Effect of homocysteine on the histological structure of femur in young male albino rats and the possible protective role of folic acid

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Abstract

Introduction: Homocysteine (HCY) is an amino acid that is formed as an intermediate during the metabolism of methionine. The role of hyperhomocysteinemia in the pathogenesis of osteoporosis has been considered a focal point.

Aim of the work: Is to investigate the structural changes that occur in the bone and epiphyseal plate of femur in young male albino rats after being treated with oral HCY and the possible protective role of folic acid.

Materials and methods: Thirty six young male albino rats were used in this experiment. They were equally divided into three group. The control group, HCY treated group (group II) received 0.6mg HCY/g b.w/day for 6 weeks and protected group (group III) received both HCY and folic acid (2 mg/kg) by oral gavages for 6weeks. At the end of experiment, the femurs of the animals were prepared for both light (hematoxylin and eosin, mallory trichrome stains and osteoprotegerin immunoreaction) and transmission electron microscopic examinations. Cortical and trabecular bone thickness were assessed using the image analyzer, in addition to counting the number of osteoclasts.

Results: HCY treated group showed significant reduction of cortical bone thickness of femur diaphysis with multiple cavity formation as well as a significant reduction in the trabecular bone thickness of distal metaphysis as compared with the control group. Additionally; the cancellous bone trabeculae were also separated by wide bone marrow rich in fat cells associated with a reduction in osteoprotegerin immunoexpression. The distal epiphyseal plate showed a lack of the orderly chondrocytes columns arrangement. Regarding the protected group, bone architecture was maintained so that the cortical and trabecular bone had nearly normal appearance in comparison with the control group. Ultrastructurally, osteocytes showed marked degenerative changes and were surrounded by irregular collagenous fibrils while the osteoclasts were increased as proved statistically.

Conclusion: Hyperhomocysteinemia induced profound histological changes in the epiphyseal plate, trabecular and cortical bone of femur in young rats. Folic acid could have a protective role against these alterations.

Keywords: Homocysteine, femur, folic acid, growing rats

Introduction

Osteoporosis is a major health problem that is characterized by low bone mineral density, deterioration of bone micro-architecture and an increased risk of fracture [1].

Osteoporotic fractures are associated with increased level

of morbidity and mortality and with substantial economic costs [2,3].

It has been hypothesized that the metabolism of homocysteine is involved in osteoporosis [4]. Homocystinuria, a rare autosomal recessive disease characterized by markedly

elevated levels of plasma homocysteine, has several clinical manifestations involving the eyes, the vasculature, and the central nervous system. The presence of homocystinuria is associated with the early onset of generalized osteoporosis [5,6]. The underlying pathophysiological mechanism for the occurrence of early osteoporosis in patients who have homocystinuria is not completely understood [7].

Several studies confirmed an association between homocysteine and bone mineral density in postmenopausal women [8,9], in patients with Crohn's disease [10], primary biliary cirrhosis [11], and hyperparathyroidism [12]. A high circulating homocysteine concentration has also been mentioned as an independent risk factor for several chronic conditions, including cardiovascular and Alzheimer disease [13]. It has been proved that the administration of homocysteine caused osteopenia in newborn rats [14]. Whereas, other studies failed to confirm such an association [15].

Homocysteine (Hcy) is a sulfur-containing amino acid formed in the metabolic pathway between methionine and cysteine [16]. Elevated levels of homocysteine known as hyperhomocysteinemia (HHcy) are associated with various bone abnormalities, such as osteopenia and osteoporosis [17,18]. The thiol groups in Hcy undergo auto-oxidation, thus triggering oxidative stress. Methionine is condensed with ATP to form S-adenosylmethionine, the universal methyl donor in transmethylation reactions and the direct precursor of Hcy *in vivo* [19].

Folate, vitamin B₆ and vitamin B₁₂ are important enzymatic cofactors in the synthesis of methionine from homocysteine (Hcy), and an elevation of Hcy can be caused by insufficiency of folate, vitamin B₆ or vitamin B₁₂ [20,21].

Numerous studies have linked high circulating Hcy levels and low concentrations of folate or vitamin B₁₂ with increased risk of low bone mineral density (BMD) in non-diabetic subjects [22,23].

Supplementation with vitamin B₁₂ and folate has recently been shown to decrease fracture risk in stroke patients [24].

Folic acid (FA) is a naturally occurring dietary component [25]. A lack of dietary folic acid leads to a folate deficiency [26].

Folic acid is essential to numerous body functions ranging from nucleotide biosynthesis to the remethylation of homocysteine [27,28]. Children and adults both require folic acid in order to produce healthy red blood cells and prevent anemia. Folic acid can be used to treat Alzheimer's disease, depression, and certain types of cancer [29].

Previous data indicated a higher bone turnover (resorption and formation) leading to decreased bone mineral density (BMD) among individuals with high Hcy levels. A high bone turnover may lead to lower BMD and is also a risk factor for fracture [30].

Osteoclasts are specialized cells that resorb bone, whereas osteoblasts are cells that synthesize new bone. The RANK ligand (RANKL), a protein expressed by the osteoblasts, plays an important role in osteoclast formation, function, and

survival through its interaction with RANK on the osteoclast [31]. Osteoprotegerin (OPG), a natural inhibitor of RANKL, interferes with RANKL and RANK association and thereby regulates osteoclast activity and resorption in the bone [32,33].

Despite this considerable evidence that identifies homocysteine as a risk factor for osteoporosis, the molecular mechanisms of how bone remodeling is hampered during hyperhomocysteinemia still remain ambiguous. The aim of the present work is to investigate the structural changes that occur in the bone and epiphyseal plate of femur in young male albino rats after being treated with oral Hcy and the possible protective role of folic acid.

Materials and methods

Experimental animals

Thirty six young male albino rats (aged 3 weeks, weighing 50-60gm at the beginning of experiment) were housed in stainless steel cages at Animal House, Faculty of Medicine, Zagazig University at room temperature, fed standard balanced diet and allowed water *ad libitum*. The experiment was performed in accordance with the "Guide for the Care and Use of Laboratory Animals" [34]. The experimental protocol was approved by the Ethical Committee of Zagazig University.

Chemicals

Homocysteine (Hcy)

Was obtained from Sigma-Aldrich, USA. The drug was dissolved in distilled water as a vehicle.

Folic acid

Was obtained from El Nasr Pharmaceutical Chemicals Co. And was dissolved in normal saline to give a suspension.

Groups

Rats were classified into three equal groups (12 animals each).

Group I (Control)

Rats were further subdivided into three equal subgroups.

Subgroup IA (negative control group)

Rats were gavaged with 1 ml of distilled water once daily for 6 weeks.

Subgroup IB (saline positive control group)

Rats were gavaged with 1 ml of normal saline {solution of 0.90%w/v of NaCl, isotonic saline} (the solvent of folic acid) once daily for 6weeks [35].

Subgroup IC (folic acid positive control group)

Rats were gavaged with folic acid at a dose of 2 mg/kg dissolved in 1 ml of normal saline for 6weeks [35].

Group II (homocysteine treated group)

In which rats received daily Hcys at a dose of 0.6 mg/kg b.wt.

dissolved in 1 ml distilled water [36] for 6 weeks through oral gavage [37].

Group III: (homocysteine+folic acid group) (protected group)

The animals in this group received concomitant administration of homocysteine at the same dose and duration as group II plus folic acid at the same dose and duration as group IC.

At the end of experiment, rats of all studied groups were anesthetized with 35mg/kg body weight sodium pentobarbital intraperitoneally [38].

Biochemical study

Blood samples were collected to undergo estimation of serum homocysteine by enzyme-linked immuno sorbent assay (ELISA) test.

With the use of a commercial enzyme-linked immunosorbent assay (ELISA kit) (Automated Chemiluminescent System, AUS: 180®, Bayer HealthCarc). Total HCY concentration was determined after protein-bound HCY was converted to free HCY, which was then enzymatically converted to S-adenosyl-L-HCY (SAH) by S-adenosylhomocysteine hydrolase enzyme. The enzyme is specific for the L-form of homocysteine, which is the only form present in the blood.

Histological study

Specimen preparation for light microscope examination

The right femurs were carefully dissected free of soft tissue. The distal part of each bone (including the diaphysis with distal metaphysis) were obtained using a sharp blade) was immediately fixed in 10% neutral buffered formaldehyde for 2 days. After fixation, preparation of decalcified specimens was performed using the chelating agent ethylene diamine tetra acetic acid (EDTA) in the form of its disodium salt (5.5 g ethylene diamine tetra acetic acid in 90 ml distilled water and 10 ml formaldehyde 37-40%). Decalcification was carried out for 3 weeks, during this time the decalcifying solution was changed every day [39]. The decalcified specimens were dehydrated and processed to form paraffin blocks. Serial coronal sections (6 µm thick) parallel to the long axis of the femur and extending from one-third to one-half the thickness of each bone were prepared and stained with hematoxylin and eosin (H&E) and Mallory trichrome stains [39].

The immunohistochemical staining for localization of osteoprotegerin protein was carried out by means of the avidin biotin-peroxidase complex method following the manufacturer's instructions. The slides were incubated with mouse monoclonal anti-OPG antibody 1:250, (Mab 8051, R&D Systems, Abingdon, UK) for 1 h, washed and incubated for 30 min with antimouse biotin-conjugated secondary antibody (30 µl/2 ml of casein solution diluted 1:50) (Vector Laboratories, Peterborough, UK). Slides were washed and developed using ABC Elite kit (Vector Laboratories), followed by diaminobenzidine (DAB) and a 20 sec counterstain with

haematoxylin. Negative control sections were incubated with PBS instead of the primary antibody [40].

Positive reaction appeared as brownish granules mainly in the cytoplasm of cells of osteoblastic cell lineage and sometimes areas of the trabecular surface [41]. Positive reaction was also detected in maturative/hypertrophic chondrocytes, and early osteocytes [42].

Specimen preparation for electron microscope examination

Small pieces from shaft of femur were immediately fixed in 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer at pH 7.4 for 2 hours at 4°C and then washed with phosphate buffer, postfixed in 1% osmium tetroxide in the same buffer for one hour at 4°C. The postfixed specimens were decalcified by EDTA solution in buffer for 3 successive days at 4°C. Then the specimens were washed in phosphate buffer and were dehydrated with ascending grades of ethanol. They were put in propylene oxide for 30 minutes at room temperature, impregnated in a mixture of propylene oxide and resin (1:1) for 24 hours and in a pure resin for another 24 hours. Then, the specimens were embedded in Embed-812 resin in BEEM capsules at 60°C for 24 hours [43].

Ultra-thin sections were obtained using Leica ultra cut UCT and stained with uranyl acetate and lead citrate [43] and were examined with JEOL JEM 1010 electron microscope in Electron Microscope Research Laboratory (EMRL) of Histology and Cell Biology Department, Faculty of Medicine, Zagazig University.

Morphometric and statistical study

The following parameters of the femur bone were quantified:

1. Outer cortical bone thickness (µm) of diaphysis: (mean width of outer cortical bone). This was measured by drawing a perpendicular line from just beneath the periosteum to the endosteum. Cortical bone thickness or width (µm) were determined at five levels (0.5mm apart), starting 0.2 mm below the lowest point of the growth plate [44].
2. Trabecular bone thickness (µm): the thickness of trabeculae in cancellous bone of distal metaphysis were measured at their midpoint away from their branching areas [45].
For estimation of outer cortical bone thickness and trabecular bone thickness, the mean values of ten non overlapping fields (at magnificationx200) from each of five different sections at different femur levels of 4 animals from each group were taken.
3. The number of osteoclasts was estimated/high power field (hpf). Osteoclasts were counted in ten random high power non overlapping fields in five sections obtained from each animal of 4animals in each group.

The standard deviation was calculated and statistical analysis was carried out. The image analyzer computer system Leica Qwin 500 (Leica Ltd, Cambridge, UK) at the Image Analyzing Unit of the Pathology Department, Faculty of Dentistry, Cairo

University (Egypt), was used. Using SPSS statistical program version 17.

Data were evaluated by using the one-way analysis of variance test. The probability values (P) less than 0.05 was considered significant and highly significant when the P values were less than 0.001.

Results

Light microscopic observations

The light and electron microscopic examinations of subgroups Ia, Ib and Ic of the control group revealed similar morphological results; hence, we chose the results of subgroup Ia (negative control) to represent the control group.

Observations of H&E stained sections

The distal metaphysis of control femur showed a network of irregular bone trabeculae and bone marrow spaces between them. The osteocytes surrounded by their lacunae were present in the bone matrix (**Figure 1A**).

The distal epiphyseal plate of control femur showed the four distinct zones of the epiphyseal growth plate with regular chondrocyte column arrangement. The growth plate showed columns of resting zone, proliferative zone, the prominent hypertrophic zone and the calcification zone, (**Figure 1B**). The cortical bone of femur diaphysis of the same group showed many haversian systems with well observed haversian canals and osteocytes in their lacunae. Basophilic osteoblasts on

Figure 1. H&E stained sections of rat femur. Control group showing (A): A network of bone trabeculae (t) at the distal metaphysis separated by bone marrow spaces (bm). Osteocytes (arrows) surrounded by their lacunae are seen in the bone matrix. (B): Distal epiphyseal plate; showing the four well distinct zones of the epiphyseal plate; resting (I), proliferative (II), hypertrophied (III) and calcified (IV) Inset: highly organized chondrocytes (arrow). (C): Cortical bone of femur diaphysis with haversian canals (H) and osteocytes in their lacunae (arrows) Cuboidal basophilic osteoblasts on smooth endosteal bone surface (curved arrow) are also observed. Treated group showing (D): Thin widely separated disconnected trabeculae (dt) of distal metaphysis and wide bone marrow spaces in between trabeculae (bm) filled with many fat cells (F). Notice the congested blood sinusoids (*). Inset: is showing an acidophilic osteoclast (arrow) housed in the eroded bone surface. (E): Epiphyseal plate with disorganized chondrocyte column arrangement (arrow). Note the presence of many acidophilic osteoclast (short thick arrow). Inset: most chondrocytes appear shrunken and atrophied with deeply stained nuclei. (F): Osteoporotic cavities (O) and multiple eroded (E) areas at the endosteal surface of the cortical bone. Protected group showing (G): The cancellous bone trabeculae (t) of femur metaphysis almost attaining their normal architecture as compared to group I with few fat cells (F) in marrow spaces. (H): The epiphyseal plate is almost identical with the control group in thickness. Inset: nearly preserved structure of chondrocytes. (I): Cortical bone of femur diaphysis: with observed reversal lines (L) and disappearance of osteoporotic cavities. (scale bar=50µm, inset scale bar=25µm).

smooth endosteal bone surface were also noticed (**Figure 1C**).

Examination of H&E stained sections at the distal metaphysis of treated femur revealed thin widely separated disconnected trabeculae, wide bone marrow spaces filled with many fat cells and congested blood sinusoids (**Figure 1D**). Osteoclasts housed in the eroded bone surface were also seen (inset, **Figure 1D**).

Examination of H&E epiphyseal plate sections obtained from homocysteine treated rats revealed disorganized chondrocyte column arrangement, chondrocytes appeared shrunken atrophied with deeply stained nuclei. Also, many acidophilic osteoclasts were noticed (**Figure 1E**). As regard, sections of the femur diaphysis of this group; osteoporotic cavities (Resorption cavities) were observed in the cortical bone. Moreover, the endosteal surface of the cortical bone appeared irregularly eroded (**Figure 1F**).

Examination of H&E stained sections at the distal metaphysis of femur of protected group showed bone trabeculae almost

attaining their normal architecture as compared with group I. Scattered adipocytes in marrow spaces were noticed (**Figure 1G**). Whereas, the epiphyseal plate was almost identical with the control group in thickness and chondrocytes attain nearly preserved structure (**Figure 1H**). The femur diaphysis of this group showed cortical bone with disappearance of osteoporotic cavities and appearance of many reversal lines (**Figure 1I**).

Observations of mallory trichrome stained sections

Trabecular bone of control group showed high content of blue collagen fibers (**Figure 2A**). As regard the epiphyseal plate; the highly organized chondrocytes were embedded in an abundant bluish extracellular matrix (**Figure 2B**). Whereas; Cortical bone of femur diaphysis had many regularly arranged blue stained collagen fibers (**Figure 2C**). Mallory trichrome stained sections of treated group showed; few collagen fibers in the trabecular bone that appeared widely separated by

Figure 2. Mallory trichrome stained sections of rat femur. Control group showing. (A): High content of collagen fibers (C) in bone trabeculae of distal metaphysis. (B): Distal epiphyseal plate showing an abundant bluish extracellular matrix (M). (C): Cortical bone of femur diaphysis with many regularly arranged blue stained collagen fibers (C). Treated group showing. (D): Few collagen fibers (C) in the trabecular bone (dt) that appeared widely separated by bone marrow rich in fat cells (F). (E): Extracellular matrix (M) of epiphyseal plate has a weak staining affinity and appeared faint basophilic. (F): Cortical bone with osteoporotic cavities (O), and few blue-stained collagen fibers (C). Note: Non homogenous matrix. Protected group (as compared with control group) showing. (G): Nearly normal content and distribution of collagen fibers (C) in the trabecular bone (t). (H): Epiphyseal plate matrix (M) with a little reduced staining affinity in comparison with control group. (I): Cortical bone with nearly normal content and distribution of blue-stained collagen fibers (C) with no cavity formation. (scale bar=50µm).

bone marrow rich in fat cells (**Figure 2D**). The epiphyseal plate extracellular matrix had weak staining affinity so it appeared faint basophilic (**Figure 2E**). Femur diaphysis showed cortical bone with osteoporotic cavities, and few blue-stained collagen fibers. Note. Non homogenous matrix (**Figure 2F**).

Mallory trichrome stained sections of protected group retained about normal content and distribution of collagen fibers in the trabecular bone in comparison with control group (**Figure 2G**). The epiphyseal plate matrix showed a little reduced staining affinity in comparison with control group (**Figure 2H**). Whereas, the cortical bone showed nearly normal content and distribution of blue-stained collagen fibers with no cavity formation (**Figure 2I**).

Osteoprotegerin immunohistochemistry

Control group of distal femur metaphysis revealed; osteoprotegerin positive expression in the cytoplasm of most osteoblasts of endosteum. Some osteoprotegerin expression is also observed on the trabecular bone surface (**Figure 3A**). Examination of distal epiphyseal plate showed most hypertrophic chondrocytes and cartilage-bone interfaces are osteoprotegerin positive (**Figure 3B**). Whereas, cortical bone of diaphysis of the same group showed that most osteocytes were osteoprotegerin negative while few osteocytes were osteoprotegerin positive (**Figure 3C**).

Examination of osteoprotegerin stained sections of treated group showed trabecular bone having weak osteoprotegerin

Figure 3. Osteoprotegerin stained sections of rat femur. Control group.

(A): Distal metaphysis; showing the cytoplasm of most osteoblasts of endosteum are osteoprotegerin positive (arrowhead). Some osteoprotegerin expression is also observed on the trabecular bone surface (arrow) of distal metaphysis. (B): Epiphyseal plate showing most hypertrophic chondrocytes (arrow head) and cartilage-bone interfaces (arrow) are osteoprotegerin positive. (C): Cortical bone of diaphysis showing most osteocytes (curved arrow) are osteoprotegerin negative. Few osteocytes are osteoprotegerin positive (arrowhead). Treated group. (D): Trabecular bone showing weak osteoprotegerin expression in most of the cells covering the trabecular surface (arrow). (E): Epiphyseal plate revealing empty lacunae (curved arrow) and few cartilage-bone interfaces (arrow) are osteoprotegerin positive. (F): Cortical bone showing; few lining cells of osteoporotic cavities (arrow) and surface cells (double arrow) are osteoprotegerin positive. Protected group. (G): Trabecular bone showing some of the cells covering the trabecular surface are osteoprotegerin positive (arrow). (H): Epiphyseal plate; a positive immunoreaction is observed in some hypertrophic chondrocytes (arrow head). (I): Cortical bone, revealing extracellular osteoprotegerin expression observed in bone matrix (arrow). (scale bar=25µm).

expression in most of the cells covering the trabecular surface (**Figure 3D**). Examination of epiphyseal plate sections revealed empty lacunae with negative expression of osteoprotegerin whereas few cartilage-bone interfaces were osteoprotegerin positive (**Figure 3E**). As regard sections at femur diaphysis, lining cells of osteoprotic cavities were osteoprotegerin positive (**Figure 3F**).

Examination of osteoprotegrin stained sections of protected group: showed some of the cells covering the trabecular surface were osteoprotegerin positive (**Figure 3G**). The epiphysal plate sections of the same group showed a positive immunoreaction observed in some hypertrophic chondrocytes (**Figure 3H**). Whereas; cortical bone revealed extracellular osteoprotegerin expression observed in bone matrix (**Figure 3I**).

Electron microscopic results

Considering examination of ultrathin sections from cortical bone of femur diaphysis of control rats revealed osteoprogenitor cells on bone surface, they contained euchromatic indented nuclei. The osteoblasts were present on the surface side by side, each cell showed many rough endoplasmic reticulum, and euochromatic nucleus with periphral heterochromatin (**Figure 4A**).

In treated group; osteocytes had irregular nuclei. They were surrounded by many irregular collagen fibrils (**Figure 4B**). The osteoclasts had irregular nuclei and many membrane bound vacuoles of different sizes. The cells were surrounded with row of osteoblasts within their osteoid product (**Figure 4C**). In protected group; osteocytes showed oval nuclei and were surrounded by few collagen fibrils in regular lamellae (**Figure 4D**).

Biochemical results

There was no significant difference between the biochemical results of group IA, group IB and IC.

There was a significant increase in the levels of serum homocysteine in treated group in comparison with the control group. On the other hand, the protected group showed a significant decrease in homocysteine level when compared with treated group. There was no significant change between the protected group and the control group (**Table 1, Figure 5A**).

Morphometric results

There was no significant difference between the morphometric results of group IA, group IB and IC.

The mean cortical and trabecular bone thickness in group II (treated group) presented a significant decrease, ($p < 0.05$) in comparison with the control group. both parameters showed a significant increase when comparing group III with group II (**Table 2, Figures 5B and 5C**). As regard the number of osteoclasts, there was a highly significant increase in treated group in comparison with the control group. whereas; there was a highly significant reduction in the number of osteoclasts ($P < 0.001$) in protected group (III) as compared to group (II) (**Table 2, Figure 5D**).

Figure 4. Electron micrographs of ultrathin sections of femur cortical bone (A) control group showing an osteoprogenitor cell (arrow) with euchromatic indented nucleus (n). An osteoblast (arrow head) is also seen having euchromatic nucleus (N) with peripheral heterochromatin and many rough endoplasmic reticulum (ER). Treated group (B): Showing an osteocyte with irregular nucleus (N) containing condensed peripheral chromatin, and surrounded with many irregular collagen fibrils (C). (C): Showing an osteoclast with irregular nucleus (N). The cytoplasm contains many membrane bound-vacuoles (v) of different sizes. A row of osteoblasts, (OS) with heterochromatic nuclei (n) surrounding themselves within their osteoid product are also seen. (D): protected group showing an osteocyte with an oval nucleus (N). It is surrounded by few collagenous fibrils (C) in regular lamellae. (Scale bar=2 μ m).

Discussion

Recent reports have indicated a higher risk of fractures among men and women with high levels of serum homocysteine (HCY) [46]. The cause of moderate hyperhomocysteinemia among adults is mainly by folate or vitamin B₁₂ deficiency [47].

However, the mechanistic role of Hcy in osteoporosis is still unknown.

Regarding, the present histomorphometric results HCY treated group showed a significant reduction of cortical bone thickness with multiple cavity formation as well as a significant reduction in the trabecular bone thickness when compared with that of control rats of group I.

Previous researchers [48] have described osteoporosis as a syndrome of excessive skeletal fragility that results from both the loss of trabecular bone mass and trabecular bone connectivity.

Regarding the epiphyseal plate of HCy treated rats of the present study showed abnormal chondrocyte column cavity, shrunken atrophied chondrocytes with deeply stained nuclei.

The extracellular matrix had weak staining affinity.

The observed results were in accordance with previous researcher findings [1] who demonstrated that homocysteine

Table 1. Mean values of serum homocysteine ($\mu\text{mol/l}$) in different groups.

	Mean $\pm$ SD				
	Group IA	Group IB	Group IC	Group II (HCY treated)	Group III (HCY+folic acid) (protected)
Serum homocysteine ($\mu\text{mol/l}$)	6.4 $\pm$ 2.36	5.9 $\pm$ 1.2 ⁿ	6.3 $\pm$ 1.25 ⁿ	11.5 $\pm$ 3.23 ^a	7.2 $\pm$ 2.26 ^b

ⁿnon significant as compared with group IA

^asignificant as compared with group IA

^bsignificant as compared with group II

Figure 5. Histogram showing (A) the mean serum homocysteine ($\mu\text{mol/l}$), (B) outer cortical bone thickness (μm), (C) trabecular bone thickness (μm), (D) number of osteoclasts/hpf in different studied groups.

Table 2. Showing the mean outer cortical bone thickness, the mean bone trabecular thickness and the number of osteoclasts/hpf in different groups.

	Mean $\pm$ SD				
	Group IA	Group IB	Group IC	Group II (HCY treated)	Group III (HCY+folic acid) (protected)
Outer cortical bone thickness(μm)	499.98 $\pm$ 67.86	498 $\pm$ 75.7 ⁿ	501 $\pm$ 83.2 ⁿ	279.42 $\pm$ 48.87 ^a	370.47 $\pm$ 20.75 ^b
trabecular bone thickness(μm)	121.43 $\pm$ 24.1	119.3 $\pm$ 21.3 ⁿ	121.4 $\pm$ 34.2 ⁿ	70.13 $\pm$ 9.85 ^a	112.52 $\pm$ 8.4 ^b
number of osteoclasts/hpf	2 $\pm$ 0.1	3 $\pm$ 0.37 ⁿ	3 $\pm$ 0.32 ⁿ	14 $\pm$ 0.26 ^c	4 $\pm$ 0.27 ^d

ⁿnon significant as compared with group IA

^asignificant as compared with group IA

^bsignificant as compared with group II

^chighly significant as compared with group IA

^dhighly significant as compared with group II

promoted growth in hypophysectomized rats, a finding, associated with increased thickness of epiphyseal plate.

In addition, other investigators [49] stated that HCY induced endoplasmic reticulum stress and reduced the secretion and expression of extracellular superoxide dismutase leading to an increase in the production of extracellular reactive oxygen species in vascular smooth muscle cells, leading to increased oxidative stress in the vascular wall [50,51].

In the present study, there was an accumulation of fat cells in the bone marrow of group II, Whereas, the protected group showed a decrease in marrow fat accumulation with the use of folic acid. Previous researchers [52] had reported an inverse relationship between fat and bone volume. This inverse relationship has been documented in bone obtained from animal models with high levels of marrow fat infiltration, such as osteoporosis and senescence-accelerated mouse. The mechanism suggested is through shifting of mesenchymal stem cells into fat cells at the expense of osteoblasts [53].

In the current work, osteoclasts of the treated group exhibited an increase in number as proved statistically with subsequent bone erosions. Coinciding with these results, clinical studies have shown that adding homocysteine to chick-bud mesenchymal micromass culture caused matrix disorganization, decreased the ability of matrix to support mineralization, increased alkaline phosphatase activity, and abnormalities in collagen cross link formation [54]. Inhibition of collagen cross-linking [55], disturbance of osteoblast function [56], and increased osteoclast activity [57], were also observed in hyperhomocysteinemia.

Osteoprotegerin (OPG), is a member of the tumor necrosis factor receptor gene superfamily [58].

Osteoprotegerin (OPG) is secreted by stromal and osteoblastic lineage cells and inhibits osteoclastogenesis by preventing the interaction of receptor activator of nuclear factor- κ B ligand (RANKL) with receptor activator of nuclear factor- κ B (RANK) [41].

Ablation of OPG by targeted gene deletion in mice leads to early onset osteoporosis, demonstrating for the first time a critical requirement for endogenous OPG in the maintenance of normal postnatal bone mass and skeletal architecture [59].

In the current study, positive immunohistochemical expression of osteoprotegerin protein in control animals appeared mainly in the cytoplasm of the osteogenic cells and osteoblasts lining the trabecular surface, whereas epiphyseal plates showed positive reaction in maturative/hypertrophic chondrocytes. Most osteocytes of cortical bone appeared negative for osteoprotegerin. The distribution of OPG was previously demonstrated by previous studies [42].

In treated animals of the current work; the trabecular bone showed weak osteoprotegerin expression in most of the cells covering the trabecular surface. Whereas, negative expression of osteoprotegerin was demonstrated in maturative/hypertrophic chondrocytes of epiphyseal plate while treated cortical bone showed positive expression of OPG in few lining

cells of osteoprotic cavities.

Similar observations were detected by previous studies [42] who also observed an extracellular immunolocalization of osteoprotegerin in the cement lines. The presence of osteoprotegerin in these site-specific areas of the trabecular bone matrix could be related to its protective action against the resorbing activity of osteoclasts.

An additional role for OPG in regulating remodeling within the cortical shafts of long bones and within bones of the skull was recorded in OPG-deficient mice [59].

It was found that osteoprotegerin administration inhibited trabecular bone loss and maintained the internal trabecular bone structure and volume in osteoprotegerin deficient mice [60].

In addition, osteoprotegerin administration in mice resulted in a decrease in the number of osteoclasts together with absence of the ruffled border denoting reduced activity. The positive effect of osteoprotegerin on bone was most probably related to its antiosteoclastogenic capacity [61].

With reference to rats treated with HCY in the current study, electron microscopic examination revealed many degenerative changes in osteoblasts and osteocytes in the form of irregular nuclei with condensation of their chromatin. The matrix appears highly affected and its fibrils were irregularly arranged. On the other hand, the osteoclasts became more active, their cytoplasm contained many membrane-bound vesicles of various sizes that represent many lysosomes or ruffled border cut in many planes. Therefore, the osteoclasts were more active while osteoblasts and osteocytes were degenerated; reflecting that the bone resorption was more than bone formation with an inaccurate bone remodeling.

Previous experimental data have suggested that high HCY levels affected both osteoclasts and osteoblasts. It was suggested a dysbalance between osteoblasts and osteoclasts in favor of osteoclasts as a major mechanism for HCY-induced bone loss and reduced bone quality *in vivo* [4,6].

The ultrastructural results of the current work also showed that HCY treatment resulted in an irregular deposition of collagen fibrils in the extracellular compartment. This could be attributed to disruption of collagen cross-links with other agents which prevent the orderly arrangement of the extracellular matrix. The abnormal deposition of collagen in the extracellular matrix may lead to abnormal cell-matrix interactions [54].

In our study, rats of group III (protected with folic acid) had shown significant lower levels of homocysteine, histologically, these animals were protected from bone loss as compared with treated rats. However, it is not clear whether folic acid directly prevents bone loss or acts via its homocysteine lowering effect. This needs to be clarified in the next studies.

Prevention of bone loss and hyperhomocysteinemia by folic acid supplements in the present study are consistent with previous studies [62,63] which stated that the serum homocysteine level is inversely associated with folate level,

and that folate supplements therapy can lower homocysteine level effectively.

Previous data suggested a role of folic acid in modulating the expression of the redox enzymes (Trx-1/NOX-4) disrupted by increased levels of HCY. These results also suggested that folic acid alleviates the oxidative stress [37].

Conclusion

The results of this study showed that hyperhomocysteinemia induced marked structural changes in the epiphyseal plate, trabecular and cortical bone of femur in young rats. These changes confirmed the association between increased homocysteine level and fracture risk. The suggested mechanism for these changes could be related to decreased osteoprotegerin expression as proved in our work.

Moreover, folic acid provided significant protection against bone changes induced by increased homocysteine levels. However, it is not clear whether folic acid directly prevents bone loss or acts via its homocysteine lowering effect. We recommend further research to clarify this point in the next studies. In addition, future research studies are needed to evaluate the potential clinical utility of folic acid as a therapeutic agent to prevent osteoporosis associated with hyperhomocysteinemia.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	RAZ	HMV
Research concept and design	✓	✓
Collection and/or assembly of data	✓	✓
Data analysis and interpretation	✓	✓
Writing the article	✓	--
Critical revision of the article	--	✓
Final approval of article	--	✓
Statistical analysis	✓	--

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