

Dietary iron supplementation reverses the effects of iron deficiency on bone formation markers in iron-deficient rats

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Abstract

Iron plays important roles in bone health as it is required as a cofactor for renal 25-hydroxyvitamin D-1 α -hydroxylase, which is involved in the production of 1,25-dihydroxyvitamin D₃ (active vitamin D), and regulates the expression of proteins involved bone formation. Herein, we investigated the effects of dietary iron supplementation on bone formation in iron-deficient rats. Male Wistar rats were fed a control (n = 12) or iron-deficient diet (n = 18) for 30 days, after which 6 rats in the iron-deficient group were switched to the control diet for 14 days. At the end of the 30-day period, hemoglobin, liver iron, serum 1,25-dihydroxyvitamin D₃, and serum osteocalcin concentrations, and femoral bone mineral density (BMD) were found to be significantly lower in iron-deficient rats than that in control rats. Following dietary iron supplementation, hemoglobin and liver iron concentrations elevated significantly in iron-deficient rats; however, they were not restored to the normal levels. Additionally, dietary iron supplementation increased serum 1,25-dihydroxyvitamin D₃ and osteocalcin concentrations and femoral mRNA expression of *Bglap* and *Col1a1* in iron-deficient rats, which were downregulated by iron deficiency, to levels similar to that in control rats. However, femoral BMD was not recovered with dietary iron supplementation. These results suggest that iron deficiency-induced downregulation of bone formation can potentially be reversed by dietary iron supplementation. Furthermore, elevated serum 1,25-dihydroxyvitamin D₃ concentrations due to dietary iron supplementation could be responsible for increased expression of bone formation markers in iron-deficient rats.

Keywords: iron deficiency, dietary iron supplementation, bone formation, 1,25-dihydroxyvitamin D₃, rats

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Introduction

Iron deficiency is one of the most common nutritional deficiencies worldwide, and reportedly affects bone health. One of the mechanisms through which iron participates in bone formation is through its role in the activation of vitamin D. Renal 25-hydroxyvitamin D-1 α -hydroxylase (1 α -hydroxylase), an enzyme that used iron as a cofactor, converts 25-hydroxyvitamin D to its active form vitamin D [1]. We previously demonstrated that dietary iron deficiency diminishes renal 1 α -hydroxylase activity, which consequently leads to a decrease in serum 1,25-dihydroxyvitamin D₃ concentration in rats [2]. Given the role of 1,25-dihydroxyvitamin D₃ in bone metabolism, lower



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serum 1,25-dihydroxyvitamin D₃ concentration could potentially result in decreased bone formation in iron-deficient rats.

Moreover, previous studies have investigated the relationship between dietary iron and bone mineral density (BMD). In human studies, dietary iron was found to be positively associated with BMD in postmenopausal women [3, 4]. Medeiros *et al.* reported that iron deficiency decreases BMD, bone mineral content, and bone strength in female weanling rats [5]. These findings were consistent with the results of our previous studies with male weanling rats [6, 7]. Furthermore, we previously showed that an iron-deficient diet causes a decrease in serum osteocalcin concentration in rats [6, 7]. In addition, dietary iron deficiency decreases serum procollagen type I N-terminal propeptide (P1NP) concentration in rats [8]. Therefore, we speculated that dietary iron deficiency inhibits osteoblastic bone formation, which could potentially account for diminished BMD in iron-deficient rats.

It has been speculated that the physiological changes caused by an iron-deficient diet can be restored by re-addition of iron into the diet. Consistently, it is speculated that the decrease in bone formation due to iron deficiency can be improved following recovery from iron deficiency. Therefore, in the present study, we investigated the effects of dietary iron supplementation on bone formation in iron-deficient rats.

Materials and Methods

Ethical approval

This study was approved by the Tokyo University of Agriculture Animal Use Committee (approval number 280070) and performed in accordance with the university guidelines for the care and use of laboratory animals.

Experimental design

Thirty 3-week-old male Wistar rats (Clea Japan, Tokyo, Japan) were individually housed in stainless-steel cages at 22 °C in a 12-hour light-dark cycle. Throughout the study period, the rats were fed a control diet or an iron-deficient diet and were given free access to deionized water. Experimental diets were prepared according to the AIN-93G formula [9]. The iron-deficient diet was prepared from a basal diet by adding iron-free AIN-93G mineral mixture instead of AIN-93G mineral mixture. Iron content of the control and iron-deficient diets were 54.5 µg/g and 8.9 µg/g, respectively.

Iron-deficient rats were fed an iron-deficient diet for 30 days. Briefly, rats (58.6 ± 2.9 g) were divided into the control (C0, n = 12) and iron-deficient (D0, n = 18) groups. The D0 group was given free access to an iron-deficient diet, and the C0 group was pair-fed the control diet at the mean intake of the D0 group for 30 days. On day 30, six rats from each group were sacrificed under anesthesia by intraperitoneal injection of anesthetic mixture (medetomidine, midazolam, and butorphanol), and blood, liver, and the left femur were collected for further analysis.

After the initial 30 day feeding period, rats in the D0 group were divided into the iron-deficient (D, n = 6) and recovery (R, n = 6) groups. Rats in D group were provided free access to an iron-deficient diet for another 14 days. Whereas, rats in the R and C0 groups (C, n = 6) were pair-fed a control diet at the mean intake of the D group. After 14 days, all rats were sacrificed under anesthesia, and blood, liver, and the left and right femurs were collected for further analysis. The hearts were removed and weighed to assess cardiac enlargement, a sign of iron-deficiency anemia.

Assessment of serum levels of bone formation markers and 1,25-dihydroxyvitamin D₃

Hemoglobin concentration in blood samples was measured using the Hemoglobin B-test Wako (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Additionally, blood samples were centrifuged at 1,500 × g for 15 min, and the supernatants were collected as serum samples to analyze the levels of osteocalcin, a bone formation marker and 1,25-dihydroxyvitamin D₃. Serum samples were stored at -80 °C until analysis. Serum 1,25-dihydroxyvitamin D₃ concentration was measured using the 1,25(OH)₂ vitamin D ELISA kit (Immundiagnostik AG, Bensheim, Germany). Serum osteocalcin concentration was measured using the Rat Osteocalcin (BGP) ELISA system (GE Healthcare Japan Corporation, Tokyo, Japan).

Assessment of iron content in the liver

Briefly, liver samples were flushed with ice-cold 0.9% NaCl through the portal vein. One gram of liver samples were dried at 100 °C for 24 hours. For measurement of iron, dried liver samples were ashed in a muffle furnace at 550 °C for 48 hours, and the minerals were diluted with 1 mol/L HCl solution. Subsequently, the iron content was analyzed using an atomic absorption spectrophotometer (ZA3300; Hitachi, Ltd., Tokyo, Japan).

Assessment of BMD

Following collection, left femur samples were placed in 70% ethanol at 4 °C until analysis, and dried at 60 °C overnight. Subsequently, BMD of the femur was measured using dual-energy X-ray absorptiometry (DXA; DSC-600EX, Hitachi Aloka Medical, Ltd., Tokyo, Japan).

Real-time PCR to assess mRNA levels of osteocalcin and collagen type I alpha 1 chain

Right femur samples were removed and stored at –80 °C until further analysis. The femur samples were homogenized in TRI Reagent (Molecular Research Center Inc., OH, USA) using ceramic bead-containing tubes and a Precellys homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) with two cycles of 7,500 rpm for 20 seconds. Total RNA was isolated from the cell lysate using the Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA). cDNA was synthesized using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR was performed on a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). The following primers were used for analysis: osteocalcin (*Bglap*), forward, 5'-GAGCTAGCGGACCACATTGG-3' and reverse, 5'-CCTAAACGGTGGTGCCATAGA-3'; collagen type I alpha 1 chain (*Col1a1*), forward, 5'-TTCACCTACAGCACGCTTG-3' and reverse, 5'-GATGACTGTCTTGCCCCAAGTT-3'; β -actin (*Actb*), forward, 5'-AGCCATGTACGTAGCCATCCA-3' and reverse, 5'-TCTCCGGAGTCCATCACAATG-3' [10, 11]. *Bglap* and *Col1a1* mRNA expression was normalized to that of the housekeeping gene, *Actb*.

Statistical analysis

Results are expressed as the mean \pm standard error for each group (n = 6 rats/group). Statistical analyses were performed using the SPSS Statistics ver.23 (IBM, Armonk, NY, USA). Homogeneity of variance between the C0 and D0 groups was assessed using the F-test, and an independent t-test was used to determine significant differences between the C0 and D0 groups. One-way analysis of variance followed by the least significant difference test was performed to evaluate significant differences among the C, D, and R groups. Differences were considered statistically significant at $p < 0.05$.

Results

Effects of dietary iron deficiency on body weight, iron status, serum 1,25-dihydroxyvitamin D₃ and osteocalcin concentrations, and femoral BMD

There was no significant difference in final body weight between the C0 and D0 groups (Table 1). However, heart weight was significantly higher in the D0 group than that in the C0 group, suggesting cardiac enlargement. Consistently, hemoglobin and liver iron concentrations were significantly lower in the D0 group than that in the C0 group. Additionally, serum 1,25-dihydroxyvitamin

Table 1. | Body weight, indicators of iron deficiency, serum 1,25-dihydroxyvitamin D₃ and osteocalcin concentrations, and BMD of the femur in the C0 and D0 groups.

| | C0 | D0 |
|--|------------------|------------------|
| Final body weight (g) | 209.5 \pm 2.5 | 199.6 \pm 4.6 |
| Heart weight (g/100 g body weight) | 0.32 \pm 0.01 | 0.56 \pm 0.02* |
| Hemoglobin (g/dL) | 11.41 \pm 0.43 | 3.59 \pm 0.10* |
| Liver iron (μ g/g dry weight) | 402.8 \pm 37.6 | 151.4 \pm 7.6* |
| Serum 1,25-dihydroxyvitamin D ₃ (pg/mL) | 112.2 \pm 4.4 | 81.0 \pm 6.5* |
| Serum osteocalcin (ng/mL) | 91.8 \pm 2.6 | 80.8 \pm 3.1* |
| Femur BMD (mg/cm ²) | 108.4 \pm 1.9 | 102.1 \pm 1.6* |

C0, control group; D0, iron-deficient group; BMD, bone mineral density. Data are presented as the mean \pm standard error (SE) for each group (n = 6).

*Significant difference compared to the C0 group, $p < 0.05$.

D₃ and osteocalcin concentrations and femur BMD were significantly lower in the D0 group than in the C0 group. These findings suggest that iron deficiency affects bone health through regulation of 1,25-dihydroxyvitamin D₃ and osteocalcin.

Effects of dietary iron supplementation on body weight and iron status of iron-deficient rats

There was no significant difference in final body weight among the C, D, and R groups (Table 2). However, heart weight was significantly higher in the D group than that in the C group and significantly lower in the R group than in the D group. Moreover, hemoglobin and liver iron concentrations were significantly lower in the D group than that in the C group and were significantly higher in the R group than that in the D group. These findings suggest that iron supplementation in iron-deficient rats cannot completely reverse the effects of iron deficiency, namely cardiac enlargement and anemia.

Table 2. | Body weight and indicators of iron deficiency in the control, iron-deficiency, and recovery groups.

| | C | D | R |
|------------------------------------|---------------------------|--------------------------|--------------------------|
| Final body weight (g) | 251.7 ± 1.5 | 230.3 ± 10.9 | 232.5 ± 4.2 |
| Heart weight (g/100 g body weight) | 0.29 ± 0.01 ^c | 0.62 ± 0.03 ^a | 0.43 ± 0.02 ^b |
| Hemoglobin (g/dL) | 14.0 ± 0.5 ^a | 3.2 ± 0.2 ^c | 11.2 ± 0.2 ^b |
| Liver iron (µg/g dry weight) | 444.1 ± 20.8 ^a | 161.9 ± 8.9 ^c | 210.4 ± 7.3 ^b |

C, control group; D, iron-deficient group; R, recovery group. Data are presented as the mean ± SE for each group of 6 rats.

^{a,b,c}The different letters denote significant differences, $p < 0.05$.

Effects of dietary iron supplementation on serum 1,25-dihydroxyvitamin D₃ and osteocalcin concentrations, femoral BMD, and femoral mRNA expression of *Bglap* and *Col1a1* in iron-deficient rats

Serum 1,25-dihydroxyvitamin D₃ concentrations were significantly lower in the D group than that in the C group and significantly higher in the R group than that in the D group (Figure 1). Serum osteocalcin concentration and mRNA expression of *Bglap* and *Col1a1* were significantly lower in the D group than that in the C group and were significantly higher in the R group than in the D group (Figure 2). Additionally, consistent with our expectations, femoral BMD was significantly lower in the D group than that in the C group. However, in contrast with the expression of bone formation markers, femoral BMD did not differ between the D and R groups. There was no significant difference in femoral BMD between the C and R groups. These findings suggest that although iron supplementation in iron-deficient rats can reverse the effect of iron deficiency on bone formation markers, but not completely reverse femoral BMD, at least in the recovery period studied herein.

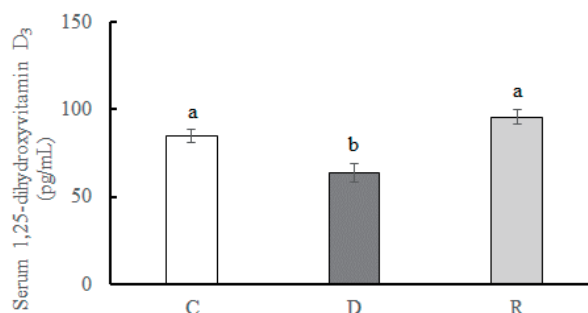


Figure 1. | Serum 1,25-dihydroxyvitamin D₃ concentration in the control (C), iron-deficient (D), and recovery (R) groups. Data are presented as the mean ± standard error (SE) for each group (n = 6 rats/group). ^{a,b}The different letters denote significant differences, $p < 0.05$.

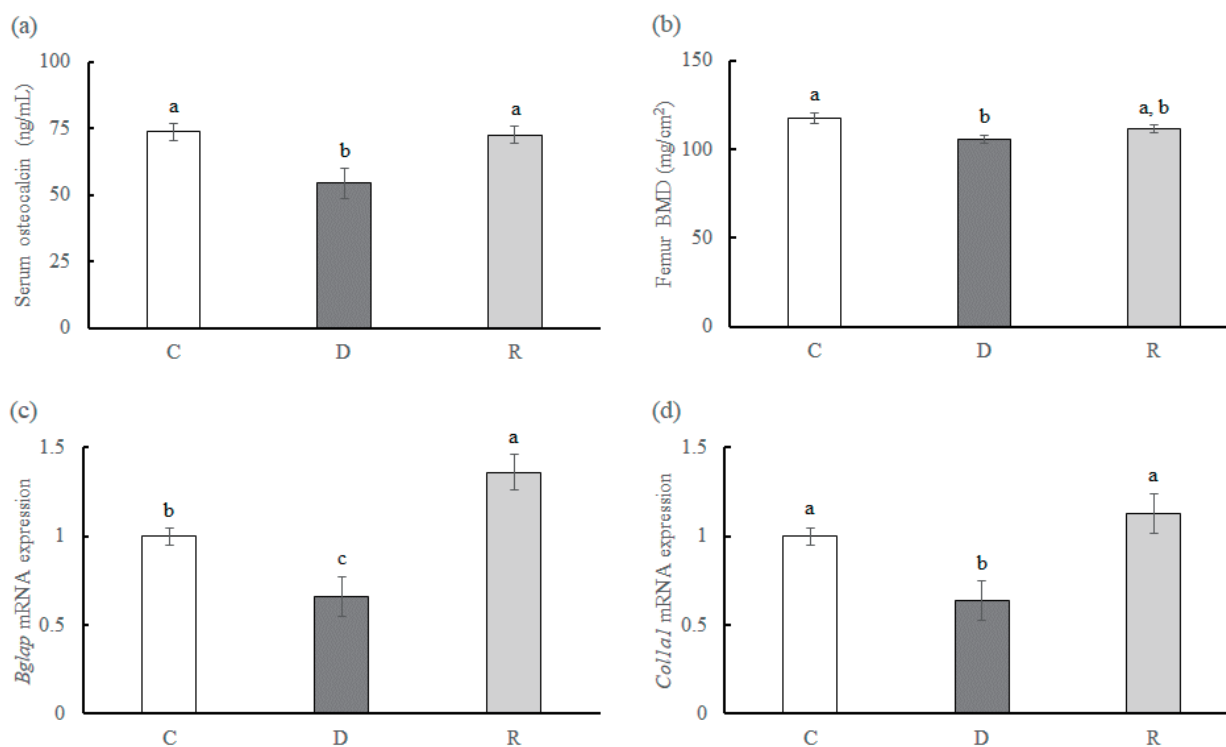


Figure 2. (a) Serum osteocalcin concentration, (b) bone mineral density (BMD) of the femur, (c) *Bglap* mRNA expression, and (d) *Col1a1* mRNA expression in the control (C), iron-deficient (D), and recovery (R) groups. Data are presented as the mean \pm SE for each group (n = 6 rats/group). ^{a,b,c}The different letters denote significant differences, $p < 0.05$.

Discussion

It has previously been reported that dietary iron deficiency can result in decreased body weight gain due to reduced food intake in rats [5-7]. Thus, an iron-deficient diet might result in iron deficiency along with other nutrient deficiencies, such as calcium and vitamin D deficiency, which would impact bone health. Furthermore, a decrease in body weight gain is thought to result in lower BMD. In the present study, we investigated the effects of iron deficiency and recovery under paired feeding conditions. Herein, an iron-deficient diet for a duration of 30 days followed by dietary iron supplementation for 14 days did not affect body weight. Therefore, in the present study, we could examine the effects of iron deficiency and iron supplementation on bone formation without the added effects of altered food intake or body weight gain.

Knutson *et al.* reported marked reductions in hemoglobin concentration, liver iron concentration, and percentage of transferrin saturation in rats fed an iron-deficient diet for 35 days, indicating severe anemia [12]. Consistently, in the present study, 30 days of an iron-deficient diet markedly reduced hemoglobin and liver iron concentrations in iron-deficient rats. Further, we previously reported that hemoglobin concentration in iron-deficient rats was reduced to approximately 25% of that in pair-fed control rats [7]. In the present study, the hemoglobin concentration in the D0 group was significantly reduced to approximately 31% of that in the C0 group. Thus, consistent with previous studies [7, 12], we were able to produce a severe iron-deficiency anemia model through a 30 day iron-deficient diet.

In the present study, dietary iron supplementation increased hemoglobin concentration in iron-deficient rats; however, the hemoglobin concentration in the R group was not restored to that in the C group. Additionally, liver iron concentration, which was decreased by iron deficiency, increased slightly following dietary iron supplementation. Consistent with our findings, Tsao *et al.* previously reported that although the decreased hemoglobin concentration caused by an iron-deficient diet increased following 2-week iron supplementation, the iron-supplemented group still had significantly lower hemoglobin concentration

when compared to the control group [13]. Furthermore, rat red blood cells have been reported to have a life span of only 60 days [14]. Therefore, taking into account the results of hemoglobin concentration and iron storage in the liver, the iron status of the R group did not recover to the normal range following iron supplementation. As a result, dietary iron supplementation in iron-deficient rats could not completely reverse the effects of iron deficiency, namely heart enlargement and anemia in this study.

Previous studies have reported that dietary iron deficiency decreases femoral BMD in rats [5-7], and similar results were observed in the present study. However, femoral BMD did not differ between the D and R groups. Also, there was no significant difference in femoral BMD between the C and R groups. These results suggest that dietary iron supplementation in iron-deficient rats may restore femoral BMD, but not fully recovered, at least in the recovery period of this study. We speculate that dietary iron supplementation can increase femoral BMD in iron-deficient rats when the supplementation period is prolonged. Hence, the effects of iron status on femoral BMD could not be fully understood in this study, and further studies should be study the same, with an extended period of dietary iron supplementation and/or different doses of iron supplementation.

The decrease in BMD induced by iron deficiency may be attributed to decreased bone formation. Previously, we reported that dietary iron deficiency decreased the levels of serum osteocalcin concentration [6, 7]. Díaz-Castro *et al.* reported that dietary iron deficiency decreased serum PINP concentration, a marker of bone formation [8]. In addition, bone histomorphometric parameters for bone formation, such as bone formation rate and osteoblast surface, were found to be decreased in iron-deficient rats [2, 7]. In the present study, dietary iron deficiency decreased serum osteocalcin concentration, suggesting that a decrease in bone formation in iron-deficient rats. Moreover, serum osteocalcin concentration in iron-deficient rats was recovered following dietary iron supplementation and was similar to that in the control rats. These results suggested that dietary iron supplementation increased bone formation, which was suppressed by iron deficiency, although the iron status was not fully restored to the normal range.

Bone matrix proteins such as type I collagen and osteocalcin are used as markers of the osteoblast differentiation. In general, *Col1a1* are early marker of osteoblast differentiation, while *Bglap* appear late, concomitantly with mineralization [15]. In the present study, femoral mRNA expression of *Bglap* and *Col1a1* was significantly decreased in iron-deficient rats compared to that in control rats. Furthermore, dietary iron supplementation increased femoral mRNA expression of *Bglap* and *Col1a1* in iron-deficient rats. We previously showed that iron deficiency downregulated *Bglap* and *Col1a1* mRNA expression in rats, along with downregulation of *Runx2* mRNA expression was observed [2]. Given that *Runx2* regulates the expression of several osteoblastic genes, including *Bglap* and *Col1a1* [16], these results suggested that diminished *Runx2* expression due to iron deficiency resulted in decreased bone formation. In the present study, we did not measure femoral *Runx2* mRNA expression; however, based on previous findings, it can be speculated that iron deficiency caused a decrease in *Runx2* mRNA expression. Consequently, decreased expression of osteoblastic genes expression due to iron deficiency may be recovered by iron supplementation, which would also regulate changes in bone formation. In addition, femoral *Bglap* mRNA expression was higher in the R group than in the C group. Previous study on recovery from zinc deficiency showed that plasma zinc concentration was relatively higher in the recovery rats than in the control rats [17]. Therefore, the *Bglap* mRNA expression, which was decreased by iron deficiency, was thought to be overexpressed by restoring iron.

Our previous studies showed that iron deficiency decreased serum 1,25-dihydroxyvitamin D₃ concentrations in rats [2, 7]. Renal 1 α -hydroxylase converts 25-hydroxyvitamin D₃ to 1,25-dihydroxyvitamin D₃, and used iron as a cofactor [1]. Therefore, iron deficiency is thought to decrease bone formation owing to a decline in vitamin D activation. Consistent with our previous studies [2, 7], in the present study, serum 1,25-dihydroxyvitamin D₃ concentration was found to be significantly decreased in iron-deficient rats. We previously demonstrated that a decrease in renal 1 α -hydroxylase activity resulted in decreased serum 1,25-dihydroxyvitamin D₃ concentration, which accounted for decreases bone formation in iron-deficient rats [2]. Although we did not measure renal 1 α -hydroxylase activity in the present study, given our previous findings, it is possible to speculate a reduction in renal 1 α -hydroxylase activity in iron-deficient rats. Additionally, in the present study, serum 1,25-dihydroxyvitamin D₃ concentration in iron-deficient rats was significantly increased following dietary iron supplementation and was similar to that in control rats, although the iron status did not fully recover to the normal range following dietary iron supplementation. Thus, an increase in the serum 1,25-dihydroxyvitamin D₃ concentration due to dietary iron supplementation could increase bone formation in iron-deficient rats. Also, 1,25-dihydroxyvitamin D₃ is responsible for transcriptional regulation of *Bglap* expression [18]. Therefore, an increase in serum 1,25-dihydroxyvitamin D₃ concentration as a result of dietary iron supplementation in the

iron-deficient rats could reflect the results of serum osteocalcin concentration and femoral *Bglap* mRNA expression observed in this study. Recently, Qiu *et al.* reported that the mRNA and protein expression of renal CYP27B1 (1α -hydroxylase) were upregulated in iron-deficient rats compared to that in control rats [19]. In the future, the relationship between the mRNA and protein expression of 1α -hydroxylase and its activity should be studied in iron-deficient rats.

Conclusion

We examined the effects of dietary iron supplementation on bone formation markers and serum 1,25-dihydroxyvitamin D₃ concentration in iron-deficient rats. Iron deficiency led to decreased hemoglobin concentration, liver iron, and serum 1,25-dihydroxyvitamin D₃ and serum osteocalcin concentrations. Although dietary iron supplementation increased hemoglobin and liver iron concentrations, the levels were not restored to normal. However, dietary iron supplementation restored serum 1,25-dihydroxyvitamin D₃ and osteocalcin concentrations and femoral mRNA expression of *Bglap* and *Col1a1* in iron-deficient rats. These results suggest that dietary iron supplementation can potentially reverse the effects of iron deficiency on bone health by increasing bone formation.

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