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Excess dietary methionine does not affect fracture healing in mice

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: An elevated serum concentration of homocysteine (hyperhomocysteinemia) has been shown to disturb fracture healing. As the essential amino acid, methionine, is a precursor of homocysteine, we aimed to investigate whether excess methionine intake affects bone repair.

Material/Methods: We analyzed bone repair in 2 groups of mice. One group was fed a methionine-rich diet (n=13), and the second group received an equicaloric control diet without methionine supplementation (n=12). Using a closed femoral fracture model, bone repair was analyzed by histomorphometry and biomechanical testing at 4 weeks after fracture. Blood was sampled to measure serum concentrations of homocysteine, the bone formation marker osteocalcin, and the bone resorption marker collagen I C-terminal crosslaps

Results: Serum concentrations of homocysteine were significantly higher in the methionine group than in the control group, while serum markers of bone turnover did not differ significantly between the 2 groups. Histomorphometry revealed no significant differences in size and tissue composition of the callus between animals fed the methionine-enriched diet and those receiving the control diet. Accordingly, animals of the 2 groups showed a comparable bending stiffness of the healing bones.

Conclusions: We conclude that excess methionine intake causes hyperhomocysteinemia, but does not affect fracture healing in mice.

key words: **methionine • homocysteine • fracture healing • mice**

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Background

During the past decades, a variety of biomechanical and biological factors that determine the outcome of bone repair have been identified [1,2]. In many patients, however, the cause of disturbed fracture repair remains unclear [3]. Accordingly, there is a need to investigate further confounding factors that might interfere with bone repair.

Recently, we showed that an elevated serum concentration of the sulfur amino acid homocysteine (hyperhomocysteinemia) affects bone repair in mice [4]. Homocysteine is the final product of the methionine cycle, in which a methyl group is transferred from methionine onto a variety of acceptors, such as different proteins, DNA and RNA. Degradation of cytotoxic homocysteine is attained by remethylation and transsulfuration requiring folate, vitamin B6, and vitamin B12 as co-enzymes (Figure 1) [5]. Thus, excess methionine intake and B vitamin deficiency are capable of increasing serum homocysteine, while vitamin B supplementation has been shown to decrease serum homocysteine [6,7]. Considering recent data showing that hyperhomocysteinemia negatively affects fracture healing, we hypothesized that vitamin B deficiency might also disturb bone repair. However, alimentary folate and vitamin B12 deficiency did not affect bone repair, although circulating homocysteine was moderately elevated. Results of another study in mice, which analyzed the atherogenic effect of excess methionine intake and vitamin B deficiency, showed that the application of a methionine-supplemented diet, but not of a B vitamin-deficient diet, induces significant atheromatous lesions [8].

Based on the fact that different models of hyperhomocysteinemia can result in different phenotypes, we hypothesized that excess methionine intake adversely affects bone repair in mice.

Material and Methods

Experimental design

The present study was approved by the local governmental animal care committee and was conducted in accordance with the German legislation on protection of animals and the NIH Guidelines for the Care and Use of Laboratory Animals.

We used 2 groups of adult CD-1 mice (body weight 30 to 40 g) to analyze the impact of excess methionine intake on bone repair. Mice of the first group received a methionine-supplemented diet (C1000 + 2.5% L-methionine, Altromin, Lage, Germany; n=13), while those of the second group were fed an equicaloric control diet (1324, Altromin, n=12). In a pilot study, we measured serum concentrations of homocysteine in animals fed with a methionine-supplemented diet for 3 weeks. These measurements revealed a hyperhomocysteinemia in all animals. Therefore, we started methionine supplementation at 3 weeks before fracture to assure that hyperhomocysteinemia was present in all animals during the whole fracture healing period. Four weeks after fracture, animals were killed and bones were resected for histomorphometric and biomechanical analyses. At the day of sacrifice, blood was sampled for the measurement of homocysteine, the bone formation marker osteocalcin (OC), and the bone resorption marker collagen I C-terminal crosslaps (CTX).

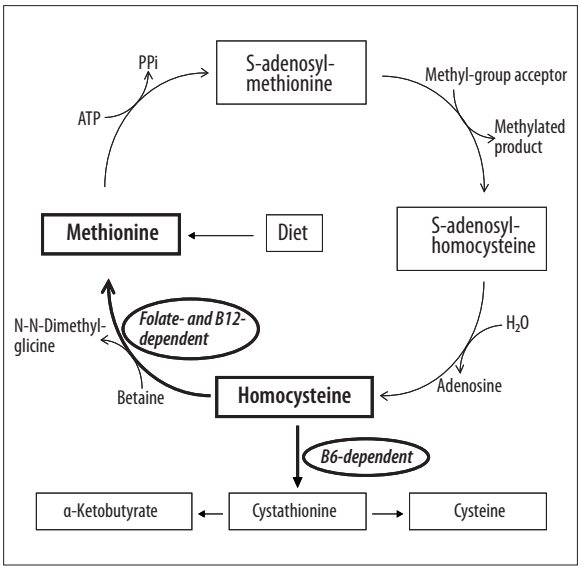

Figure 1. Schematic overview of the methionine homocysteine metabolism. The essential amino acid methionine is activated by condensation with ATP to the ubiquitous methyl donor S-adenosyl-methionine. During the transmethylation process S-adenosylhomocysteine is formed, which is a potent competitive inhibitor of most transmethylation reactions. Homocysteine is the cytotoxic end-product of the methionine pathway and must be removed, which is achieved by either folate- and vitamin B12-dependent remethylation to methionine or irreversible vitamin B6-dependent transsulfuration.

Surgical procedure

For surgery, the mice were anesthetized by intraperitoneal (i.p.) injection of ketamine (75 mg/kg body weight; Pharmacia GmbH, Erlangen, Germany) and xylazine 2% (25 mg/kg body weight; Rompun, Bayer, Leverkusen, Germany). The right femur of each animal was fractured by a 3-point bending device and stabilized by an intramedullary compression screw as described previously [4,9,10]. Fracture configuration (type A2-A3 according to the AO-OTA classification) [11], and implant position were documented by X-rays (Inside IP-21 high- resolution dental films, Kodak) (Figure 2).

Serum analyses

Blood samples were taken by vena cava puncture. After 30 min of clotting, serum was separated from cellular compartments by centrifugation (3400 g for 3 min) and stored at –80°C until batched analysis. Serum homocysteine was determined by gas chromatography mass spectrometry, as reported previously [12]. OC and CTX were analyzed using the OC ELISA kit DEPT470 (Demeditec Diagnostics, Kiel, Germany) and the CTX ELISA kit 4CRL4000 (Nordic Bioscience Diagnostics, Herlev, Denmark). Intra- and inter-assay coefficients of variation (CV) were 3.2% and 6.7% for homocysteine, 6.0% and 8.0% for OC, and 5.1% and 6.5% for CTX, respectively.

Biomechanical testing

For biomechanical testing, the intramedullary screws were removed. The distal ends of the femurs were embedded in

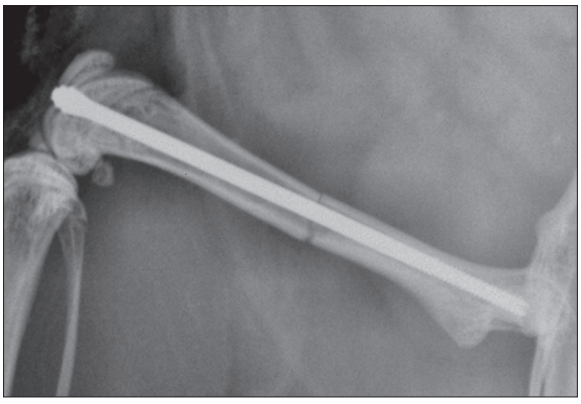

Figure 2. Representative x-ray of a mouse femur after closed fracture and stabilisation by an intramedullary compression screw.

aluminium tubes (10 mm in diameter) using polymethylmethacrylate (Technovit 3040, Kulzer, Wehrheim, Germany), and fixed in a 3-point testing device. The proximal ends of the femurs were mounted on a support, as described previously [4]. Using a materials testing machine (Zwick 1454, Ulm, Germany), a dorsal deflection was generated at the level of the callus with a constant velocity of 1 mm/min and a maximum force of 10 N. The stiffness of the healed femurs was calculated from the slope within the linear part of the load-deflection curve.

Histomorphometric analyses

After biomechanical testing, femurs were fixed in 4% formalin, dehydrated with ethanol, and embedded in polymethylmethacrylate (VLC 7200, Kulzer) for histology. Undecalcified longitudinal sections of 70 µm thickness were cut, grinded, and stained with paragon (toluidine blue and basic fuchsin). Using a digital calliper (Digit-Cal 15P 118704, Tesa, Renens, Switzerland), size and tissue composition of the callus (bone, fibrocartilage or connective tissue including bone marrow) were analyzed at a magnification of 200× (Axiophot Light Microscope, Zeiss), as described previously [4].

Statistics

All data are given as means ± standard deviation (SD). As some variables, such as osteocalcin and CTX, were not normally distributed, the experimental groups were compared by ANOVA on ranks, followed by Mann-Whitney U test. A Bonferroni adaptation for multiple testing was included. Statistical analyses were performed using the SAS-software package (JMP, SAS Institute). A p-value <0.05 was considered to indicate significant differences.

Results

Serum analyses

Serum analyses demonstrated a markedly increased homocysteine concentration in the methionine group when compared to the control group (Figure 3). Considering reference values applied in humans, mice of the methionine group showed an intermediate hyperhomocysteinemia, while animals of the control group were normohomocysteinemic [13].

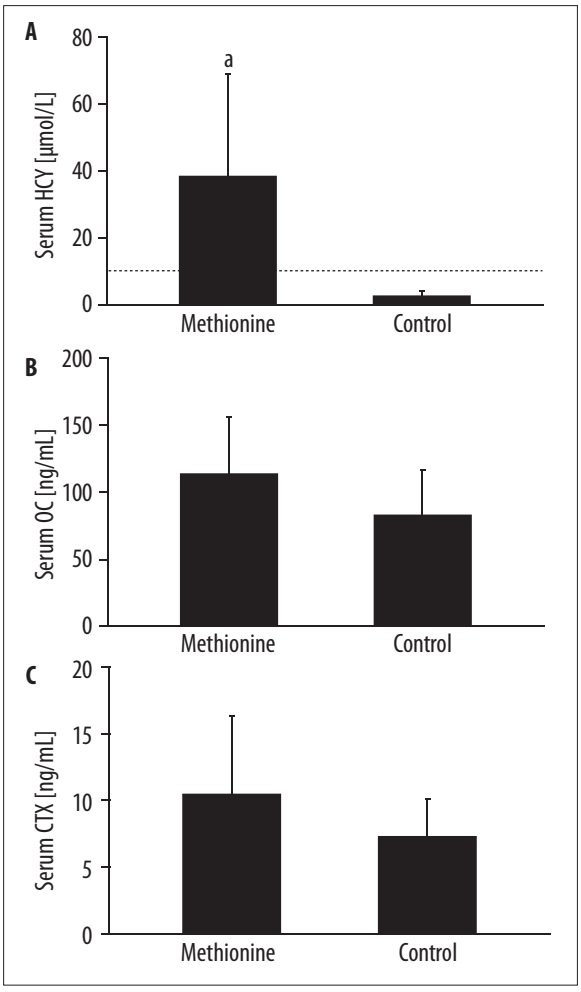

Figure 3. Serum analyses of homocysteine (HCY; (**A**)), osteocalcin (OC; (**B**)), and collagen I C-terminal crosslaps (CTX; (**C**)) in animals, which were fed the methionine-supplemented diet and in animals, which received the control diet. Data are given as means ± standard deviation (SD). [a] P<0.05 *vs.* control. The dotted line (**A**) demonstrates the cut-off value of serum homocysteine indicating hyperhomocysteinemia in humans.

Serum analyses of OC and CTX revealed no significant differences in bone turnover between treatment and control animals (Figure 3).

Biomechanical testing

Biomechanical testing showed no significant differences in bending stiffness of the healed femurs when comparing animals that were fed the methionine-supplemented diet and those receiving the according control diet (Figure 4).

Histomorphometric analyses

Histomorphometry demonstrated in all animals a typical pattern of secondary fracture healing with osseous bridging of the fracture gap after 4 weeks of bone repair. In some animals callus was still evident, whereas in other animals remodelling was very advanced. These different stages of fracture healing could be observed in animals of both groups without differences in the pattern and quality of bone repair

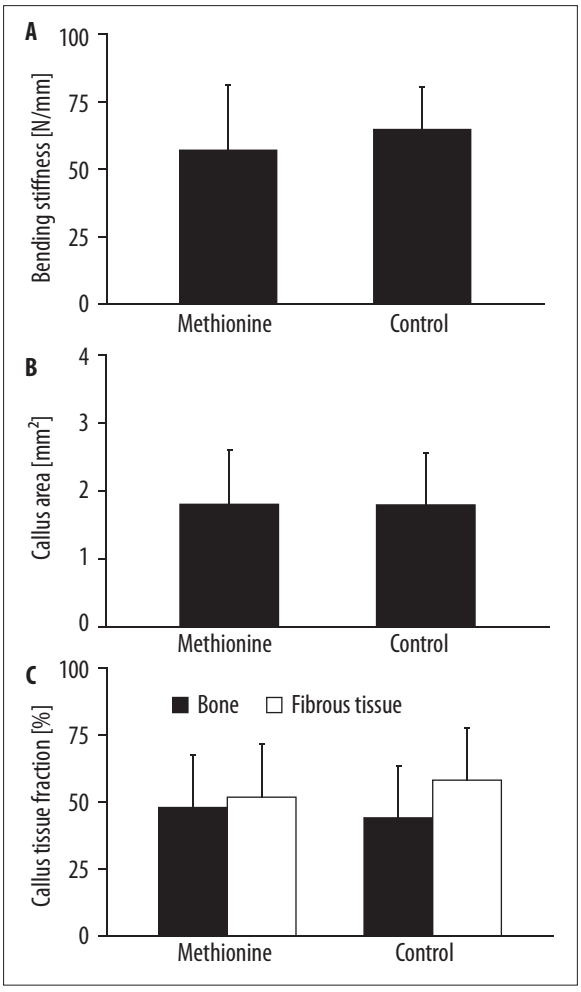

Figure 4. Biomechanical analysis of the bending stiffness of the healed femurs (**A**) as well as histological analysis of the size (**B**) and tissue composition (**C**) of the fracture callus in animals, which were fed the methionine-supplemented diet and in animals, which received the control diet. Data are given as means ± standard deviation (SD).

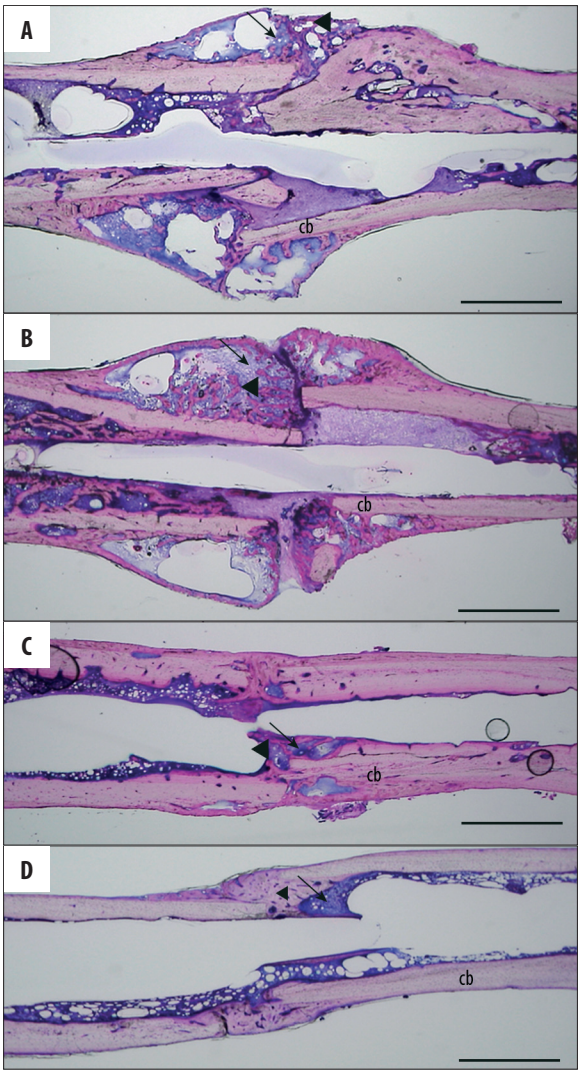

Figure 5. Representative histological sections of femurs of methionine-treated animals (**A, C**) and controls (**B, D**). All samples show complete osseous bridging of the fractured cortical bones (cb) by new woven bone (arrow head), which is surrounded by fibrous tissue (arrow). In some animals (**A, B**) callus is still evident, whereas in other animals (**C, D**) remodelling is very advanced. These different stages of fracture healing can be observed in animals of both groups without differences in the pattern and quality of bone repair between methionine-treated animals and controls.

between methionine-treated animals and controls (Figure 5). Quantitative analysis did not show any significant difference in size and tissue composition of the callus between methionine-treated animals and controls (Figure 4).

Discussion

The present study did not show a significant effect of excess methionine intake on bone repair in mice. This result is surprising, as recent data demonstrated that dietary homocystine intake significantly alters fracture healing [4]. Both homocystine- and methionine-rich diets have been found to increase serum homocysteine. However, the increase of serum homocysteine induced by a homocystine-supplemented diet was more than twice as high as that observed in animals receiving a methionine-rich diet (102 µmol/L *vs.* 38 µmol/L). Our results are in line with those of another study, in which moderate hyperhomocysteinemia (15 µmol/L) induced by vitamin B deficiency did not interfere with bone repair [10]. Considering the results of existing studies, it appears that the degree of hyperhomocysteinemia and the model by which hyperhomocysteinemia is induced are essential for the effect of homocysteine on bone repair. We feel that this finding is of high clinical significance, because it shows that that the outcome of bone repair is determined by the underlying cause of hyperhomocysteinemia. Many foods and some medications contain the essential amino acid methionine, whereas the ingestion of homocystine is an artificial model not representing the typical cause of hyperhomocysteinemia in practice. Thus, the results of the present study indicate that hyperhomocysteinemia in general does not represent a risk factor for delayed fracture healing.

We cannot exclude an effect of methionine on the early stages of fracture healing. However, we decided to evaluate bone

repair at 4 weeks after fracture for 2 reasons. First, we intended to continue our previous research on the influence of hyperhomocysteinemia on fracture repair [4,10]. The effect of an alimentary hyperhomocysteinemia and the effect of vitamin B deficiency on bone repair were studied at 4 weeks after fracture. To compare data of the present study with results of these previous 2 studies, we decided to use the 4-week time point in the present study. The second reason why we studied bone repair at 4 weeks was the fact that at this time point non-disturbed fracture healing in mice is completed [14]. Therefore, we feel that this time point is appropriate to study factors that are thought to delay bone repair.

Several mechanisms have been reported by which homocysteine might deteriorate bone metabolism [15]. On the cellular level, homocysteine has been indicated to stimulate the proliferation of osteoclasts, and to inhibit the activity of osteoblasts inducing a misbalance between bone formation and resorption]15–20]. Measurements of serum markers of bone resorption and formation have been proven as a powerful tool to analyze the impact of different metabolic conditions on bone turnover [15]. The results of the present study did not show a significant association between the moderately increased serum homocysteine and markers of bone turnover during fracture healing.

The lack of significant effects on fracture healing in the present study can at least to some extent be explained by mechanisms that are not related to homocysteine. S-adenosylmethionine (SAM), the activated form of methionine, acts as an universal methyl group donor, transferring methyl-groups to a wide spectrum of acceptors, such as DNA, RNA, and a variety of different proteins [21]. Of interest, it has recently been demonstrated that an inhibition of SAM-dependent methylation impairs the differentiation of osteoblasts [22]. In turn, excess dietary methionine might be capable of stimulating osteoblasts. Therefore, a potential negative effect of serum homocysteine on bone formation in the present study might be masked by a SAM-dependent stimulation of osteoblasts.

Another important point to consider is that results from animal studies do not always reflect the situation in humans. The induction of hyperhomocysteinemia by excess homocysteine intake is an artificial model that does not represent the typical cause of hyperhomocysteinemia in humans. Vitamin B deficiency and renal impairment are by far the most common reasons for high serum concentrations of homocysteine in the clinical situation [23,24]. In humans, intake of large amounts of methionine has been reported to have different effects. Excess methionine intake has been reported to be associated with a rapid, but short, increase of serum homocysteine, reaching a peak concentration around 8 hours after intake [25,26]. On the other hand, dietary methionine has been shown to be unrelated, or even negatively related, to baseline serum homocysteine [27,28]. These seemingly conflicting effects of methionine on serum homocysteine might be due to the fact that meat and foods rich in animal proteins are the principal dietary source of both methionine and B vitamins. Thus, the effect of methionine on the serum concentration of homocysteine in humans might be masked by the protective properties of B vitamins [29]. The protective effect of B vitamins was missing in the diet used in the present animal study, which might explain why the supplementation of methionine significantly increased baseline serum homocysteine. This hypothesis is supported by the results of another animal study in mice, which showed that the combination of a methionine-supplemented and vitamin B-deficient diet causes an intermediate hyperhomocysteinemia, while the application of a methionine- and vitamin B-supplemented diet is not capable of increasing serum homocysteine [8]. The fact that most foods that are rich in methionine are also the primary source of B vitamins raises the question of whether the use of a methionine-supplemented, but not vitamin B-supplemented, diet represents an artificial model, too. We are aware that excessive methionine intake is associated in most cases with profuse ingestion of B vitamins. However, it has to be considered that methionine also is used as a medication for the treatment of recurrent or relapsing urinary tract infections [30,31].

Another limitation of animal studies that always needs to be discussed is the difference in metabolism, anatomy, and tissue morphology between animals and humans. While the methionine metabolism is quite comparable between mice and humans, bone morphology and anatomy show significant differences between species. In addition, the time course of fracture healing is faster in mice than in humans [32]. Nevertheless, we feel that the results of the present study are of potential clinical relevance because the different stages, as well as the cellular and molecular events, during secondary fracture healing are very similar in mice and humans [32].

Conclusions

In conclusion, the results of the present study show that excessive methionine intake leads to moderate hyperhomocysteinemia, but does not affect fracture healing in mice.

Conflicts of interest

All authors have no conflicts of interest.

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