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ORIGINAL ARTICLE

Protective effect of folic acid on cyclosporine-induced bone loss in rats

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Summary

Hyperhomocysteinemia is seen in patients with decreased bone mineral density. Cyclosporine can cause alveolar bone loss and osteopenia. It is also associated with elevated serum homocysteine levels. We aimed to investigate the effect of cyclosporine on serum homocysteine level, bone volume, and bone density, and determine whether folic acid had a protective effect against bone loss. In an experimental study, 40 male Sprague–Dawley rats were randomly assigned to five groups and received dietary supplementation for 6 weeks with olive oil (Group A), cyclosporine (Group B), folic acid (Group C), and cyclosporine plus folic acid (Group D), or no supplementation (Group F, control). Serum homocysteine, calcium, alkaline phosphatase, total bone volume, periodontal ligament volume, and volume density of bone were compared between groups. Mean serum homocysteine level (10.84 ± 0.93 μmol/l) was significantly higher in group B (cyclosporine supplementation) compared with the other groups ($P = 0.001$). Mean total mandibular volume was 46.3 ± 13.6 mm^3 in rats treated with cyclosporine, 80.4 ± 15.70 mm^3 in rats treated with folic acid ($P = 0.004$), and 73.9 ± 21.3 mm^3 in rats treated with cyclosporine plus folic acid ($P = 0.028$). In our experimental model, cyclosporine increased serum homocysteine levels and decreased bone volume and density. Folic acid may have a preventive role against bone loss in rats treated with cyclosporine.

Introduction

Cyclosporine, an immunosuppressive agent discovered in 1970, interferes with interleukin-2 synthesis and has inhibitory effects on T-lymphocyte-dependent immune responses [1]. It was initially used to prevent rejection episodes among transplant patients, but now it is being used to manage several illnesses including psoriasis, rheumatoid arthritis, multiple sclerosis, myasthenia gravis, and other autoimmune disorders [2–4]. Like other drugs, cyclosporine has side effects. With expanding indications for its application, it is not surprising that its adverse effects are being seen increasingly among treated patients. Nephrotoxicity, hypertension, and dyslipidemia are among the important side effect of this medication [5].

One of the other proposed side effects of cyclosporine is bone loss after long-term use. Although it is a controversial topic, it was recently shown that cyclosporine causes alveolar bone loss in rats [6]. Increased osteoclasia and decreased bone formation were also observed in periodontal sites in the alveolar bones of cyclosporine-treated rats [7,8]. Another animal model study confirmed that high-dose cyclosporine caused osteopenia with decreased bone mass and serum osteocacin [9]. Furthermore, cyclosporine treatment is associated with elevated serum homocysteine, and predisposes patients to cardiovascular

complications [10]. In addition, several studies have shown that hyperhomocysteinemia was associated with higher bone turnover, poor physical performance and lower bone mineral density [11], as well as higher rates of osteoporosis and increased risk of fracture [12].

Thus, we hypothesized that cyclosporine-associated bone loss and osteoporosis is mediated by elevated serum homocysteine levels. We conducted this study to assess the effect of cyclosporine on bone volume and serum homocysteine, and also to evaluate the possible preventive effects of folate supplements on these factors in male rats.

Materials and methods

Animals

Forty male Sprague–Dawley rats, 2–2.5 months old, with an average body weight of 200 ± 20 g were randomly distributed into five groups each consisting of eight rats. Group A received a dietary supplement of olive oil, Group B received cyclosporine, Group C received folic acid, Group D received cyclosporine plus folic acid, and Group F (controls) received no dietary supplementation. All animals were housed in our animal lab located in the Endocrine and Metabolism Research Center of Shiraz University of Medical Sciences, Iran, under the same situation (ambient temperature of 25 °C, 12 h light, and 12 h night cycles). They were handled at all times in accordance with our center's guidelines for experimental animals and current national law regarding the ethical handling of research animals. They were all fed a customized rat chow diet (Pars Dam Co, Tehran, Iran) containing 60% vegetable starch, 10% corn oil, and 30% animal protein. Cumulative food and water intake were almost identical in all groups, and the animals had free access to chow and water. Cyclosporine is an immunosuppressive agent, and the rate of mortality among our animals increased during the first days of study. The animals were weighted biweekly, and drug doses were adjusted according to body weight.

Study protocols

Cyclosporine was obtained from (Novartis Pharma AG, Basel, Switzerland). Folic acid tablets and olive oil were prepared according to previous animal studies. Considering the basal metabolism in the rat body, the therapeutic dose of cyclosporine was considered to be 15 mg/kg. Cyclosporine 15 mg/kg was dissolved in 2 ml olive oil, and was administered daily via gastric feeding using insulin syringes. Daily folic acid (20 mg/kg) was given to the rats using the same method. The study lasted for 6 weeks. Serum samples were obtained from all the animals before and after the experimental feeding period to determine serum calcium, alkaline phosphatase, and homocysteine.

After food and water were withheld for 12 h, blood samples were taken from a tail vein of rats in each group at the start of study and 6 weeks later. Blood samples were collected on ice; serum samples were immediately separated and stored at −20 °C until analysis. Homocysteine was measured using enzyme-linked immunoassay (Axis-Shield Diagnostics, Dundee, UK).

At the end of study and after blood was sampled, the animals were killed, and the mandibles were removed, cleaned of soft tissues, and fixed in buffered formalin 4% for a week. The bone was decalcified with nitric oxide 25%, and the mandibles were divided into two parts (A and B) below the level of eruption of each incisor [13]. Periodontal ligament (PDL) and bone sections were obtained from parts A and B of the mandibles. Terminal molars were removed from part A, and incisors (above the alveolar crest) were resected from part B, and were embedded in paraffin. Serial paraffin sections of 6 μm and 20 μm were obtained from both parts of mandibles and stained using the Heidenhain Azan rapid technique [14].

Volume measurement

Volumetric measurements were calculated manually using the Cavalieri method [15]. In this method, the cut surface areas of the sections are calculated, and multiplying the total cut surface area by the mean section thickness provides an estimate of the volume of the examined object [16]. The cut surface area of each section or slab was estimated with point-counting grids [17]. The point-counting grid, which has point arrayed in different densities on a transparent sheet, is superimposed on sections randomly, and the points that lie on the cut surface are counted [18]. Finally, the volume of the object is estimated using the following formula:

$$\text{Total Volume(TV)} = t * a/p * \Sigma P$$

where, ΣP is the total number of the points hitting the cut surface areas, t is the section thickness, and a/p is the area of each point on the point-counting grid.

Volume density was measured according to Delesse's principles using the formula:

$$\text{VD} = P(\text{Structure})/P(\text{Ref}) * 100$$

where, P (Structure) is the number of points on tissue section and P (Ref) is the number of points on the whole mandible.

Absolute volume (AV) was calculated by multiplying volume density (VD) for each point by total volume (TV) of the bone or periodontal ligament using the formula

$$\mathrm{AV}\left(\mathrm{mm}^3\right) = \mathrm{VD} * \mathrm{TV}$$

The number of cavities was calculated using a dissector technique in 20-μm sections. Total volume of mandible (mm^3), absolute volume of mandible (mm^3), absolute volume of cavities (mm^3), volume density of bone, volume density of cavities, and total volume of PDL (mm^3), were calculated using above mentioned methods.

Statistical analysis

All variables are expressed as the mean ± SEM. The differences between the experimental and control groups were tested statistically using the unpaired Student's *t*-test for variables with a parametric distribution and the Mann–Whitney test for variables with a nonparametric distribution. *P* value of <0.05 was considered statistically significant.

Results

Of the 40 rats at the start of the experiment, data were analyzed for 32. Eight rats died during the study. The group statistics are summarized in Table 1. Mean serum homocyteine level (10.84 ± 0.93 μmol/l) was significantly higher in group B (cyclosporine) compared with the other groups ($P = 0.001$). Mean serum alkaline phosphatase and calcium did not differ significantly between groups ($P > 0.05$).

Total mandibular volume, absolute bone volume, and absolute volume of cavities, were significantly lower in rats treated with cyclosporine (group B) compared with rats treated with folic acid (group C) (Table 2). Total mandibular volume, absolute bone volume, and absolute volume of cavities were also significantly lower in rats treated with cyclosporine (group B) in comparison to rats treated with cyclosporine and folic acid (group D) (Table 3). Total PDL volume and bone volume density showed no significant differences between groups.

The comparisons between rats receiving olive oil (group A) versus cyclosporine group (group B), and rats receiving cyclosporine (group B) versus cyclosporine + folic acid group (group D) were outlined in Fig. 1.

Table 2. Comparison between rats treated with cyclosporine (group B) and rats treated with folic acid (group C).

	Group B	Group C	*P* value
TV (mm^3)	46.3 ± 13.6	80.4 ± 15.70	0.004
AMV (mm^3)	36.6 ± 10.2	58.7 ± 18.03	0.020
AVC (mm^3)	9.5 ± 3.5	17.1 ± 4.1	0.014
VBD	79.7 ± 3.3	77.2 ± 4.8	0.386
VDC	20.8 ± 3.7	21.3 ± 3.7	0.841
TV(pdl) (mm^3)	0.36 ± 0.06	0.55 ± 0.21	0.118
Alk.ph1 (U/l)	407 ± 74.3	413.6 ± 93.2	0.892
Alk.ph2 (U/l)	499.2 ± 162.1	381.5 ± 101.1	0.095
Ca1 (mg/dl)	7.9 ± 0.5	8.5 ± 1.1	0.498
Ca2 (mg/dl)	7.5 ± 0.4	6.3 ± 0.9	0.158

Group B: cyclosporine, Group C: folic acid.
Alk.ph1, alkaline phosphates before experiment; Alk.ph2, alkaline phosphates after experiment; AMV, absolute volume of mandible; AVC, absolute volume of cavities; Ca1, calcium before experiment; Ca2, calcium after experiment; Hcy, homocysteine; TV, total volume of mandible; TV (pdl), total volume of periodontal ligament; VBD, volume density of bone; VDC, volume density of cavities.

Discussion

Our results show that hyperhomocysteinemia occurred in rats fed cyclosporine in the diet. However, elevated serum

Table 1. Bone and mineral variables in the five groups of rats.

	Group A	Group B	Group C	Group D	Group F
TV (mm^3)	73.1 ± 26.1	46.3 ± 13.6	80.4 ± 15.70	73.9 ± 21.3	77.7 ± 23.4
AMV (mm^3)	57.2 ± 21.9	36.6 ± 10.2	58.7 ± 18.03	56.2 ± 17.7	62.6 ± 19.9
AVC (mm^3)	15.8 ± 5.3	9.5 ± 3.5	17.1 ± 4.1	17.3 ± 5.2	14.8 ± 3.4
VBD	75.6 ± 6.7	79.7 ± 3.3	77.2 ± 4.8	75.8 ± 5.04	80.3 ± 2.7
VDC	23.05 ± 6.5	20.8 ± 3.7	21.3 ± 3.7	23.9 ± 5.2	19.6 ± 2.7
TV(pdl) (mm^3)	0.4 ± 0.2	0.36 ± 0.06	0.55 ± 0.21	0.5 ± 0.17	0.68 ± 0.17
Alk.ph1 (U/l)	379.5 ± 105.1	407 ± 74.3	413.6 ± 93.2	437.7 ± 91.0	416.7 ± 141.3
Alk.ph2 (U/l)	411.7 ± 107.9	499.2 ± 162.1	381.5 ± 101.1	475.4 ± 139.8	399.5 ± 125.9
Ca1 (mg/dl)	8.6 ± 1.2	7.9 ± 0.5	8.5 ± 1.1	8.5 ± 1.3	8.6 ± 1.4
Ca2 (mg/dl)	8.7 ± 1.1	7.5 ± 0.4	8.2 ± 1.06	8.1 ± 0.9	9.2 ± 0.9
Hcy (μmol/l)	5.4 ± 0.6	10.8 ± 0.9	6.3 ± 0.9	5.9 ± 0.5	5.8 ± 0.4

Group A: dietary supplement of olive oil, Group B: cyclosporine, Group C: folic acid, Group D: cyclosporine plus folic acid, and Group F (controls): no dietary supplementation.
Alk.ph1, alkaline phosphates before experiment; Alk.ph2, alkaline phosphates after experiment; AMV, absolute volume of mandible; AVC, absolute volume of cavities; Ca1, calcium before experiment; Ca2, calcium after experiment; Hcy, homocysteine; TV, total volume of mandible; TV (pdl), total volume of periodontal ligament; VBD, volume density of bone; VDC, volume density of cavities.

Table 3. Comparison between rats treated with cyclosporine (group B) and rats treated with cyclosporine and folic acid (group D).

	Group B	Group D	*P* value
TV (mm^3)	46.3 ± 13.6	73.9 ± 21.3	0.028
AMV (mm^3)	36.6 ± 10.2	56.2 ± 17.7	0.042
AVC (mm^3)	9.5 ± 3.5	17.3 ± 5.2	0.028
VBD	79.7 ± 3.3	75.8 ± 5.04	0.123
VDC	20.8 ± 3.7	23.9 ± 5.2	0.372
TV (pdl)	0.36 ± 0.06	0.5 ± 0.17	0.202
Alk.ph1 (U/l)	407 ± 74.3	437.7 ± 91.0	0.740
Alk.ph2 (U/l)	499.2 ± 162.1	475.4 ± 139.8	0.935
Ca1 (mg/dl)	7.9 ± 0.5	8.5 ± 1.3	0.625
Ca2 (mg/dl)	7.5 ± 0.4	8.1 ± 0.9	0.141

Group B: cyclosporine, Group D: cyclosporine plus folic acid.
Alk.ph1, alkaline phosphates before experiment; Alk.ph2, alkaline phosphates after experiment; AMV, absolute volume of mandible; AVC, absolute volume of cavities; Ca1, calcium before experiment; Ca2, calcium after experiment; Hcy, homocysteine; TV, total volume of mandible; TV (pdl), total volume of periodontal ligament; VBD, volume density of bone; VDC, volume density of cavities.

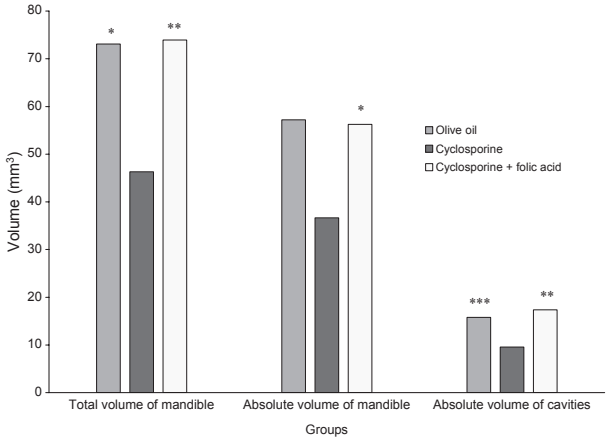

Figure 1 Total volume of mandible, absolute volume of mandible, and absolute volume of cavities in rats receiving olive oil (group A), cyclosporine (group B), and cyclosporine + folic acid (group D). $*P = 0.04$; $**P < 0.05$; $***P = 0.03$.

homocysteine levels were not found in rats treated with folic acid. There was also a significant reduction in total mandibular volume, absolute bone volume, and bone volume density in cyclosporine-treated animals, whereas rats treated with cyclosporine plus folic acid were protected. These findings support the hypothesis that cyclosporine-associated bone loss and osteoporosis are mediated by elevated serum homocysteine levels. By increasing number of patients receiving cyclosporine, clinicians encountered its numerous debilitating side effects. Our findings, if confirmed in large population human studies, will have important influence in prevention of bone loss as one of the side effects of cyclosporine.

Osteoporosis is defined as ''a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture'' [19]. It is a common and costly disease with a considerable annual health system burden all over the world. Morbidity and mortality related to this disease and its associated fragility fractures are also increasing [19]. It is estimated that 200 million women have osteoporosis worldwide [20]. So, the prevention and treatment of osteoporosis can potentially reduce costs to health systems and improve the quality of life of affected patients.

Risk factors for osteoporosis can be classified according to clinical, medical, nutritional, behavioral, and genetic variables [21]. Among medical risk factors, exposure to certain medications can exacerbate osteoporosis. These include glucocorticoids, anticonvulsants, and cytotoxic and immunosuppressant drugs [21]. Cyclosporine is an immunosuppressive agent that is widely used in transplant patients and diseases of the immune system such as rheumatoid arthritis and psoriasis [22,23]. Hyperlipidemia, hypertension, and metabolic syndrome are among the complications of cyclosporine [5,24]. Several animal studies showed that cyclosporine can contribute to bone loss and osteopenia [25,26]. Bone loss was seen in trabecular bone more than cortical bone [27]. The first evidence of bone loss associated with cyclosporine treatment in humans was obtained from patients receiving cyclosporine as an immunosuppressive agent after heart transplantation [28,29]. Subsequently, others showed that the long-term use of cyclosporine had negative effects on bone mineral density in patients with rheumatoid arthritis [30]. Bone mineral density was diminished in patients treated with oral cyclosporine for eczema [31]. Movsowitz *et al.* [32] showed that the effect of cyclosporine on bone mineral metabolism is dependent on the dose and duration of administration. The exact mechanism by which cyclosporine causes bone loss is not clear. Cyclosporine inhibits osteoblast differentiation, osteocalcin production, and collagen synthesis, reducing the bone replaced in each remodeling cycle [33]. Some have proposed that the role of T lymphocytes via receptor activator of NFκ B ligand (RANKL) is essential for triggering cyclosporine-associated bone loss; however, the exact mechanism for this action is still obscure [34].

Elevated serum homocysteine levels were reported in patients treated with cyclosporine [10]. Homocysteine is an amino acid well known for its proposed role in cardiovascular disease [35,36]. The first evidence that hyperhomocysteinemia can affect bone quality appeared in patients with homocystinuria, an inborn error of metabolism [37]. These patients present with very high levels of homocysteine, early ospteoporosis, and skeletal

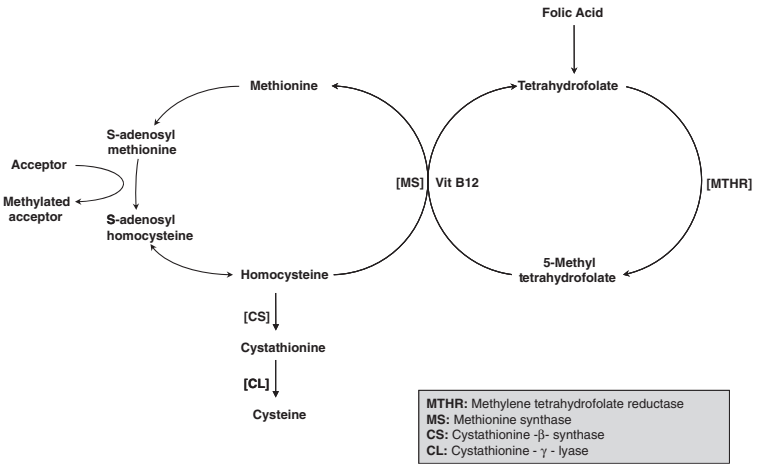

Figure 2 The folic acid-Homocysteine metabolism pathway.

disproportion because of excess growth of the long bones [38]. Nevertheless, this topic remains controversial. Several studies confirmed an association between homocysteine and bone mineral density in postmenopausal women [39,40], in patients with Crohn's disease [41], primary billiary cirrhosis [42], and hyperparathyroidism [43]. However, other studies failed to confirm such an association [44]. We have previously showed that the administration of homocysteine caused osteopenia in newborn rats [45]. The mechanisms that elevated homocysteine level causes bone loss has not been clearly identified yet. Khan *et al.* showed 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 [46]. Inhibition of collagen cross-linking [47], disturbance of osteoblast function [48], and increased osteoclast activity [49], were also observed in hyperhomocysteinemia.

Another study at our center showed that hyperhomocysteinemia, secondary to folate deficiency, is associated with lower bone mineral density and may contribute to the pathogenesis of osteoporosis in postmenopausal women [50]. Prevention of bone loss and hyperhomocysteinemia by folic acid supplements in the present study provides other evidence that homocysteine-folate metabolism pathway may involve in cyclosporine-induced bone loss. The homocysteine metabolism pathway and several factors associated with serum homocysteine level were outlined in Fig. 2. The pathway showed the close association of folate metabolism with homocysteine metabolism. Serum homocysteine is inversely associated with folate level, and folate supplements therapy can lower homocysteine level effectively. In our study, rats treated with folic acid and cyclosporine had significantly lower levels of homocysteine, and were protected from bone loss compared with cyclosporine only 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. A newly published study that potentiates our theory showed that folic acid supplements ameliorates bone loss in a cystathionine-β-synthase heterozygous (CBS±) mice as a genetic model of hyperhomocysteinemia-induced bone loss [51].

Our study is the first to propose that cyclosporine-induced hyperhomocysteinemia may be associated with bone loss and may be preventable using oral folate supplements. Cyclosporine is now widely used in patients with immune based disease. Patients undergoing solid organ transplantation are long-term users of cyclosporine to prevent allograft rejection. Glucocorticoids are other components of immunosuppressive regimens after transplantation. It has been established that even low doses of glucocorticoids are accompanied with significant bone loss. Glucocorticoids induce apoptosis of osteoblasts and osteocytes, and decrease replication and differentiation of osteoblasts. They also inhibit expression of genes for type I collagen, osteocalcin, transforming growth factor β (TGF β), and receptor activator for NF κ B- ligand (RANK-L) [52]. There is lack of data comparing glucocorticoids and cyclosporine regarding their negative effects on bone; however, it is clear that organ transplant patients are more susceptible to bone loss using these two drug categories. Therefore, understanding underlying mechanisms and new treatment modalities of cyclosporine-associated bone loss can minimize one of the most important complications after transplantation. Placebo-controlled trials should be conducted to evaluate the benefits of folate supplementation in preventing cyclosporine-induced bone loss in patients on long-term cyclosporine treatment.

Authorship

AM, LO, LRO: participated in the performance of the research and design of the study. FK: participated in the design of the study. AE: participated in the performance of the research, data analysis, and writing the paper. ZA: participated in the performance of the research. GRO: participated in the design and performance of the research, data analysis, and writing the paper.

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