Research article

Chemical and biomechanical characterization of hyperhomocysteinemic bone disease in an animal model

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Abstract

Background: Classical homocystinuria is an autosomal recessive disorder caused by cystathionine β-synthase (CBS) deficiency and characterized by distinctive alterations of bone growth and skeletal development. Skeletal changes include a reduction in bone density, making it a potentially attractive model for the study of idiopathic osteoporosis.

Methods: To investigate this aspect of hyperhomocysteinemia, we supplemented developing chicks (n = 8) with 0.6% dl-homocysteine (hCySH) for the first 8 weeks of life in comparison to controls (n = 10), and studied biochemical, biomechanical and morphologic effects of this nutritional intervention.

Results: hCySH-fed animals grew faster and had longer tibiae at the end of the study. Plasma levels of hCySH, methionine, cystathionine, and inorganic sulfate were higher, but calcium, phosphate, and other indices of osteoblast metabolism were not different. Radiographs of the lower limbs showed generalized osteopenia and accelerated epiphyseal ossification with distinct metaphyseal and suprametaphyseal lucencies similar to those found in human homocystinurics. Although biomechanical testing of the tibiae, including maximal load to failure and bone stiffness, indicated stronger bone, strength was proportional to the increased length and cortical thickness in the hCySH-supplemented group. Bone ash weights and IR-spectroscopy of cortical bone showed no difference in mineral content, but there were higher Ca²⁺/PO₄³⁻ and lower Ca²⁺/CO₃²⁻ molar ratios than in controls. Mineral crystallization was unchanged.

Conclusion: In this chick model, hyperhomocysteinemia causes greater radial and longitudinal bone growth, despite normal indices of bone formation. Although there is also evidence for an abnormal matrix and altered bone composition, our finding of normal biomechanical bone strength, once corrected for altered morphometry, suggests that any increase in the risk of long bone fracture in human hyperhomocysteinemic disease is small. We also conclude that the hCySH-supplemented chick is a promising model for study of the connective tissue abnormalities associated with homocystinuria and an important alternative model to the CBS knock-out mouse.
Background

Classical homocystinuria is an inborn error of sulfur aminoacid metabolism caused by cystathionine β-synthase (CBS) deficiency [1]. The excess homocysteine (hCySH) that accumulates because of this defect is exported into the circulation and spills into the urine. In humans, CBS deficiency manifests as a distinctive spondylo-epimeta-physal dysplasia, characterized by accelerated skeletal growth, osteopenia, elongated appendicular skeleton and flattening of the vertebral bodies [2,3]. The overall risk of osteoporosis has been reported to be 50% by age 16 [4], and bone mineral density measured by dual X-ray absorptionmetry is reduced in affected children [5]. However, there are no good estimates of the frequency or severity of fractures, and interactions with late-onset physiologic bone loss have not been reported. Some of the osseous manifestations are attributable to a disturbance in collagen cross-links, but there is still little known about the molecular mechanisms generating the dysmorphic skeletal phenotype [6], and the CBS-deficient knock-out mouse offers few clues. [7]. The role of intermolecular collagen cross-links in bone has been adduced in part from studies of lathyrism caused by the compound, β-amino-propionitrile (BAPN), found in the sweet pea (Lathyrus odorum). BAPN irreversibly inhibits lysyl oxidase, and blocks initial collagen cross-link formation [8,9].

The fast-growing chick has been introduced as an animal model for detailed study of soft connective tissue abnormalities resulting from controlled perturbations of the transsulfuration pathway [10–12]. In animals receiving aminoacetoniitrile, a less toxic analog of BAPN, electron microscopy of long bones shows significant enlargement of Type I collagen fibrils while a similar but less severe abnormality is observed in vitamin B6-deficiency and in hyperhomocysteinemia induced by hCySH added to the diet [13]. Changes in collagen solubility and collagen cross-linking are hallmarks of connective tissue changes associated with these dietary manipulations [14–16].

The mechanical integrity of bone is dependent on both collagenous matrix (which allows for plastic deformation of the tissue) and mineral (which allows for the elastic deformation) [17]. Lathyrism induced by BAPN affects both components of bone [18–21]. In our hands, vitamin B6 deficiency has been a useful alternative model to study the effect(s) of altered intermolecular collagen cross-links on whole bone, because the mineral component is unaltered [15]. In growing chicks fed a B6-deficient diet, there were no changes in bone length, bone diameter, or moment of inertia, but mechanical performance was altered. Moreover, histomorphometric and radiological studies showed low-turnover osteopenia [14].

Table 1: Basal diet

| Constituents | Composition (w/w %) |
|--------------|---------------------|
| Soyamin      | 23.40               |
| Corn         | 33.00               |
| Cerelose (glucose monohydrate) | 28.00 |
| Corn Oil     | 4.90                |
| Mineral salts2 | 5.35               |
| Vitamins3    | 2.00                |
| Trace minerals4 | 2.00            |
| dl-homocysteine | 0.30             |
| Glycine      | 0.30                |
| Choline chloride | 0.15             |

1 Metabolizable energy calculated as 13.8 MJ (3000 kcal) per kg diet.
2 Salt mixture (g/kg diet): CaHPO4, 20.0; CaCO3, 15.0; KH2PO4, 11.0; NaCl, 4; MgSO4, 3.5. 3 Vitamin premix (mg/kg diet): retinyl palmitate, 5.49; cholecalciferol, 0.016; all-rac-α-tocopherol acetate, 55.0; mendione sodium bisulfite, 1.52; thiamine • HCl, 1.5; nicotinic acid, 50; pyridoxine • HCl; 3; calcium-d-pantothenate, 20; cyanocobalamine, 0.02; folic acid, 6.0; biotin, 0.6. 4 Trace minerals (mg/kg diet): C24H22FeO4 • 2H2O, 700; MnSO4 • 7H2O, 170; ZnO, 65; CuSO4 • 5H2O, 20; Na2MoO4 • 2H2O, 5; CoCl2 • 6H2O, 1.5; Na2SeO3 • 5H2O, 0.5; KI, 0.7.

The objectives of the present study were to investigate the radiologic, biochemical, and biomechanical properties of bone in the growing chick made hyperhomocysteinemic by excess dietary hCySH and to evaluate those changes in relation to skeletal abnormalities characterizing human CBS-deficiency homocystinuria.

Methods

Animals and diets

Male day-old broiler chicks purchased from the Miami International Hatchery (Miami FL) were randomly assigned to control (n = 10) or experimental (n = 8) groups. They were fed normal diet and diet supplemented with 0.6 % w/w of dl-homocysteine, respectively, over eight weeks. Both diets were supplied by ICN Pharmaceuticals (Aurora, OH) and were identical in terms of protein and essential amino acid content. Both were also identically supplemented with vitamins and minerals to fulfill all nutritional requirements, including specific needs for vitamin B6, a coenzyme in the transsulfuration pathway, and menadione (vitamin K), which is essential for the synthesis of osteocalcin, involved in bone formation (Table 1). The vitamin D intake was kept constant and the Ca:P molar ratio optimal for chick bone growth was identical in each diet. The dl-hCySH supplement (98% pure) was obtained from Sigma (St. Louis MO).

The animals were kept in a temperature-controlled environment and fed ad libitum and a 12 hr constant light cycle was maintained. Chicks were weighed at the beginning of
the experiment, and then on a weekly basis, and the growth curve was used to monitor the health status throughout the experiment. Experimental procedures were reviewed and approved by the research animal care committee of VA Medical Center, Miami FL, in accordance with current National Institutes of Health policies.

**Blood biochemistry**

At the end of the eight week experiment, samples of fasting blood were collected by jugular venipuncture using heparin or EDTA. The blood was centrifuged and plasma collected for analysis. Erythrocytes were resuspended in an equal volume of isotonic saline and centrifuged. The heparin supernatant was discarded and the erythrocytes analyzed for pyridoxal-5'-phosphate (PLP) according to the methods of Mahuren and Coburn [22]. HPLC with pulsed integrated amperometry was used to measure total hCySH, methionine, cystathionine, and glutathione, as previously described [23]. Plasma free inorganic sulfate was measured by microassay using controlled-flow anion chromatography [24,25]. Plasma calcium, phosphate and bone alkaline phosphatase (ALP: orthophosphoric monoester phosphohydrolase, alkaline EC 3.1.3.1) were assayed on an automated COBAS-BIO autoanalyser (Hoffmann LaRoche, Switzerland). Plasma ALP bone isoenzyme was determined by using bromotetramisole as inhibitor [26]. Plasma osteocalcin, an index of bone formation, was measured in serially diluted samples of plasma by radioimmunoassay [27,28] Circulating IGF-1 level was measured by radioimmunoassay, as described by Zhao et al. [29].

**Bone measurements, radiographic evaluation, and mechanical testing**

The chick tibiotarsus bone (the tibia) was selected for further analysis because it is known to be the most rapidly growing and, by inference, the bone most susceptible to mechanical stress [30]. Immediately after sacrifice by cervical dislocation, left tibias were dissected, cleaned of soft tissue, wrapped in saline-soaked gauze, and stored at -70°C until testing. Radiographic and biomechanical studies were followed by chemical analyses of mineral and collagen composition.

Whole bones were thawed after 10–20 days of storage (a procedure known not to affect bone biomechanical properties) and maintained in a wet condition while they were weighed and their length measured. They were then immediately tested with 3-point bending, anterior cortex on the tensile side with the 3-point contact centered on the mid-shaft of the bone [31–34]. These tests were performed in ambient air on an MTS closed-loop servohydraulic apparatus at a displacement rate of 6.25 mm/sec. Testing to failure was performed in the medio-lateral plane, using an outer support span of 33 mm and an inner span of 11 mm. Load versus displacement of the loading ram was recorded on computer. Specimens were selected randomly for testing and were kept wet with distilled water throughout testing. The whole bone properties of fracture load were determined directly from the load vs. displacement curve for each specimen. Prior to failure testing, each specimen was loaded elastically to 5.62 N at a constant rate of 2.81 N/sec until fracture. Tibias were radiographed (60 kV, 4.5 min) before and after fracture in the anterior-posterior (AP) and medial-lateral (ML) planes.

Typical load/deflection curves with both the elastic (linear) and plastic components separated by the yielding point as described by Burstein et al. [17] were obtained, enabling graphic determination of the following mechanical properties: maximal load, bone stiffness, and energy at failure. To consider the influence of diaphyseal geometrical variation on whole bone mechanical properties, inner endosteal and outer periosteal AP (antero-posterior) and ML (medio-lateral) diameters on X and Y axes, respectively, of the tibial fracture section were measured from the radiograph by digital caliper. Two geometric characteristics – moment of inertia of the cross-section in relation to the horizontal axis ($I_x$) and cross-sectional area (A) – were calculated as shown in Figure 1. Moment of inertia is an indicator of the architectural efficiency of the tibial cross-sectional design [33].

**Bone evaluation: mineral and matrix composition**

Following mechanical testing, bone samples from the fracture site were lyophilized, ground in a Spex freezer mill (Metuchen, NJ) at liquid nitrogen temperature, and three sets of aliquots set aside for mineral analyses. For analysis of mineral content, triplicate aliquots of 2 to 4 mg
each were transferred to weighed dry crucibles. Dry weight was determined following heating to constant weight at 110°C. Ash weight was determined following heating to constant weight at 600°C. Mineral content was calculated as the ratio of ash weight to dry weight. The ash was dissolved in 1 N HCl, as described for our vitamin B₆ deficiency model [15]. To calculate the Ca²⁺/PO₄³⁻ ratio, the calcium content was measured by atomic absorption spectrophotometry and the phosphate concentration determined spectrophotometrically.

Aliquots of ground bone obtained near the fracture site were also subjected to wide-angle X-ray diffraction using CuKα radiation. The line width at half-maximum of the c-axis 002 reflection was measured as an index of crystalline size and perfection [35]. Each assay was repeated in triplicate. Two milligram aliquots of ground bone were mixed with 200 mg KBr, and pellets prepared for infrared spectroscopy. The pellets were analysed at 4 cm⁻¹ resolution on a Mattson Cygnus Fourier Transform Infrared (FT-IR) spectrometer. The spectra were analyzed to provide information on the relative mineral to matrix ratio; that is, the ratio of integrated areas of the phosphate ν₁, ν₃ mode (900–1200 cm⁻¹) to the amide I band (1580–1650 cm⁻¹) and the carbonate (840–890 cm⁻¹) to phosphate (900–1200 cm⁻¹) ratio [15].

An additional bone sample was dissected from the diaphyseal section, ground as described above, and aliquots used for analysis of hydroxyproline to estimate collagen content [36]. Hydroxyproline analysis was performed on HCl hydrolysates (6 N, 18 h, 110°C) of EDTA-demineralized ground bone.

**Data presentation and statistical analysis**

Data are reported as mean ± standard deviation (SD) for 8 experimental and 10 control animals. Differences between groups for biochemical data and biological, chemical and biomechanical parameters of bone were assessed by unpaired Student t-test, or non-parametric Mann-Whitney test when data displayed a significantly non-Gaussian distribution. The Bartlett test was used to exclude heteroscedasticity. The two-tailed significance level was set at 0.05. Analysis of covariance was used for bone biomechanical data to adjust for statistical differences in biological variables.

**Results**

Charted body weights of experimental and control chicks from day 1 to 8 week-old are shown in Figure 2. Mean weights of hCySH-fed chicks increased at a faster rate than in the controls, and were significantly (P < 0.01) greater at the end of the experiment. Tibial weights in animals fed a hCySH-rich diet were proportionally (12%) greater compared to controls, but the difference did not reach a significance (Figure 2 – Inset A); however, the positive difference for length (Figure 2 – Inset B) was significant (P < 0.01).

Table 2 gives biochemical data regarding sulfur amino acid and mineral metabolism for each group. Mean plasma total hCySH concentration in the experimental group was 8 times that of controls (P < 0.0001) and two other sulfur amino acids of interest, cystathionine and methionine, were also increased significantly (P < 0.0001 and P < 0.001, respectively). Cystathionine concentrations were elevated and there was a 30% increase in plasma inorganic sulfate (SO₄²⁻) (P < 0.001) in the experimental group under otherwise identical dietary sulfur conditions. However, there was no evidence for disturbance of glutathione metabolism, which is regulated independently of the transsulfuration pathway. There was also no difference in erythrocyte PLP levels. Methionine was increased by nearly 20%, indicating functional remethylation characteristic of CBS-deficient homocystinuria.

Plasma calcium and phosphate were normal in both groups (Table 2), and markers of bone formation – osteocalcin and bone specific ALP – did not vary significantly. Circulating IGF-1 was also not different, and values in both groups are comparable to chick data in the literature [35].
Radiologic AP views of distal tibia from representative control (N) and hCySH-fed animals are seen in Figures 3A and 3B. It can be seen that there is better visualization of the trabeculae in control bone, while the hCySH-treated bone showed less radio-opacity. A well-defined radiolucency overlying the proximal metaphysis is seen in the hCySH-treated bone. Figures 3C and 3D are representative lateral views of whole tibia after loading to failure. At the proximal epiphysis of tibia are the Tuberositas tibia and Cristae tibiae protuberances, to which the frequently ossified Ligamenta recta patellae are attached. At the distal epiphyses, the two joint ridges corresponding to the Ossa tarsi can be distinguished. The ovoid lucency visible in the metaphysis (black arrow) corresponds to an unmineralized, avascular collagen "plug", typical of chondrodysplasia seen on histological analysis (unpublished observation). In hyperhomocysteinemic animals (Figures 3B and 3D), the presence of multiple transverse lines of increased density at both proximal and distal ends (horizontal arrows) suggests more advanced ossification of the epiphyses. Increased cortical thickness is evident in both AP and LM views of hCySH-treated bone, particularly in the upper diaphyses. At the fracture site (Figure 3D), the jagged rupture pattern suggests that the bone from the hyperhomocysteinemic animal is more brittle than the control (Figure 3C).

As expected, AP outer (periosteal) diameters (Y axis) of the tibiae at mid-shaft were significantly greater than LM outer diameters (X axis) in both control (P < 0.001) and experimental (P < 0.01) groups (Figure 4A). The mean difference between the two axes was smaller in hCySH-fed animals. The outer AP diameter of their diaphyseal tibiae was significantly (P < 0.01) greater than in controls. Medullary cavity and cortical cross-sectional areas of hCySH-treated bone were approximately 10% greater than those in controls (Figures 4B and 4C). Consequently, moments of inertia of hCySH-treated tibiae were greater, suggesting greater architectural efficiency of cross-sectional design (Figure 4D). However, none of these differences was statistically significant. The hCySH-treated bone looked bigger to the naked eye, a subjective observation confirmed by morphometric measurement (Figure 5). Differences in tibial diaphyseal profiles influenced overall shape of cortical bone in hyperhomocysteinemic animals, who displayed an abnormally eccentric and more elliptical medullary cavity. The thicker cortex on one side of the hCySH-treated bone as compared to control was also noticeable on both AP and ML radiographs (Figures 3B and 3D).

Statistical analysis of biomechanical data also indicates that the hCySH-treated bone is stronger. Mean maximal load, stiffness and energy-at-failure were all greater than the values in controls (Figure 6). The increase for energy-at-failure was significant (P < 0.01). Analysis of covariance, correcting for morphometric differences, showed no significant differences in structural biomechanical properties when bone length and cortical thickness were included as covariates.

Table 3 describes the mineral and chemical properties of the hCySH-treated tibiae in comparison to controls. Ashing of diaphyseal tibial bone segments revealed that the proportion of mineral was not significantly different between groups. Infrared spectroscopy of hCySH-treated cortical bone showed no difference in terms of the mineral-to-matrix ratio when compared to controls. The collagen content of this bone, assessed by hydroxyproline content, was similar to controls. However, qualitative
changes in mineral composition were observed. The molar Ca\(^{2+}/\text{PO}_4\,^3-\) ratio was significantly higher \((P < 0.001)\) in the experimental group and the CO\(^3-/\text{PO}_4\,^3-\) ratio, as revealed by FT-IR, was reduced \((P < 0.01)\). X-ray diffraction analysis revealed no difference in the size and symmetry of mineral crystals, as measured by line broadening \((\beta_{002})\).

**Discussion**

In the present study, the hCySH-rich diet administered to fast-growing chicks induces a bone disease bearing a striking similarity to early CBS-deficiency homocystinuria, including accelerated skeletal growth, epiphyseal growth plate lesions, and cortical bone chemical alterations [1–4,6]. The sulfur metabolite in the transsulfuration path-
way first associated with both accelerated skeletal growth and abnormal extracellular matrix was homocysteic acid [37,38], an oxidation product of homocysteine found in the urine of CBS-deficient patients [39]. In 1976, Clopath et al [38] demonstrated that homocysteic acid promoted growth of hypophysectomized rats, a finding associated with increased thickness of epiphyseal cartilage of the tibia and greater tail growth. We did not evaluate homocysteic acid directly, but we note that hCySH may be stimulatory in vitro when homocysteic acid is not [40], indicating the need for further studies to identify the key metabolite(s). Pyeritz and others [6] postulate that a fibrillin defect may be central to the skeletal overgrowth phenotype, since the inherited fibrillin deficiency of Marfan syndrome induces connective tissue changes strikingly similar to that of CBS-deficiency homocystinuria. They have also been able to show that cysteine deficiency, which may occur in CBS-deficiency homocystinuria, can induce the fibrillin defect in culture [41], which has been reproduced in the developing chick [42]. Dietary cysteine deficiency in our model is unlikely because the chicks were fed a diet enriched in methionine (Table 1) and showed high circulating concentrations of the cysteine precursor, cystathionine, and the cysteine product, sulfate. However, the possibility that tissue insufficiency of cysteine (through competition with homocysteine [43], for example) may have specific hard tissue effects in vivo [44] could be tested in our model.

Figure 5
Mean mid-shaft tibial cross-sectional contours for control and homocysteine (hCySH)-treated animals. hCySH-treated cortical bone is asymmetric with an eccentric medullary cavity. Its greater thickness on one side of the X axis was also noticeable on radiographs (Figure 4B). A: anterior cortex of tensile side; P: posterior cortex of compressive side; MC: medullary cavity; - - - - - periosteal cortical bone - - - - - - endosteal cortical bone. Bar = 1 mm.

Figure 6
Biomechanical properties of the tibia. Top Panel – Maximum load (N); Middle Panel – Stiffness (N/mm); and Lower Panel – Energy at failure (N.mm). Unadjusted data (mean ± SD) for controls (CON) and hyperhomocysteinemic animals (hCySH) are shown by open and shaded bars, respectively. Bars with hatching show values statistically adjusted for length and cortical thickness by analysis of covariance.

The major skeletal changes noted in hyperhomocysteinemic animals in this study were the increased width and length of the tibia, features not present in the vitamin B₆ deficiency model [15]. However, cystathionine is the predominant sulfur amino acid elevated in that model [45],
not hCySH. In the present study, all three amino acids of the transsulfuration pathway – hCySH, cystathionine and methionine – were elevated (Table 2). Also unlike vitamin B6 deficiency, qualitative alterations in the cortical bone mineral composition were observed. The bone carbonate to phosphate ratio was slightly but significantly ($P < 0.01$) decreased (Table 3), a finding associated with high turnover and remodeling rates [46]. The increased cross-sectional area of the medullary cavity (Figure 4B) may also reflect increased endosteal resorption.

The markers of bone formation we studied – osteocalcin and bone-specific ALP – were unchanged (Table 2), but these measures were recorded only at the end of the experiment when the bony abnormality had been established. Levels of IGF-1, a paracrine and autocrine stimulus of bone growth [47–51], were also normal, consistent with recent observations in human CBS deficiency [52]. The increased bone length we observed could be related to changes in the growth cartilage at the cellular level. Whether the proliferative zone of the ossification front or the number or size of hypertrophic chondrocytes is increased warrants further investigation.

The non-collagenous constituents of the epiphyseal matrix are likely important in the pathophysiology of accelerated bone growth. McCully demonstrated that addition of hCySH to culture medium of normal skin cells produced histological changes in proteoglycan structure similar to those found in CBS-deficient cell cultures, along with an increase in radioiodinated sulfate incorporation [53]. Although it was postulated that homocysteic acid may act as a sulfate source for phosphoadenosine phosphosulfate (PAPS), the substrate necessary for sulfoster and glycosaminoglycans synthesis, no direct confirmation of this concept has been reported [54]. The increased Ca/P ratio found on bone mineral analysis may parallel an elevation of extracellular calcium associated with a greater production of non-aggregated proteoglycans similar to those seen in vitro by others [53,55], but further study is needed. Because of the strongly polyanionic nature of their sulfate and carboxylate groups, proteoglycans have a considerable capacity to form calcium complexes, which may serve as a readily accessible source of mineral for hydroxyapatite deposition [56]. In the osteochondrodystrophy induced by vitamin B6 deficiency in the growing chick, connective tissue proteoglycans were found to be more extractable [45], consistent with the structural changes seen by light and electron microscopy in arteriosclerotic wall arteries [53]. Mineral abnormalities in a defective connective tissue matrix, particularly calcium, are also relevant to the role of hyperhomocysteinemia in arteriosclerosis [53,57].

Unlike osteogenesis imperfecta (OI) with its defective collagen synthesis [58,59], chemical changes and associated alterations in collagen morphology in this model [13] do not substantially alter biomechanical properties of long bone and the mineralization process itself, as assessed by the size and degree of symmetry in the mineral crystal analysis ($\beta_{002}$ line broadening) (Table 3). However, the increased moment of inertia of hyperhomocysteinemic bone (Figure 4D) and enhanced cortical thickness on one side (Figure 5) may compensate for abnormally asymmetric and weakened bone due to defective cross links [60] — hence, the lack of significant difference when size covariants such as length and cortical thickness were included.

This report presents a dietary chick model of severe hyperhomocysteinemia as a superior alternative to the CBS-knockout mouse [7] for study of connective tissue abnormalities. The homozygous knock-out mice display plasma hCySH concentrations forty times normal and succumb prematurely after suffering from growth retardation and marked liver disease, neither of which is typical of human CBS deficiency. The heterozygote knock-out animals have total homocysteine concentrations only twice that of wild-type, compared to a difference of more than 5-fold increase that is typical of human CBS deficiency [43] and seen in our experimental model.

| Group                                | Control          | Experimental     |
|--------------------------------------|------------------|------------------|
| Ash (% of net weight)                | 61.2 ± 1.4       | 60.1 ± 1.4       |
| Calcium:Phosphate (molar ratio)      | 1.79 ± 0.16      | 2.11 ± 0.15***   |
| Hydroxyproline (µg/mg dry wt)        | 28 ± 3           | 29 ± 2           |
| Mineral Matrix FT-IR peak area ratio | 4.37 ± 0.35      | 4.43 ± 0.14      |
| CO$_3$PO$_4$ FT-IR peak area ratio   | 0.0190 ± 0.001   | 0.0174 ± 0.001***|
| $\beta_{002}$ (degrees)              | 0.531 ± 0.018    | 0.527 ± 0.024    |

*** $P < 0.001$  ** $P < 0.01$
Conclusions
In summary, this chick model demonstrates that changes in bone mineral composition, tibial cross-sectional contour, and radiological changes are induced by a hyperhomocysteinemia diet and associated with abnormal bone matrix. The hyperhomocysteinemia causes greater radial and longitudinal bone growth with more advanced ossification of epiphyses and greater architectural efficiency of the diaphyseal cross-sectional design, despite the collagen defect [13], chondrodysplastic cartilage, and the chemical abnormalities of bone. The cause of accelerated skeletal growth is an important subject for further study. Moreover, delineation of the factors maintaining normal biomechanical strength in osteopenic bone will be of direct relevance to the homocystinuric patient and the larger population at risk for idiopathic osteoporosis alike [61].

Competing interests
None declared.

Authors’ contributions
Masse and Howell contributed equally to the study concept and design, preparation of the animal materials for analysis, and the basic biochemical measurements. Donovan performed assays of IGF-1 and Haucshka measured osteocalcin. Ziv did the biomechanical study and Boskey performed the bone chemistry measurements. Cole performed the sulfate and sulfur amino acid analyses and contributed to the statistical analysis and interpretation of the data as well as the primary editing of the final manuscript. All authors contributed to the written contents of this manuscript.

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