. Quantitative analysis was done based on $n=100$ unit measurements per sample. Values are mean ± s.e.m.; $n=4$. *Statistical significance with vehicle control; **statistical significance with HHcy day 7 bone. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-16-0562>.

changes are shown in Fig. 3A. We observed a significant increase in mRNA levels of all these genes only on day 30 after homocysteine administration. The following changes were observed: *Pdpr* showed 2.5-fold increase ($P=0.012$), *Dmp1* showed 4.5-fold increase ($P=0.01$), *Sost* showed 4.3-fold increase ($P=0.016$), *Phex* showed 3-fold increase ($P=0.012$), *Ahsg* showed 6-fold increase ($P=0.013$), *Col1a1* showed 2-fold decrease ($P=0.016$), *Tnfrsf11* showed 2-fold increase ($P=0.012$) and *Runx2* showed 3-fold increase ($P=0.011$) in gene expression with respect to vehicle control. No significant changes in mRNA levels of these genes were found on HHcy day 7 bone samples with respect to vehicle control except *Bglap*. The mRNA level of *Bglap* was seen to progressively increase with time (Fig. 3A).

Changes in the protein levels of sclerostin and Dmp1 in cortical bone with administration of homocysteine

Vehicle control bone showed positive immunostaining for Sost and Dmp1 inside osteocyte lacunae by immunohistochemistry (IHC) confirming cells as osteocytes (Fig. 3). Positive immunostaining for sclerostin was not found in the osteoid matrix of vehicle control (Fig. 3Ba). No significant changes in the number of sclerostin-positive osteocyte lacunae were seen between groups (Fig. 3Bd). However, significant changes in the intensity of immunostaining were observed between groups. HHcy day 30 bone samples also showed the highest immunostaining for sclerostin per osteocyte (Fig. 3Be). The negative control did not show any sclerostin positive osteocyte lacunae.

Figure 3

Changes in osteocyte markers during HHcy. (A) Changes in mRNA levels of genes by quantitative PCR. Data are shown as fold-changes with respect to vehicle control and mean of three independent PCR experiments done in triplicate normalized to expression of beta-actin. (B) Immunohistochemical staining of cortical bone sections. (a) Sclerostin, (b) Dmp1 and (c) cleaved caspase 3. Scale = 150 μ m. (d) Histogram showing changes in average number of Sost⁺, Dmp1⁺ and cleaved caspase⁺ osteocyte lacunae population among groups. (e) Histogram showing mean intensity of Sost, Dmp1 and cleaved caspase 3 immunostaining per osteocyte. Quantitative analysis was done based on $n=50$ unit measurements per sample. (C) Changes in carboxylated osteocalcin and sclerostin in serum by ELISA. Values are mean $\pm$ S.E.M.; $n=4$. *Statistical significance with vehicle control; **statistical significance with HHcy day 7 bone.

IHC analysis of HHcy day 7 samples showed a rise in the number of Dmp1-positive osteocytes (Fig. 3Bd).

HHcy day 30 samples showed the same number of Dmp1-positive osteocytes as vehicle control (Fig. 3Bd). However, the intensity of Dmp1 immunostaining per osteocyte was significantly higher ($P=0.02$) in HHcy day 30 bones as compared to HHcy day 7 samples (Fig. 3B). Negative controls did not show any Dmp1 immunostaining.

Changes in the protein levels of apoptotic marker in cortical bone with administration of homocysteine

Cleaved caspase-3, an apoptotic marker, showed a slow yet time-dependent increase in caspase-3 immunostaining upon HHcy. The labeling indices were ~10% in HHcy day 7 bone samples and ~49% in HHcy day 30 samples (Fig. 3Bc and e). The negative antibody control did not show caspase-3-positive staining (Fig. 3Bd).

Changes in carboxylated osteocalcin and sclerostin in serum of mice upon HHcy

Analysis of the level of carboxylated osteocalcin in serum showed that the protein peaked on day 7 of homocysteine administration and returned toward baseline values on day 30 (Fig. 3C). A statistical difference in serum osteocalcin was found between HHcy day 7 and HHcy day 30 mice.

Serum sclerostin was found to be significantly high ($P=0.01$) only in the late phase of HHcy (Fig. 3C). No significant change in serum sclerostin level was observed between HHcy day 7 mice and vehicle control.

Homocysteine induced changes in the biomechanical stability of mouse cortical bone

The integrity of HHcy bones was tested via biomechanical testing (Fig. 4). All parameters relating to biomechanical testing like load, stress, strain and Young's modulus were

Figure 4

Changes in mechanical property of the bone after HHCY induction. (A) Ultimate load, (B) ultimate stress, (C) ultimate strain and (D) Young's modulus. Values are mean $\pm$ S.E.M.; $n=4$.

*Statistical significance with vehicle control;

**statistical significance with HHCY day 7 bone.

low on day 7 and did not show any significant change from this status with time. Results showed that prior to the fracture, the ultimate load (N) that correlates to the stiffness of the bone was significantly low ($P=0.011$) for both HHCY day 7 and day 30 bones as compared to their respective age-matched vehicle controls (Fig. 4A). The ultimate stress that relates to the mineral content of the bone was also significantly lower ($P=0.011$) in HHCY day 7 and day 30 bone samples with respect to their age-matched controls (Fig. 4B). In concordance to ultimate load and ultimate stress, ultimate strain that is directly proportional to the collagen content was also significantly low ($P=0.01$) in HHCY day 7 and even lower on day 30 samples when compared to age-matched vehicle controls (Fig. 4C). Young's modulus that is stress by strain was significantly lower ($P=0.01$) on day 7 and day 30 HHCY bones when compared to their corresponding age controls (Fig. 4D).

Discussion

Literature cites only few examples that link homocysteine with bone fragility and the most modern and accredited one is that of Thaler and coworkers (Thaler *et al.* 2011). The study demonstrated that suppression of Lox or 'lysyl oxidase', a collagen cross-linker enzyme by homocysteine via CpG methylation, is a reason for loss of bone matrix quality during HHCY. Conversely, experimental and clinical studies that investigated whether HHCY affects bone mineral density have not yet yielded any conclusive results and the question whether homocysteine affects bone mineralization or not still remains unanswered (van Meurs *et al.* 2004, Gjesdal *et al.* 2006, Bucciarelli *et al.* 2010).

In the current study, we investigated whether homocysteine affected tissue mineral density (TMD) of cortical bone and the role of osteocytes in this regard. Femoral cortices (and not the whole bone) harvested from mice after 7, 15 and 30 days of homocysteine administration demonstrated temporal changes in tissue mineral density (TMD) and osteocyte lacunar density. The changes were dynamic that transpired via two phases: (i) an 'early phase' that involved loss of TMD and increase in osteocytogenesis and (ii) a 'late phase' that involved osteocyte reprogramming and reinstatement of TMD. The decline in cortical TMD after 7 days of homocysteine administration shows that cortical bone is highly vulnerable to increase in circulatory homocysteine. Nonetheless, the decline in TMD upon homocysteine exposure (on day 7) was not permanent, and TMD innately reinstated with time to baseline values (on day 30) without improving the biomechanical property of cortical bone. The major questions we addressed at this point were (a) how did TMD re-establish with time and (b) why the biomechanical property of HHCY day 30 bone was not improved with increase in TMD?

Molecular analysis by real-time PCR and IHC showed that HHCY day 30 bone displayed increased mRNA levels of osteocyte marker genes such *Pdpn*, *Dmp1*, *Sost*, *Ahsg*, *Phex*, *Bglap*, *Colla1*, *Tnfrsh11* and *Runx* and high protein levels of *Sost* and *Dmp1* in the osteocyte lacunae. According to literature, these changes denote anomalous osteocyte maturation and possible induction of lacunar mineralization (Busse *et al.* 2010). We also found that HHCY day 30 bone samples exhibited an increase in the number of *Dmp1*⁺ osteocytes when available mineral content was low. Interestingly, as *Dmp1* expression increased significantly, TMD of cortical bone was restored.

It is well documented that under conditions of low mineral availability, mineral crystals 'grow' instead of 'forming newer crystals' as the former is energetically favorable (Boskey 2001). In the bone, such growth processes are mediated by certain proteins of the SIBLING (small integrin-binding ligand N-linked glycoprotein) family like Dmp1 (He *et al.* 2004, Qin *et al.* 2004, Ling *et al.* 2005). Dmp1 is a hydroxyapatite nucleator with high affinity for calcium and apatite. The protein is naturally secreted to extracellular matrix during bone formation and plays a predominant role in the initial phase mineralization and osteocyte maturation by regulating phosphate homeostasis (Asamiya *et al.* 2016). However, when overexpressed, Dmp1 can increase mineralization and induce bone crystallinity, which compromises bone quality (Bhatia *et al.* 2012). In the HHcy day 30 bone, the improvement in biomechanical stability may not have occurred owing to untoward mineralization caused by overexpression of Dmp1. Such changes were however time based and may be the reason for incongruities between experimental and clinical data and more importantly among clinical data. At the late stage of HHcy, cortical TMD resembled vehicle control though molecular and biomechanical analyses proved otherwise.

The other notable finding in HHcy bone was the induction in *Phex* and *Ahsg* gene expressions alongside high Dmp1 gene expression in late HHcy bone. *Phex* and *Ahsg* are physiological regulators of Dmp1 expression and mineralization in bone. Fundamentally, *Phex* is a metalloproteinase that binds to MEPE (a SIBLING member), an inhibitor of Dmp1 while *Ahsg* or FetuinA (originally discovered in the liver) is a regulator that slows down the process of calcification in matrix region surrounding developing osteocytes (Set *et al.* 2012, Rowe 2013). Our presumption is that the high expression of regulatory genes such as *Phex* and *Ahsg* occurred in the late HHcy conceivably to avert overgrowth of minerals owing to low mineral availability in the milieu.

Another protein that was seen to be upregulated alongside Dmp1 in the osteocyte lacunae of HHcy bone was Sost or sclerostin. Both mRNA and protein levels of sclerostin were found to be increased in the late phase of HHcy indicating that the protein stabilized during HHcy. The simultaneous expressions of Sost and Dmp1 have been previously reported in mineralizing bone scenarios (Sutherland *et al.* 2004, Atkins *et al.* 2009, Ryan *et al.* 2013). Besides its role in mineralization, sclerostin has a role on bone remodeling process as well. Sclerostin produced by osteocytes acts a paracrine signal for inhibiting bone

formation. Sclerostin produced by osteocytes also elicits RANKL, a potent stimulus that induces osteoclastogenesis (Wijenayaka *et al.* 2011). Herein, we observed disturbed cortical bone remodeling status got disturbed in the late phase of HHcy when sclerostin expression was maximum. There was an apparent decline in the level of serum-carboxylated osteocalcin and induction in *Tnfsf11* gene (or RANKL) when Sost expression was maximum i.e. the late phase of HHcy. The amount of carboxylated osteocalcin that appears in circulation is normally the protein that escapes from the bone during bone remodeling process. Carboxylated osteocalcin therefore is a clinical marker that represents bone formation status (Rathore *et al.* 2016). RANKL is a protein secreted by differentiating osteoblasts and osteocytes that bind to RANK receptor on pre-osteoclasts to induce differentiation (Wijenayaka *et al.* 2011). From the results obtained, it is obvious that HHcy hampered normal bone homeostasis via sclerostin, a protein with potential implications on bone remodeling. An interesting point to note here is that HHcy induced sclerostin both in osteocyte lacunae as well as circulation. However, unlike serum carboxylated osteocalcin that directly correlates with bone formation, sclerostin in circulation is not a direct reflection of the changes occurring in cortical bone. Nonetheless, sclerostin is related to erroneous phosphate metabolism involving Dmp1-FGF23, which are osteocyte by-products and therefore represent a functional status of bone (Feng *et al.* 2009, Asamiya *et al.* 2016).

A notable sequential change that occurred in the HHcy bone, which also related TMD with osteocyte, was the concomitant rise in the number of empty lacunae upon reinstatement of TMD. Studies have shown that disappearance of osteocyte from lacunae elicits mineralization in the lacuna and induces filling by a process referred to as fossilization or micropetrosis (Frost 1960, Busse *et al.* 2010). Klein-Nulend and Bonewald (2008) reported that lacunar mineralization can reduce the energy-absorbing/-dissipating capacity of bone. A major limitation of this study is that we were not able to employ quantitative backscattering electron imaging as suggested by Carpentier and coworkers (Carpentier *et al.* 2012) to validate lacunar occlusions. Nonetheless, typical changes that occurred in the cortical bone during late HHcy such as (i) increase in TMD by microCT50, Alizarin staining, calcium and phosphorus estimation and % ash content; (ii) overall reduction in osteocyte lacunar density by microCT50; (iii) disappearance of nucleated osteocytes and appearance of empty lacunae by H&E staining; (iv)

induction of atypical osteocyte proteins like sclerostin and Dmp1 in osteocyte lacunae by immunohistochemistry and (v) increase in osteocyte apoptosis by caspase 3 staining are information adequate enough to understand that lacunar mineralization is elicited upon HHcy.

In 2011, Carpentier and coworkers demonstrated that accumulation of apoptotic osteocytes hastens bone mineralization. Fundamentally, osteocyte apoptosis occur in response to injury, toxic stimuli, loss of cellular attachment, trophic signals, stress, total hip arthroplasty, withdrawal of estrogen, alcohol induction, fatigue and glucocorticoid therapy (Weinstein *et al.* 2000). We identified a significant increase in the number of cleaved caspase 3+ apoptotic osteocytes during late phase of HHcy. Caspase 3 denotes dying osteocytes and not osteocyte remnants. Erstwhile studies have shown that apoptotic osteocytes are not 'debris' but vital regulators of 'targeted remodeling,' which signal neighboring cells or 'by-stander cells' to release RANKL, VEGF, ATP, sphingosine-1-phosphate and chemokines for endothelial cell activation and recruitment of osteoclasts and osteoblasts to the site of injury to facilitate repair via BMU-mediated remodeling process (Weinstein *et al.* 2000, Cardoso *et al.* 2009). Herein, we noted a time-dependent increase in cleaved caspase 3-positive apoptotic osteocytes indicating that homocysteine-mediated changes in osteocyte turnover were rather slow.

There are also reports that associate osteocyte density with biomechanical quality of the bone (Qiu *et al.* 2005, Busse *et al.* 2010). Our results showed that HHcy reduced mechanical properties of the cortical bone as evidenced by decreased Young's modulus, ultimate stress and ultimate load parameters in HHcy bone with respect to vehicle control. The changes occurred at the early stage of HHcy itself and did not recover even though osteocyte lacunar density and TMD reinstated toward baseline values. These results pinpoint that reinstatement of lacunar density and TMD alone cannot recuperate bone quality. Qiu and coworkers (Qiu *et al.* 2005) demonstrated that osteocyte density correlates with the initiation and propagation of microdamage. Osteocyte lacunae and in particular empty lacunae serve as triggers for microcrack initiation. When osteocyte density decreases, the length of cracks in bone due to mechanical insults increases. In addition, the canalicular fluid flow decreases and cuts the ability of bone to detect microdamage (Busse *et al.* 2010). From the results obtained herein, we strongly believe that apart from changes such as incongruous distribution of mineral, presence of empty lacunae and occurrence of osteocyte death, 'reduction in viable osteocytes' is another

important factor that alters the biochemical properties of the bone. A point that deserves mention is that the 'ultimate stress' parameter in biomechanical testing that corresponds to collagen content was not further changed with time upon HHcy though molecular analysis showed that Col1a1 mRNA level decreased upon HHcy. Previously Lubec and coworkers (Lubec *et al.* 1996) stated that alterations in post-translational modification of collagen induce bone fragility during HHcy. HHcy bones also show weak collagen staining (Vijayan *et al.* 2013). We are not sure why ultimate stress parameter did not decline further especially when Col1a1 level decreased during HHcy. Further investigations are required to investigate why alterations in collagen content did not further weaken the mechanical property of HHcy bone.

To summarize, HHcy alters three facets of the cortical bone geometry: its porosity, mineral density and biomechanical strength. Interestingly, osteocytes are associated with all these three aspects. HHcy affects cortical pore density by changing both the 'closed pore' as well as the 'open pore' populations in the cortical bone. The open pores that include vascular channels like Haversian system were studied using s.e.m. During the early phase of HHcy, vascular pores did not increase in number but only in size. With time, both reduction in vascular pore sizes as well as overall reduction in density of vascular pores were seen in HHcy bone. Likewise, HHcy also affected the closed pore population, i.e., osteocyte lacunae (segregated from open pores by size). Lacunar density increased during early HHcy and reverted to baseline values in late HHcy. Taking both results into consideration, it can be concluded that decline in open pores is the reason for the reduction in cortical porosity during late HHcy bone. The osteocyte lacunar population at large may not affect the overall cortical porosity, but the sustainability of osteocyte lacunae affects the tissue mineral density of cortical bone. A greater percentage of empty lacunae and reduced population of nucleated osteocyte lacunae in HHcy bone alongside high expression of Dmp1, Sost and caspase 3 in osteocyte lacunae are reasons that perturb the biomechanical property of bone during HHcy. It was interesting to find how temporal changes in tissue mineral density in HHcy bone resulted in generation of SOS signals, which in this case is 'osteocyte apoptosis' that can trigger a new set of events. Thus, the late phase of HHcy that appears 'inactive' with normalized cortical porosity, thickness and mineralization is otherwise 'active' with osteocyte reprogramming and osteocyte apoptosis.

To conclude, HHcy induces pathogenesis in cortical bone. The effect is mediated by osteocytes through (i)

anomalous expression of mineralization genes such as Dmp1 and Sost and (ii) induction of apoptosis. Both features are reported causes of osteocyte lacunar mineralization and interestingly in the HHCY bone, both features occur after a decline in TMD, which means decline in cortical TMD is the initial step in bone pathogenesis during HHCY. After decline in TMD, anomalous expression of osteocalcin-specific genes by osteocytes and osteocyte apoptosis induce mineralization that affects the biomechanical stability of bone. Our findings thus provide an interesting avenue for future research into the role of osteocytes in disease-mediated changes in bone mineralization.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S G funded this work and corrected the manuscript. V V designed the study, performed experiments, interpreted the data and wrote the manuscript.

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