Experimental Hyperhomocysteinemia Reduces Bone Quality in Rats

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Background: Recently, hyperhomocysteinemia (HHCY) has been suggested as a new risk factor for osteoporosis. This study investigated if HHCY is a causal osteoporotic factor in vivo.

Methods: We used 3 groups of rats: a control group (n = 20), a moderate HHCY group (induced by a 2.4% methionine-enriched diet, n = 10), and an intermediate HHCY group (induced by a 2% homocystine-enriched diet, n = 10). We measured bone fragility [maximum force of an axial compression test (F\text{max})], bone area as percentage of total area (BA\text{T}/TAr, histomorphometry), and biochemical bone turnover markers [osteocalcin (OC) and collagen I C-terminal crosslaps (CTx)].

Results: Compared with controls, 3 months of moderate or intermediate HHCY increased mean (SD) bone fragility at the femoral neck by 18% (6%) in methionine-fed (P = 0.001) and 36% (13%) in homocystine-fed rats (P < 0.001). Mean (SD) BA\text{T}/TAr at the distal femur in methionine and homocystine groups was decreased by 45% (21%; P = 0.001) and 93% (9%; P = 0.001), respectively. At the femoral neck, BA\text{T}/TAr was decreased by 19% (11%; P < 0.001) and 55% (19%; P < 0.001). At the lumbar spine, the reduction of BA\text{T}/TAr was 17% (23%; P = 0.099) and 44% (19%; P < 0.001). Plasma OC (bone formation marker) was decreased by 23% (20%; P = 0.006) and 34% (21%; P < 0.001). Plasma CTx (bone resorption marker) did not differ between groups.

Conclusion: Bone quality is consistently decreased in the presence of increased circulating homocysteine. The results provide evidence that HHCY is a causal osteoporotic factor.

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Osteoporosis is a widespread problem that frequently has devastating health consequences through its association with fragility fractures (1, 2). In a 10-year period, postmenopausal white women in the US will experience 5.2 million fractures of the hip, spine, or distal forearm, which will lead to 2 million person-years of fracture-related disability (3). A comparable situation can be found in Europe (4, 5). The total number of fractures, and hence the cost to society, will increase dramatically over the next 50 years as a result of demographic changes in the number of elderly people (1). Thus, prevention of osteoporosis by identifying risk factors or risk indicators and development of new treatment strategies are major issues.

Important risk factors for osteoporosis include advancing age, female sex, early menopause, low body weight, cigarette smoking, alcohol consumption, low calcium intake, low physical activity, and a prior low-trauma fracture as an adult (4, 5). Recent epidemiologic investigations suggest that increased plasma homocysteine (HCY) could be a new common and independent risk factor for osteoporotic fractures (6–8). Moreover, hyperhomocysteinemia (HHCY) seems to be associated with higher circulating concentrations of bone turnover markers, suggesting a disturbance of bone metabolism (9–12).

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HHCY in elderly adults, when not attributable to impaired renal function, is mainly caused by deficiencies of folate, vitamin B_{12}, or vitamin B_{6} (13, 14). The importance of HHCY as a risk factor for osteoporosis relates particularly to the possibility of simply and safely lowering HCY by supplementation with these B vitamins. In the 1st prospective double-blind intervention trial, which enrolled 628 individuals, the vitamin treatment group had a dramatic reduction of hip fractures (15). However, none of the existing studies provides evidence for a causal role of HHCY in osteoporosis.

In the present study, we investigated the effect of HHCY on bone quality and bone metabolism. On the basis of existing data, we hypothesized that an isolated HHCY can reduce bone quality in a concentration-dependent manner and induce osteopenia in healthy adult rats. Consequently, we investigated bone fragility, histomorphometric bone quality, and biochemical bone turnover markers in healthy adult rats after 3 months of moderate or intermediate HHCY.

Materials and Methods

**ANIMALS AND STUDY DESIGN**

After 7 days of acclimatization, 40 10- to 12-week-old female Wistar rats (180–292 g; Charles River) were randomized into 3 groups: controls (n = 20) and 2 HHCY groups (methionine-enriched diet, n = 10; homocystine-enriched diet, n = 10). Animals were maintained on a 12-h:12-h light:dark cycle in our institutional facility with free access to food and water. HHCY was induced by 2 different diets (Altromin) that were administered for 12 weeks: methionine at 24 g/kg food and homocystine at 20 g/kg food. All diets were based on identical composition that did not contain homocystine or methionine (C1000, Altromin). Blood was collected at the beginning and after 12 weeks of feeding. At the end, animals were killed. The Animal Care and Use Committee of the Saarpfalz-Kreis (Germany) approved the study.

We monitored body weight, food consumption, and fluid intake weekly. Capillary blood sampling using heparin-coated capillaries from the retrobulbar plexus was performed before and at the end of the 12-week feeding period. Immediately after blood sampling, plasma was separated by centrifugation (10 min at 2000g) and stored at −80 °C until analysis. For blood sampling, animals were anesthetized using 1 mL/L ketamine hydrochloride (Ketavet; Pharmacia GmbH) and 20 mL/L xylazine (Rompun; Bayer Healthcare). After blood sampling, both femurs and the first lumbar vertebral body were explanted. The right femur from each animal was deep-frozen immediately at −20 °C and used for biomechanical testing. The left femur and the vertebral body were fixed and used for histomorphometric analyses. In the blood specimens we measured HCY, biochemical bone turnover markers, folate, vitamin B_{12}, and creatinine. All analyses were performed in a blinded fashion.

**BIOMECHANICAL TESTING**

Femurs were cleaned from all soft tissue and embedded at their distal end in a 2-mL plastic cup (Eppendorf) using polymethylmethacrylate (Technovit 3040; Heraeus Kulzer GmbH) for stable fixation of the specimens during the biomechanical testing. We subjected these samples to a standardized axial compression test to measure the force required to induce a femoral-neck fracture. The axial compression test was performed by use of a material testing machine (Zwick GmbH). We loaded bone specimens with a compression force at the femoral head with a displacement rate of 1 mm/min starting with 1 newton (N) preload. Load and displacement were continuously measured and recorded as a diagram. From the load-displacement curve, we determined the force to fracture the bone (F_{max}). All specimens were tested under wet conditions at room temperature.

**HISTOMORPHOMETRY**

The left femurs and the first vertebral bodies were fixed for 1 day [composition of 1 L fixative: 324 mL formaldehyde solution (370 mL/L, Hedinger GmbH), 550 mL ethanol (990 mL/L, Merck KG), 130 mL 0.1 mol/L barbitonal-sodium buffer, pH 7.3 (Dr. K. Hollborn & Söhne GmbH), and 6 g glucose (Sigma-Aldrich)] and dehydrated by sequential changes of increasing concentrations of ethanol (700, 800, 960, and 1000 mL/L). Fat was removed by immersion in xylol for 1 day at room temperature. We embedded samples in methylmethacrylate (Technovit 9100 NEU; Heraeus Kulzer GmbH) and prepared 6-μm frontal sections from standardized locations of the proximal and distal femur and vertebral body by use of a microtome (Leica SM 2500; Leica Instruments GmbH). We stained sections with von Kossa stain and measured histomorphometric bone characteristics with a digitized morphometry system. The morphometry system consisted of a microscope (Leica DMRB; Leica Instruments GmbH), a digital camera (Zeiss AxiomCam; Zeiss GmbH), and a personal computer with morphometry software (Zeiss KS 400; Zeiss GmbH). A standardized rectangle, so-called region of interest (ROI), was placed in the same position of each sample and location. First, bone area/total area (BAr/TAr) was analyzed. The area of the entire ROI was set as 100% and the area of the black-stained, trabecular bone in the ROI was expressed as percentage of the total ROI. In addition, the total trabecular perimeter, trabecular number, thickness, and separation were measured in the ROI according to the recommendations of the American Society of Bone and Mineral Research (16).

**BLOOD MEASUREMENTS**

Plasma samples were used to measure plasma HCY, osteocalcin (OC), collagen I C-terminal crosslaps (CTX), folate, vitamin B_{12}, and creatinine. We analyzed HCY by use of an enzymatic fluorescence polarization immunoassay on an Axsym analyzer (Abbott). Intra- and interassay
CVs of this method were 4.5% and 5.1%, respectively. We measured OC and CTx by use of ELISA (RatLaps® and Rat-Mid Ostecalcin®, Nordic Biosciences Diagnostics). Intra- and interassay CVs were 3.6% and 6.6% for OC and 5.6% and 10.5% for CTx. We measured folate and vitamin B12 by use of chemiluminescence immunoassay on an ACS Centaur analyzer (Bayer Healthcare). Intra- and interassay CVs were 4.0% and 4.4%, respectively, for vitamin B12 and 5.3% and 5.5% for folate, respectively. We measured creatinine by use of a colorimetric assay with the Jaffé method on a Hitachi 917 analyzer (Roche Diagnostics). Quality control materials recommended by the manufacturers were used for all analytes.

STATISTICS
Based on results of a Kolmogorov–Smirnov test, all variables, except the trabecular separation, were gaussian distributed. Consequently, results of the descriptive statistics are expressed as mean (SD). We performed group comparisons of gaussian-distributed variables using one-way ANOVA with least-squares difference post hoc test. We compared trabecular separation, which was not gaussian distributed, using a Kruskal–Wallis and a Mann–Whitney test. We calculated Pearson correlation analyses for gaussian-distributed variables. All calculations used the SPSS 11.0 software package (SPSS).

Results
ANIMAL MODELS
At the beginning of the study, animals had comparable baseline characteristics. Mean (SD) body weight was 233 (27) g and mean plasma HCY concentration was 4.3 (1.8) μmol/L. After 12 weeks of treatment, methionine- and homocystine-fed animals exhibited significant HHHCY (Table 1). The methionine group developed moderate HHHCY and the homocystine group intermediate HHHCY.

At the end of the treatment period, 3 animals from the control group had to be excluded because they exhibited spontaneously increased HCY >10 μmol/L. Additionally, 2 animals from the homocystine group were excluded because their HCY concentration at the end of the treatment was <10 μmol/L, indicating that the diet was not effective in these animals. Creatinine was not significantly higher in any of the treatment groups compared with controls, excluding impaired renal function as a major confounder for the evaluation of plasma HCY and bone metabolism. Folate and vitamin B12 are 2 additional major factors that affect circulating HCY. All animals exhibited high plasma concentrations of folate and vitamin B12 (Table 1). Body weight was lower in methionine [259 (17) g, P <0.01] and homocystine [254 (28) g, P <0.01] groups compared with controls [322 (30) g]. Heart rate and blood pressure did not differ between groups.

BIOMECHANICAL TESTING
Control animals exhibited a mean Fmax of 97 (15) N (Fig. 1). In HHHCY animals, the mean Fmax was decreased by 18% in methionine and 36% in homocystine animals. Fmax showed a highly significant negative correlation with plasma HCY (r = 0.554, P = 0.001).

HISTOMORPHOMETRY
At the end of the study, histomorphometric analyses revealed dramatic effects in hyperhomocysteinemic animals. Compared with control animals, mean (SD) BAr/TAr at the distal femur was decreased by 93% (9%) in homocystine animals (Fig. 2A). The spongiosa was almost completely absent. In the methionine group, BAr/TAr was decreased by 45% (21%), P <0.001.

Methionine and homocystine animals exhibited a pronounced lowering of BAr/TAr at the proximal femur: methionine vs control: −19% (11%), P <0.001, and homocystine vs control: −52% (19%), P <0.001 (Fig. 2B). Decreases were also seen at the lumbar spine: methionine vs control: −17% (23%), P <0.105, and homocystine vs control: −44% (19%), P <0.001 (Fig. 2C). At the femoral neck, the magnitude of the reduction in BAr/TAr was comparable to that observed for Fmax in the methionine and homocystine groups. Accordingly, there was also a highly significant correlation between circulating HCY and BAr/TAr (Fig. 2D).

Similar to BAr/TAr, trabecular perimeter (Fig. 3A) and number (Fig. 3B) decreased with increasing HCY plasma concentrations at the distal femur and the lumbar spine. Accordingly, at these locations trabecular separation increased stepwise in methionine and homocystine animals (Fig. 3C). At the proximal femur, significant changes of trabecular perimeter, number, and separation were detected only in homocystine animals.

BIOCHEMICAL BONE TURNOVER MARKERS
The mean concentration of the bone formation marker OC was 155 (30) μg/L in controls and decreased dependent on concentration in HHHCY animals (Fig. 4). Compared with controls, OC concentrations decreased to 119 (31) μg/L and 102 (32) μg/L in methionine and homocystine

| Table 1. Biochemical findings in animals at the end of the treatment period. |
|---------------------------------|-----------------|-----------------|
| Control                         | Methionine      | Homocystine     |
| HCY, μmol/L                     | 5.6 (1.9)       | 27.3 (8.8)<sup>b</sup> | 54.0 (46.0)<sup>c</sup> |
| Folic acid, μg/L                | 50 (13)         | 56 (9)          | 64 (13)<sup>c</sup>     |
| Vitamin B<sub>12</sub>, ng/L    | 1267 (194)      | 1132 (248)      | 1177 (353)               |
| Creatinine, μmol/L             | 46.4 (7.3)      | 56.6 (13.3)     | 36.2 (5.3)<sup>c</sup>  |

<sup>a</sup> Data are mean (SD). <sup>b</sup> P <0.001 vs control; <sup>c</sup> P <0.05 vs control.
Discussion

The present study demonstrates a consistent relationship between diet-induced HHCY and increased bone fragility as well as decreased histological bone quality. The reduction of bone quality was accompanied by disturbed bone turnover, as indicated by lower OC concentrations. At the femoral neck, the magnitude of the observed effects was comparable for Fmax, BAr/TAr, and plasma OC.

The study had several limitations. Although the mean HCY concentration in the methionine group was similar to that reported by others (17) (27.3 vs 25.3 μmol/L), excessive methionine supplementation in rats is known to have toxic effects, such as hemolytic anemia and growth suppression (18). HCY appears to be among the most plausible explanations for these toxic effects (18), but it is possible that methionine-induced effects might reflect systemic toxicity. We thus substantiated our observations by another, biochemically different model of HHCY consisting of a homocystine-enriched diet, and achieved mean HCY concentrations similar to those reported by Joseph et al., who used a 10 g/kg homocystine diet. A second possible limitation is that growth suppression occurred in our animals. The lower weight in methionine and homocystine animals might have contributed to several of the effects observed. Third, 3 control animals exhibited spontaneously high HCY plasma concentrations, exceeding the reference interval of 3–9 μmol/L.
Because plasma HCY ranged between 11 and 13 μmol/L in these animals and folate as well as vitamin B12 was normal, we assume that genetic polymorphisms or a mild renal impairment in the creatinine-blind range are the most probable explanations for this phenomenon. Finally, in the homocystine group there were 2 nonresponders. The reason is obscure; however, it can be speculated that the absorption of dietary homocystine was disturbed. Because these 3 control and 2 homocystine animals were outside the scope of the corresponding group, we excluded them from further analysis.

Femoral neck fractures are one of the most common complications of osteoporotic patients. Consequently, the induction of a standardized femoral neck fracture was selected as an appropriate tool to monitor the effects of HHCY on biomechanical bone properties of the femoral neck. Our results demonstrate a strong increase of bone fragility, by up to 36%, after 12 weeks of HHCY. In humans, such a change usually takes years or decades. Even in the methionine group, with a moderate HHCY, femoral neck fragility increased by 18%, which is still an impressive effect for such a short treatment period. A relation between HHCY and osteoporotic fractures in humans has previously been described in large epidemiologic investigations (6–8,19). However, these studies cannot prove causality. Moreover, the existence of a recent study from Sweden (n = 996 women), which could not demonstrate a significant increase of fracture risk in individuals with high HCY concentrations, has to be mentioned (12). The discrepancy between this study and all other trials illustrates the limitations of epidemiologic studies, where sample size and inclusion and exclusion criteria may strongly affect the outcome. The study from Sweden included only women, whereas all other studies enrolled men and women. Results from the Framingham study revealed a less-pronounced relation between HCY and fracture risk in women than in men (7). Animal studies offer the possibility to investigate the variation of a single variable (e.g., HCY) in healthy individuals with identical baseline characteristics. Even if animal data cannot be directly transferred to the situation in humans, our data substantiate the relation between HHCY and fracture risk. Our findings are supported by a recent double-blind placebo-controlled trial, supplementing 628 patients for 2 years of placebo or 5 mg/d of folate and 1500 μg/d vitamin B12 (16). The vitamin-treated group exhibited a 75% reduction of femoral neck fractures, and the overall fracture rate was strongly diminished. However, a high incidence of hip fractures in the controls and a high mean concentration of circulating HCY (19.9 μmol/L) limit the generalizability of these results (20). In addition, the low number of 33 hip fractures confers a low power to this study.

The histomorphometric analyses substantiate the biomechanical findings by a dramatic loss of cancellous bone,
which was most pronounced at the distal femur. In the homocystine group, almost all trabecules disappeared, and mean BAr/TAr was decreased by 93%. At the femoral neck, the reduction of BAr/TAr was less pronounced, but at −52% still very strong. Taking into consideration that the trabecular network is fundamental for the biomechanical properties of cancellous bone (21, 22), these structural changes are most probably the explanation for the increased bone fragility. The significant correlation between \( F_{\text{max}} \) and the histomorphometrical BAr/TAr confirms this suggestion. Tommasini et al. (22) have also shown a good correlation between histomorphometry and biomechanical bone quality. Considering the loss of spongiosa, it is not surprising that the other histomorphometric features were changed accordingly.

Our findings seem to be in contrast to clinical studies, where no or only weak relations were found between circulating HCY and bone mineral density (6, 10, 23–25). However, it has repeatedly been shown that bone mineral density measurement by dual-energy X-ray absorptiometry does not provide reliable information about the 3-dimensional structure of the spongiosa (26). Consequently, our results cannot be compared with dual-energy X-ray absorptiometry data.

The pronounced effects presented in Fig. 2 suggest a marked disturbance of bone metabolism. The bone formation marker OC showed a strong decrease of up to 34%. OC is a noncollagenous bone matrix protein that is considered a specific osteoblast activity marker (27). Because the collagen I degradation marker CTx did not reveal any difference between groups, the lower OC concentrations in the HHCY groups suggest decreased osteoblast activity in the presence of an unchanged osteoclast activity as a potential explanation for the observed microscopic and biomechanical changes. However, the CTx and OC data have to be handled with care since the bone turnover of a mature human skeleton cannot be compared with a growing skeleton of rats. In addition, the lower OC concentrations in HHHCY animals could simply be due to the quantitative reduction of cancellous bone. Another hypothetic mechanism of HHCY-related effects on bone quality could be disturbed collagen cross-linking (28). However, disturbed collagen cross-linking would result in a decreased quality of collagen I rather than a decreased quantity of cancellous bone. Nevertheless, recent data from Saito et al. (29) indicate a significant negative correlation between HCY and collagen cross-linking.

In conclusion, the present study provides strong evidence that HHCY is a major causal osteoporotic factor with a strong clinical impact. Future studies will be required to clarify the mechanisms of the HHCY effect.

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