

## Changes in bone metabolism-related mRNA expression in iron-deficient rats

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### Abstract

To determine the underlying mechanism of iron deficiency-induced bone loss, we investigated the effects of dietary iron deficiency on bone metabolism-related mRNA expression in rats. Weanling male Wistar rats were divided into three groups, each comprising six rats. Rats in two of the three groups were given free access to either a control (C group) or an iron-deficient (D group) diet for 4 weeks. Rats in the PF group were pair-fed the control diet with rats in the D group. Hemoglobin concentration, serum osteocalcin concentration, femoral bone mineral content and density, and femoral *Igf1*, *Bmp2*, *Runx2*, *Sp7*, *Spp1*, *Bglap*, and *Col1a1* mRNA expression were significantly lower in the D group than in the C and PF groups. Additionally, urinary deoxypyridinoline excretion and femoral *Acp5* mRNA expression were significantly lower in the D group than in the C and PF groups. These results suggested that dietary iron deficiency reduced osteoblastogenesis by decreasing the expression of insulin-like growth factor-1 and bone morphogenetic protein 2. Furthermore, bone resorption parameters indicated a decrease in bone resorption in iron-deficient rats.

**Keywords:** dietary iron deficiency, insulin-like growth factor-1, bone morphogenetic protein 2, bone formation, bone resorption

**Statements about COI:** The authors declare no conflict of interest associated with this manuscript.

### Introduction

Osteoporosis is a bone disorder affected by many factors, such as sex, age, and the environment. Nutritional status has been identified as a risk factor for osteoporosis, and a deficiency or proportional change in calcium, phosphorus, and magnesium may lead to bone loss [1]. In addition, iron deficiency induces bone loss, and dietary iron deficiency decreases bone mineral content (BMC) and bone mineral density (BMD) in rats [2-4]. In postmenopausal women, dietary iron intake is associated with BMD [5, 6].

An imbalance in bone turnover between osteoblastic bone formation and osteoclastic bone resorption results in bone loss. The concentrations of bone formation markers, such as serum osteocalcin and procollagen type I N-terminal propeptide (PINP) concentrations, are decreased in iron-deficient rats [2-4, 7]. Additionally, bone histomorphometric parameters have shown that dietary iron deficiency decreases the bone formation rate and osteoblast surface in rats [3, 4]. Therefore, decreased

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bone formation is thought to cause dietary iron deficiency-induced bone loss. Runt-related transcription factor 2 (Runx2) and Osterix (also known as Sp7) are essential transcription factors for osteoblast differentiation [8, 9]. Dietary iron deficiency decreases femoral *Runx2* and *Sp7* mRNA expression, suggesting that iron deficiency-induced downregulation of Runx2 and Osterix might influence osteoblastic bone formation [4]. However, the mechanisms by which Runx2 and Osterix are regulated in iron-deficient rats remain unclear.

Díaz-Castro *et al.* demonstrated that dietary iron deficiency increases the serum C-terminal telopeptide of type I collagen (CTx) and tartrate-resistant acid phosphatase (TRAP) concentrations in rats [7]. Consistently, urinary CTx excretion was increased in iron-deficient rats [2, 3]. These results indicated that dietary iron deficiency increases bone resorption. However, dietary iron deficiency decreases urinary deoxypyridinoline (DPD) excretion and reduces osteoclast numbers in rats [3, 4]. The reason for this discrepancy remains unclear.

This study aimed to identify the factors through which dietary iron deficiency inhibits osteoblast differentiation and reduces bone formation. Therefore, we investigated the effects of dietary iron deficiency on bone formation-related mRNA expression in rats. Furthermore, to determine whether dietary iron deficiency increases or decreases bone resorption, we measured bone resorption-related mRNA expression in iron-deficient rats.

## Materials and Methods

### Ethical approval

This study was approved by the Animal Use Committee of Tokyo University of Agriculture (approval number 280069) and performed in accordance with the university's guidelines for the care and use of laboratory animals.

### Experimental design

Eighteen 3-week-old male Wistar rats were purchased from Clea Japan (Tokyo, Japan) and housed individually in stainless-steel cages at 22 °C with a 12-hour light-dark cycle. During the acclimatization period, all rats were fed a control diet for 3 days. Subsequently, the rats were randomly divided into three experimental groups comprising six rats each. The rats in two of the three groups were given free access to either a control (C group) or an iron-deficient (D group) diet. Rats in the third group (PF group) were pair-fed the control diet with the D group. The control diet was prepared according to the AIN-93G formula [10], and the iron-deficient diet was prepared in a similar way, except for the use of an iron-free AIN-93G mineral mixture. Throughout the experimental period, all rats had free access to deionized water. Urine was collected using metabolic cages with providing their respective diet over the last 24 h of the experimental period. After 4 weeks, all rats were sacrificed under anesthesia, and blood and femur samples were collected for analysis. Hemoglobin concentration was measured using a hemoglobin B-test kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). In addition, hearts were removed and weighed to assess heart enlargement, a sign of iron-deficiency anemia.

### Measurement of bone turnover markers

Blood samples were centrifuged at  $1,500 \times g$  for 15 min after coagulation and the supernatants were collected to analyze the levels of serum osteocalcin, a bone formation marker. Serum and urine samples were stored at  $-80\text{ }^{\circ}\text{C}$  until analysis. The serum osteocalcin concentration was measured using the rat osteocalcin/bone gla protein enzyme-linked immunosorbent assay system (GE Healthcare Japan Corporation, Tokyo, Japan). Urinary CTx was measured using the RatLaps<sup>TM</sup> enzyme-linked immunosorbent assay (Immunodiagnostic Systems Nordic A/S, Herlev, Denmark). Urinary DPD excretion was measured using the METRA DPD EIA kit (Quidel, San Diego, CA, USA). Urinary creatinine excretion was measured using the Jaffé reaction [11].

### Measurement of femoral BMC and BMD

Left femur samples were stored in 70% ethanol at 4 °C until analysis. Subsequently, the BMC and BMD of the femur were measured using dual-energy X-ray absorptiometry (DSC-600EX; Hitachi Aloka Medical, Ltd., Tokyo, Japan).

### Isolation of total RNA and subsequent real-time PCR to measure bone metabolism-related mRNA expression

Right femur samples were stored at  $-80\text{ }^{\circ}\text{C}$  until analysis. The samples were homogenized in TRI Reagent (Molecular Research Center Inc., Cincinnati, OH, USA) using a Precellys homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) in ceramic

bead-containing tubes for two 20 s cycles at 7,500 rpm. Total RNA was isolated from the lysate using the Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Complimentary DNA 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 the PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). Specific primers were used for the analysis of insulin-like growth factor-1 (IGF-1) (*Igf1*: forward 5'-TTCAGTTCGTGTGTGGACCAAG-3', reverse 5'-GATCACAGCTCCGGAAGCAA-3'), bone morphogenetic protein 2 (BMP2) (*Bmp2*: forward 5'-ACCGTGCTCAGCTTCCATCAC-3', reverse 5'-TTCCTGCATTTGTTCCCGAAA-3'), Runx2 (*Runx2*: forward 5'-GCACCCAGCCCATAATAGA-3', reverse 5'-TTGGAGCAAGGAGAACCC-3'), Osterix (*Sp7*: forward 5'-GCCTACTTACCCGTCTGACTTT-3', reverse 5'-GCCCCTATTGCCAACTGC-3'), osteopontin (*Spp1*: forward 5'-TGAGACTGGCAGTGGTTTGC-3', reverse 5'-CCACTTTCACCGGGAGACA-3'), osteocalcin (*Bglap*: forward 5'-GAGCTAGCGGACCACATTGG-3', reverse 5'-CCTAAACGGTGGTGCCATAGA-3'), collagen type I alpha 1 chain (*Col1a1*: forward 5'-TTCACCTACAGCAGCTTGTG-3', reverse 5'-GATGACTGTCTTGCCCCAAGTT-3'), TRAP (*Acp5*: forward 5'-CAGCCTTATTACCGTTTGC-3', reverse 5'-GAATTGCCACACAGCATCAC-3'), cathepsin K (*Ctsk*: forward 5'-TGTCTGAGAACTATGGCTGTGG-3', reverse 5'-ATACGGGTAACGTCTTCAGAG-3'), and  $\beta$ -actin (*Actb*: forward 5'-AGCCATGTACGTAGCCATCCA-3', reverse 5'-TCTCCGGAGTCCATCACAATG-3') [12-16]. Their amplification specificity was confirmed by melting curve analysis. mRNA expression levels were normalized to that of the housekeeping gene, *Actb*.

### Statistical analysis

Results are expressed as the mean  $\pm$  standard error for each group of 6. All statistical analyses were performed using SPSS Statistics ver.23 (IBM Corp., Armonk, NY, USA). One-way analysis of variance, followed by the Fisher's protected least significant difference test, was performed to evaluate significant differences among the groups. Differences were considered statistically significant at  $p < 0.05$ .

## Results

### Body weight, food intake, and indicators of iron deficiency

Final body weight and food intake were significantly lower in the D and PF groups than in the C group (Table 1), and these parameters did not differ significantly between the D and PF groups. Hemoglobin concentration was significantly lower and heart weight was significantly higher in the D group than in the C and PF groups. Hemoglobin concentration and heart weight did not differ significantly between the C and PF groups.

**Table 1.** | Body weight, food intake, and indicators of iron deficiency in the C, D, and PF groups.

	C	D	PF
Initial body weight (g)	51.1 $\pm$ 1.4	51.1 $\pm$ 0.7	50.9 $\pm$ 1.2
Final body weight (g)	240.5 $\pm$ 8.0 <sup>a</sup>	167.0 $\pm$ 6.1 <sup>b</sup>	183.9 $\pm$ 2.8 <sup>b</sup>
Food intake (g/4 weeks)	422.3 $\pm$ 12.6 <sup>a</sup>	314.4 $\pm$ 10.8 <sup>b</sup>	312.6 $\pm$ 0.3 <sup>b</sup>
Hemoglobin concentration (g/dL)	12.0 $\pm$ 0.4 <sup>a</sup>	3.4 $\pm$ 0.2 <sup>b</sup>	12.6 $\pm$ 0.2 <sup>a</sup>
Heart weight (g/100g body weight)	0.29 $\pm$ 0.01 <sup>b</sup>	0.64 $\pm$ 0.05 <sup>a</sup>	0.29 $\pm$ 0.01 <sup>b</sup>

C, control group; D, iron-deficient group; PF, pair-fed group. Data are presented as mean  $\pm$  standard error for each group (n = 6).

<sup>a,b</sup>The different letters denote significant differences,  $p < 0.05$ .

### Bone turnover markers and femoral BMC and BMD

Serum osteocalcin concentration was significantly lower in the D group than in the C and PF groups and was significantly lower in the PF group than in the C group (Table 2). Urinary CTx excretion was significantly higher and urinary DPD excretion was significantly lower in the D group than in the C and PF groups. There were no significant differences in urinary CTx and DPD excretion between the C and PF groups. Femoral BMC and BMD were significantly lower in the D group than in the C and PF groups, and significantly lower in the PF group than in the C group.

**Table 2.** | Bone turnover markers and femoral bone mineral content and bone mineral density in the C, D, and PF groups.

	C	D	PF
Serum osteocalcin (ng/mL)	130.7 ± 1.6 <sup>a</sup>	89.5 ± 2.9 <sup>c</sup>	116.3 ± 2.3 <sup>b</sup>
Urine CTx (µg/mmol creatinine)	45.6 ± 2.0 <sup>b</sup>	70.4 ± 8.1 <sup>a</sup>	30.8 ± 4.2 <sup>b</sup>
Urine DPD (nmol/mmol creatinine)	621.2 ± 58.6 <sup>a</sup>	396.4 ± 27.2 <sup>b</sup>	545.5 ± 35.5 <sup>a</sup>
Femoral BMC (mg)	143.8 ± 1.7 <sup>a</sup>	122.9 ± 3.8 <sup>c</sup>	134.7 ± 2.7 <sup>b</sup>
Femoral BMD (mg/cm <sup>2</sup> )	90.7 ± 1.0 <sup>a</sup>	78.5 ± 1.0 <sup>c</sup>	87.0 ± 1.0 <sup>b</sup>

C, control group; D, iron-deficient group; PF, pair-fed group; CTx, C-terminal telopeptide of type I collagen; DPD, deoxypyridinoline; BMC, bone mineral content; BMD, bone mineral density. Data are presented as mean ± standard error for each group (n = 6).

<sup>a,b,c</sup>The different letters denote significant differences,  $p < 0.05$ .

### Femoral bone formation-related mRNA expression

Femoral mRNA expression levels of *Igf1*, *Bmp2*, *Runx2*, *Sp7*, *Spp1*, *Bglap*, and *Col1a1* were significantly lower in the D group than in the C and PF groups (Table 3). *Bglap* expression was significantly lower in the PF group than in the C group. There were no significant differences in the expression of *Igf1*, *Bmp2*, *Runx2*, *Sp7*, *Spp1*, or *Col1a1* between the C and PF groups.

**Table 3.** | Bone formation-related mRNA expression in the femur in the C, D, and PF groups.

	C	D	PF
<i>Igf1</i>	1.00 ± 0.05 <sup>a</sup>	0.65 ± 0.01 <sup>b</sup>	0.86 ± 0.09 <sup>a</sup>
<i>Bmp2</i>	1.00 ± 0.06 <sup>a</sup>	0.59 ± 0.05 <sup>b</sup>	1.04 ± 0.06 <sup>a</sup>
<i>Runx2</i>	1.00 ± 0.15 <sup>a</sup>	0.56 ± 0.04 <sup>b</sup>	1.25 ± 0.12 <sup>a</sup>
<i>Sp7</i>	1.00 ± 0.15 <sup>a</sup>	0.38 ± 0.04 <sup>b</sup>	1.38 ± 0.15 <sup>a</sup>
<i>Spp1</i>	1.00 ± 0.05 <sup>a</sup>	0.67 ± 0.03 <sup>b</sup>	0.98 ± 0.03 <sup>a</sup>
<i>Bglap</i>	1.00 ± 0.03 <sup>a</sup>	0.60 ± 0.04 <sup>c</sup>	0.88 ± 0.04 <sup>b</sup>
<i>Col1a1</i>	1.00 ± 0.07 <sup>a</sup>	0.55 ± 0.03 <sup>b</sup>	0.93 ± 0.04 <sup>a</sup>

C, control group; D, iron-deficient group; PF, pair-fed group. Data are presented as mean ± standard error for each group (n = 6). The value of the C group was considered to be 1.00.

<sup>a,b,c</sup>The different letters denote significant differences,  $p < 0.05$ .

### Femoral bone resorption-related mRNA expression

Femoral *Acp5* mRNA expression was significantly lower in the D group than in the C and PF groups (Table 4). There was no significant difference in *Acp5* expression between the C and PF groups. Femoral *Ctsk* mRNA expression was significantly lower in the D and PF groups than in the C group. There was no significant difference in *Ctsk* expression between the D and PF groups.

**Table 4.** | Bone resorption-related mRNA expression in the femur in the C, D, and PF groups.

	C	D	PF
<i>Acp5</i>	1.00 ± 0.09 <sup>a</sup>	0.50 ± 0.03 <sup>b</sup>	0.92 ± 0.11 <sup>a</sup>
<i>Ctsk</i>	1.00 ± 0.10 <sup>a</sup>	0.63 ± 0.05 <sup>b</sup>	0.78 ± 0.03 <sup>b</sup>

C, control group; D, iron-deficient group; PF, pair-fed group. Data are presented as mean ± standard error for each group (n = 6). The value of the C group was considered to be 1.00.

<sup>a,b</sup>The different letters denote significant differences,  $p < 0.05$ .

### Discussion

Undernutrition is a cause of bone loss, and dietary iron deficiency reduces BMD and bone strength. In this study, dietary iron deficiency decreased hemoglobin concentration and femoral BMC and BMD, as previously reported [2-4]. Since hemoglobin concentration and femoral BMC and BMD were lower in the D group than in the PF group, dietary iron deficiency could be the dominant cause of anemia and bone loss, independent of decreases in food intake and body weight, similar to our previous studies [3, 4].

Decreased osteoblastic bone formation is one of the reasons dietary iron deficiency induces lower BMC and BMD. The serum P1NP concentration is decreased in iron-deficient rats [7]. Furthermore, in this study, the serum osteocalcin concentration was significantly lower in the D group than in the C and PF groups, as previously reported [3, 4]. These results suggest that dietary iron deficiency causes a decline in bone formation. Dietary iron deficiency decreases the serum 1,25-dihydroxyvitamin D<sub>3</sub> concentration, which is caused by a decline in renal 25-hydroxyvitamin D-1 $\alpha$ -hydroxylase activity [4]. Thus, although we did not measure the serum 1,25-dihydroxyvitamin D<sub>3</sub> concentration in this study, it is possible that a lower serum 1,25-dihydroxyvitamin D<sub>3</sub> concentration due to dietary iron deficiency might lead to decreased bone formation.

Runx2 and Osterix are essential transcription factors for osteoblast differentiation [8, 9]. In this study and our previous study [4], dietary iron deficiency decreased femoral *Runx2* and *Sp7* mRNA expression, indicating that decreased *Runx2* and *Sp7* mRNA expression may cause a decrease in osteoblast differentiation in iron-deficient rats. Furthermore, Runx2 regulates the expression of several osteoblastic genes, including *Spp1*, *Bglap*, and *Col1a1* [17]. In this study, femoral *Spp1*, *Bglap*, and *Col1a1* mRNA expression levels were significantly lower in the D group than in the C and PF groups, suggesting that a decrease in Runx2 expression downregulates the expression of these osteoblastic genes. However, decreased osteoblast number could reduce the expression of osteoblastic genes without downregulating these gene expression per osteoblast. Therefore, further studies may be needed to clarify the downregulation of osteoblastic genes in osteoblastic cells of iron-deficient rats.

In this study, we measured femoral *Bmp2* and *Igf1* mRNA expression. BMP2 is a potent inducer of bone formation that stimulates osteoblast differentiation and regulates Runx2 and Osterix expression [18, 19]. IGF-1 upregulates osteogenic-related genes such as *Bmp2*, *Runx2*, *Spp1*, and *Bglap* [20]. Furthermore, BMP2 and IGF-1 regulate Osterix expression during osteoblast lineage progression [21]. In this study, femoral *Bmp2* and *Igf1* mRNA expression levels were significantly lower in the D group than in the C and PF groups. These results suggest that dietary iron deficiency decreases BMP2 and IGF-1 expression, leading to reduced Runx2 and Osterix expression. Furthermore, 1,25-dihydroxyvitamin D<sub>3</sub> enhances IGF-1 production in osteoblast-like cells *in vitro* [22]. Therefore, we hypothesized that decreased 1,25-dihydroxyvitamin D<sub>3</sub> due to dietary iron deficiency reduces

IGF-1 expression, which, in part, is related to a decrease in osteoblast differentiation. Consequently, our results revealed that dietary iron deficiency decreased IGF-1 and BMP2 expression, which decreased bone formation through downregulation of osteoblastogenesis-related genes.

Dietary iron deficiency decreased urinary DPD excretion in this study, as previously reported [2, 3]. Furthermore, according to previous studies based on bone histomorphometry, osteoclast numbers are reduced in iron-deficient rats [3, 4]. Therefore, we hypothesized that dietary iron deficiency would reduce bone resorption. In this study, femoral *Acp5* and *Ctsk* mRNA expression levels were significantly lower in the D group than in the C group, supporting the finding of decreased osteoclastic bone resorption due to dietary iron deficiency. *In vitro* studies have shown that iron chelation by deferoxamine inhibits osteoclast differentiation in murine bone marrow macrophages [23, 24]. Finally, our results showed that dietary iron deficiency inhibited osteoclastic bone resorption. However, Díaz-Castro *et al.* demonstrated that dietary iron deficiency increased serum CTx and TRAP concentrations in rats [7]. The reason for this discrepancy is unknown, and detailed examinations are necessary to clarify the conditions under which dietary iron deficiency induces increased bone resorption.

## Conclusion

We examined the effects of dietary iron deficiency on bone metabolism-related mRNA expression in rats and found decreased femoral *Igf1*, *Bmp2*, *Runx2*, *Sp7*, *Spp1*, *Bglap*, *Col1a1*, *Acp5*, and *Ctsk* mRNA expression. Our study revealed that dietary iron deficiency reduced the expression of BMP2 and IGF-1 as well as decreased bone formation. The decreased expression of BMP2 and IGF-1 by iron deficiency could decrease osteoblast differentiation and osteoblastic bone formation. Furthermore, osteoclastic gene expression indicated a decrease in osteoclastic bone resorption in iron-deficient rats.

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