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Dietary phosphate supplementation delays the onset of iron deficiency anemia and affects iron status in rats

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ABSTRACT

Inorganic phosphate (Pi) plays critical roles in bone metabolism and is an essential component of 2,3-diphosphoglycerate (2,3-DPG). It has been reported that animals fed a low-iron diet modulate Pi metabolism, whereas the effect of dietary Pi on iron metabolism, particularly in iron deficiency anemia (IDA), is not fully understood. In this study, we hypothesized the presence of a link between Pi and iron metabolism and tested the hypothesis by investigating the effects of dietary Pi on iron status and IDA. Wistar rats aged 4 weeks were randomly assigned to 1 of 4 experimental dietary groups: normal iron content (Con Fe) + 0.5% Pi, low-iron (Low Fe) + 0.5% Pi, Con Fe + 1.5% Pi, and Low Fe + 1.5% Pi. Rats fed the 1.5% Pi diet for 14 days, but not for 28 days, maintained their anemia state and plasma erythropoietin concentrations within the reference range, even under conditions of low iron. In addition, plasma concentrations of 2,3-DPG were significantly increased by the 1.5% Pi diets and were positively correlated with plasma Pi concentration ($r = 0.779$; $P < .001$). Dietary Pi regulated the messenger RNA expression of iron-regulated genes, including divalent metal transporter 1, duodenal cytochrome B, and hepcidin. Furthermore, iron concentration in liver tissues was increased by the 1.5% Pi in Con Fe diet. These results suggest that dietary Pi supplementation delays the onset of IDA and increases plasma 2,3-DPG concentration, followed by modulation of the expression of iron-regulated genes.

Abbreviations: 2,3-DPG, 2,3-diphosphoglycerate; ATP, adenosine triphosphate; Con Fe, normal iron content; EPO, erythropoietin; DcytB, duodenal cytochrome B; DMT1, divalent metal transporter 1; FGF23, fibroblast growth factor 23; Hb, hemoglobin; IDA, iron deficiency anemia; iFGF23, intact fibroblast growth factor 23; iPTH, intact parathyroid hormone; Low Fe, low iron; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; mRNA, messenger RNA; Pi, inorganic phosphate; PTH, parathyroid hormone; RBC, red blood cell; Tf, transferrin.

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1. Introduction

Inorganic phosphate (Pi) is an essential nutrient in the processes of glycolysis, gluconeogenesis, mineral metabolism, and other diverse cellular functions that involve intermediary metabolism and energy transfer mechanisms [1,2]. Serum Pi concentration is maintained through a complex interplay between intestinal absorption, exchange with intracellular and bone storage pools, and renal tubular reabsorption [3,4]. Absorption of Pi in the intestine and its transport in the kidney are mediated by several sodium-dependent phosphate cotransporters [4-6]. Parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23), and 1α,25-dihydroxyvitamin D_3 are hormones that are well recognized for their roles in regulating Pi metabolism [7]. We have previously reported the effects of Pi intake on circulating FGF23 and PTH as well as vascular endothelial function in human and animal studies [8-10].

Iron deficiency anemia (IDA) is characterized by a reduction or absence of iron stores; low serum concentrations of iron, hemoglobin (Hb), and ferritin; decreased hematocrit; increased platelet count; reduced transferrin (Tf) saturation; and a marked increase in total iron-binding capacity [11,12]. Total body iron stores are maintained through dietary absorption by duodenal enterocytes. Dietary nonheme iron exists mainly in ferric form (Fe^{3+}). Fe^{3+} must be reduced by apical membrane–bound ferric reductase activity via duodenal cytochrome B (DcytB) [13,14]. Duodenal cytochrome B is a candidate enterocyte ferric iron reductase, but its physiological importance is not clear. Reduced iron (Fe^{2+}) then enters the enterocytes via the divalent metal transporter 1 (DMT1) located in the apical membrane of enterocytes [15-17]. Furthermore, the control of iron absorption is regulated by hepcidin, a polypeptide hormone secreted by the liver in response to a high concentration of iron in the body [18,19]. Hepcidin binds to ferroportin, an iron exporter on the surface of nucleated cells, leading to its internalization and degradation, thereby inhibiting iron export from intracellular pools [20].

Recent studies have provided evidence of a relationship between iron and Pi metabolism. Iron deficiency stimulates the expression of hypoxia-inducible factor 1 α, leading to an increase in the transcription of *Fgf23* [21]. In addition, duodenal *Npt 2b* (Slc34a2), which primarily mediates intestinal Pi absorption, was down-regulated in rats by a low-iron diet [22]. A low-iron diet has also been shown to induce hypophosphatemia in neonatal wild-type mice [23]. On the other hand, hypophosphatemia caused by a Pi imbalance reduced red blood cell (RBC) adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG) contents [24,25]. 2,3-Diphosphoglycerate is an erythrocyte-specific metabolite that decreases the oxygen-binding affinity of Hb and thereby induces oxygen release from Hb [26-29]. A strong correlation has been reported between the serum Pi and 2,3-DPG concentrations [30]. However, little is understood about the effect of Pi supplementation on 2,3-DPG concentration in IDA. Acute hemolytic anemia was associated with serum Pi concentrations less than 1.0 mg/dL, whereas parenteral Pi administration corrected the hemolytic anemia and restored low 2,3-DPG and ATP concentrations to a reference range [31]. Based on these studies, it was clear that a low-iron diet could modulate Pi metabolism and that Pi administration corrected hemolytic anemia, whereas the effects of dietary Pi on iron metabolism and IDA, in particular, are not fully understood.

We hypothesized that Pi administration may also correct IDA and tested the hypothesis by determining the effect of dietary Pi supplementation on diet-induced IDA in rats. Young rats were used to ensure induction of anemia.

2. Methods and materials

2.1. Animals

Male Wistar rats aged 4 weeks (Japan SLC, Inc, Shizuoka, Japan), weighing approximately 80 g, were individually caged in a climate-controlled room (23°C ± 1°C, humidity 70%-75%; specific pathogen free) with a 12-hour light/12-hour dark cycle. Rats of equivalent mean body weight were distributed among 4 dietary treatment groups and given free access to food and deionized water. At the end of the experiment, all rats were killed by exsanguination after being anesthetized, and blood and tissue samples were collected for analysis. Tissue samples were stored at –80°C until analysis. The breeding and handling of all animals in this study were approved by the Animal Experimentation Committee of the University of Tokushima.

2.2. Diets

The compositions of the experimental diets are shown in Table 1. The rats were divided into 4 experimental groups of 5 rats per group. Each group was fed 1 of the 4 different diets for 14 days: normal iron content (Con Fe) + 0.5% Pi, low iron (Low Fe) + 0.5% Pi, Con Fe + 1.5% Pi, and Low Fe + 1.5% Pi. The experimental diets were based on the AIN-93G formulation [32]. The basal diet was supplemented with iron citrate ($FeC_6H_5O_7$) and potassium dihydrogenphosphate (KH_2PO_4) (Wako Pure Chemical Industries Co, Osaka, Japan). The Con Fe diet contained 250 ppm iron and the Low Fe diet contained 5 ppm iron. For the long-term experiment, rats were fed the same experimental diets for 28 days.

2.3. Blood parameters

For biochemical analysis, blood was collected into tubes containing heparin or EDTA-2K after puncture of inferior vena cava. Concentrations of Pi, calcium (Ca), and iron were measured using assay kits (Wako Pure Chemical Industries Co) [33]. To calculate the degree of Tf saturation, plasma total iron-binding capacity was determined using an unsaturated iron-binding capacity kit (AKJ Global Technology Co, Chiba, Japan). Red blood cell count and Hb concentration were measured at 14 and 28 days using a blood corpuscle analyzer (Mitsubishi Chemical Medicine, Tokyo, Japan). The concentrations in plasma of intact PTH (iPTH), intact FGF23 (iFGF23), and 2,3-DPG were determined using the Rat Intact PTH ELISA Kit (Immutopics, San Clemente, CA, USA), the FGF23 ELISA Kit (Kinos, Tokyo, Japan), and the Rat 2,3-diphosphoglycerate ELISA Kit (Cusabio Biotech, Hubei, China), respectively [33]. Concentrations of plasma erythropoietin (EPO) were measured using the radioimmunoassay 2-antibody method (SRL Co, Tokyo, Japan).

Table 1 – Composition of the experimental diets

	Diets, g/kg			
	Con Fe + 0.5% Pi	Low Fe + 0.5% Pi	Con Fe + 1.5% Pi	Low Fe + 1.5% Pi
Casein	200.00	200.00	200.00	200.00
L-Cysteine	3.00	3.00	3.00	3.00
Cornstarch	397.49	397.49	397.49	397.49
α-Cornstarch	132.00	132.00	132.00	132.00
Sugar	82.84	83.92	38.36	39.44
Soybean oil	70.00	70.00	70.00	70.00
Cellulose	50.00	50.00	50.00	50.00
Vitamin mix	10.00	10.00	10.00	10.00
Choline bitartrate	2.50	2.50	2.50	2.50
Tert-butylhydroquinone	0.01	0.01	0.01	0.01
Mineral mix changed[1]	15.65	15.65	15.65	15.65
$CaCO_3$	14.64	14.64	14.64	14.64
KH_2PO_4	20.78	20.78	65.26	65.26
$FeC_6H_5O_7{\cdot}H_2O$	1.09	0.01	1.09	0.01

The mineral mix did not contain $CaCO_3$, KH_2PO_4, and $FeC_6H_5O_7{\cdot}H_2O$.
Con Fe, normal iron content (Fe 250 ppm); Low Fe, low iron (5 ppm).

2.4. *Quantitative polymerase chain reaction analysis*

Quantitative real-time polymerase chain reaction (PCR) analysis was performed by using the Light Cycler (Roche Diagnostics, Mannheim, Germany). Extraction of total RNA, complementary DNA synthesis, and real-time PCR were performed as previously described [34]. The amplification products were then analyzed using a melting curve, which confirmed the presence of a single PCR product in all reactions (apart from negative controls). The PCR products were quantified by fit-point analysis, and results were normalized to those for β-actin. For PCR amplification, the primer sequences are shown in Table 2.

2.5. *Measurement of hepatic iron*

To measure hepatic iron, the liver was dried, ashed, and subsequently demineralized with 1 mol/L HCL as previously described [35]. The concentration of iron in the liver was determined using an inductively coupled plasma optical atomic emission spectrometer model iCAP 6300 (Thermo Electron Corporation, Waltham, MA, USA).

2.6. *Statistical analyses*

The values are means ± SEM. Significance was determined by 1-way analysis of variance followed by the Turkey-Kramer post hoc test. Significance was defined at $P < .05$. Pearson correlation coefficient analysis was used to assess the relationship between plasma Pi and 2,3-DPG concentrations. All data were analyzed using STATCEL2 (OMS, Saitama, Japan), an add-in application for Excel (Microsoft, Redmond, WA, USA). Power analysis was not used to determine the sample size in each experimental group, as these were preliminary feasibility studies.

3. Results

3.1. *Body weight and food intake*

Two groups of rats were fed a Low Fe (5 ppm) diet, plus either 0.5% or 1.5% dietary Pi. Mean body weight decreased in the groups fed 1.5% Pi compared with those fed 0.5% Pi, despite there being no significant differences in food intake among the rats in the different groups (Table 3).

3.2. *Blood biochemistry*

The blood biochemical parameters are shown in Fig. 1. The plasma concentrations of Pi, iPTH, and iFGF23 were higher in the groups fed 1.5% Pi compared with those fed 0.5% Pi, whereas the plasma Ca concentration was lower in the Low Fe + 1.5% Pi group than in the Low Fe + 0.5% Pi group.

To examine the impact of dietary Pi on IDA, a complete blood count was performed in peripheral blood. In the 0.5% Pi

Table 2 – Sequence of oligonucleotides for quantitative real-time PCR analysis

Gene name	Sense primer	Antisense primer	Accession Number
Dmt1	5′-AAGTCCTGCTGAGCGAAGAT-3′	5′-TGGTCCCTAAATGCAGTCTG-3′	NM_013173
DcytB	5′-AACAGTGATTGCCACGGTCC-3′	5′-CCAAAAGATGAGGGCTCCAA-3′	NM_001011954
Ferroportin	5′-GTCTACGGGTTGGTGGTGGCAG-3′	5′-TCTGAACCACCAGGGACGTCTG-3′	NM_133315
Epo	5′-CAAGCCATCAGTGGGCTAC-3′	5′-CTGTGAGTGTTCGGAGTGGAG-3′	NM_017001
Hepcidin	5′-GGCAACAGACGAGACAGACT-3′	5′-AGAGGCATATGGGGAAGTTG-3′	NM_053469
β-Actin	5′-CTAAGGCCAACCGTGAAAAGA-3′	5′-TGGTACGACCAGAGGCATACA-3′	NM_031144

Table 3 – Effects of dietary Pi on body weights and food intake in IDA rats after 14 days

Experimental diets	Con Fe + 0.5% Pi	Low Fe + 0.5% Pi	Con Fe + 1.5% Pi	Low Fe + 1.5% Pi
Body weight, g	165.3 ± 25.7	152.0 ± 4.4	116.7 ± 16.5 *	110.3 ± 12.7 †
Food intake, g	20.6 ± 0.5	18.6 ± 0.6	18.7 ± 3.0	17.7 ± 2.9

Values are expressed as means ± SEM. The results were representative of 3 separate experiments; n = 5 rats per group.
* $P < .05$ vs Con Fe + 0.5% Pi.
† $P < .05$ vs Low Fe + 0.5% Pi.

diet group, a Low Fe diet significantly decreased the RBC count and Hb concentration and altered the hematocrit compared with the Con Fe diet group. Interestingly, the groups of rats fed the 1.5% Pi diet for 14 days did not exhibit anemia even while consuming the Low Fe diet (Fig. 2A-C). The mean corpuscular volume (MCV) was significantly lower in the Low Fe + 1.5% Pi group than in the Low Fe + 0.5% Pi and Con Fe + 1.5% Pi groups (Table 4). Mean corpuscular hemoglobin (MCH) and MCH concentration were not significantly different among the 4 treatment groups. To test the long-term effect of dietary Pi on IDA, rats were fed the same experimental diets for 28 days. As a result, the Low Fe + 1.5% Pi diet significantly decreased the RBC count and Hb concentration and altered the hematocrit compared with the Con Fe + 1.5% Pi diet group (Table 5). We could not measure the anemia state of the Low Fe + 0.5% diet group at 28 days, as they had severe anemia and coagulated blood.

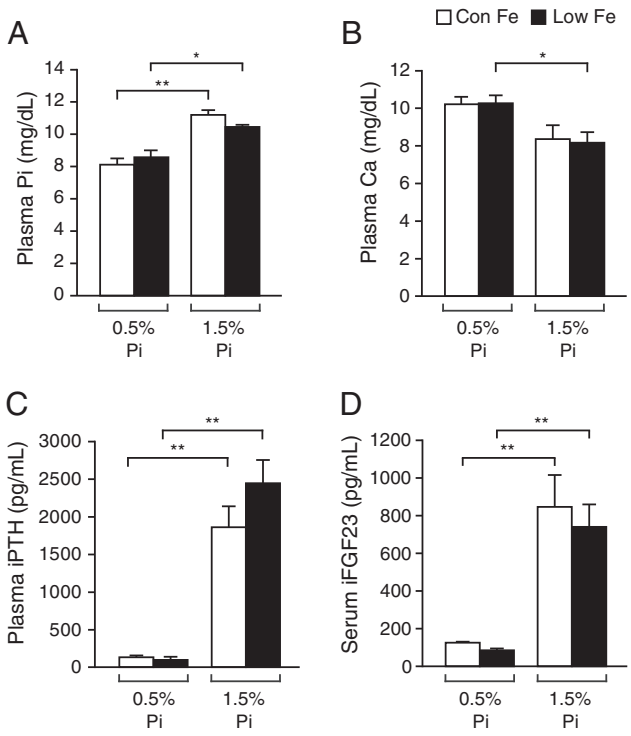

Fig. 1 – Biochemical parameters in plasma. Plasma levels of Pi (A), Ca (B), iPTH (C), and serum iFGF23 (D) in rats fed experimental diets for 14 days. Values are expressed as means ± SEM. The results are representative of 3 separate experiments. n = 4-5 rats per group. *P < .05; **P < .01.

We evaluated biochemical markers of iron status. Plasma iron concentrations and Tf saturation were lower in the Low Fe + 0.5% Pi group than in the Con Fe + 0.5% Pi group, whereas these parameters were higher in the Low Fe + 1.5% Pi group than in the Low Fe + 0.5% group (Fig. 2D and E). Feeding 1.5% Pi to rats averted IDA and maintained the blood iron concentration.

3.3. *Correlation between plasma Pi and 2,3-DPG*

A Low Fe diet did not affect the circulating concentration of 2,3-DPG in plasma (Fig. 3A). However, plasma 2,3-DPG concentrations were higher in the rats fed the diet containing 1.5% Pi than in those fed a diet with 0.5% Pi. In addition, a strong correlation ($r = 0.779$; $P < .001$) was observed between the plasma 2,3-DPG and Pi concentrations (Fig. 3B).

3.4. *Regulation of EPO expression by dietary Pi in IDA*

The production of RBCs in the bone marrow is determined by the plasma concentration of EPO, a hormone released from the kidneys and up-regulated by anemia, hypoxia, and iron chelators [36]. We analyzed the concentration of EPO in plasma and renal messenger RNA (mRNA) expression. The group fed the Low Fe + 0.5% Pi diet had much higher plasma EPO concentrations than the Con Fe + 0.5% Pi diet group, although there were no significant differences between the Con Fe + 1.5% Pi group and the Low Fe + 1.5% Pi group (Fig. 4A). Quantitative real-time PCR analysis showed suppression of renal *Epo* expression in the rats given 1.5% Pi in their diet (Fig. 4B).

3.5. *Effects of dietary Pi on expression of iron-regulated genes in IDA*

Next, we analyzed duodenal iron-regulated gene expressions to elucidate the effect of dietary Pi on iron metabolism in IDA. Real-time PCR analysis showed that duodenal mRNA levels of *Dmt1*, *DcytB*, and *ferroportin* were significantly greater in the groups of rats fed a Low Fe diet than in those fed the Con Fe diet (Fig. 5A-C). Interestingly, the diet with 1.5% Pi inhibited the mRNA expression of *Dmt1* and *DcytB* associated with the Low Fe diet but did not affect the *ferroportin* mRNA concentrations. Notably, the Con Fe + 1.5% Pi diet resulted in lower duodenal *DcytB* mRNA levels than those detected in rats fed the Con Fe + 0.5% Pi diet (Fig. 5B).

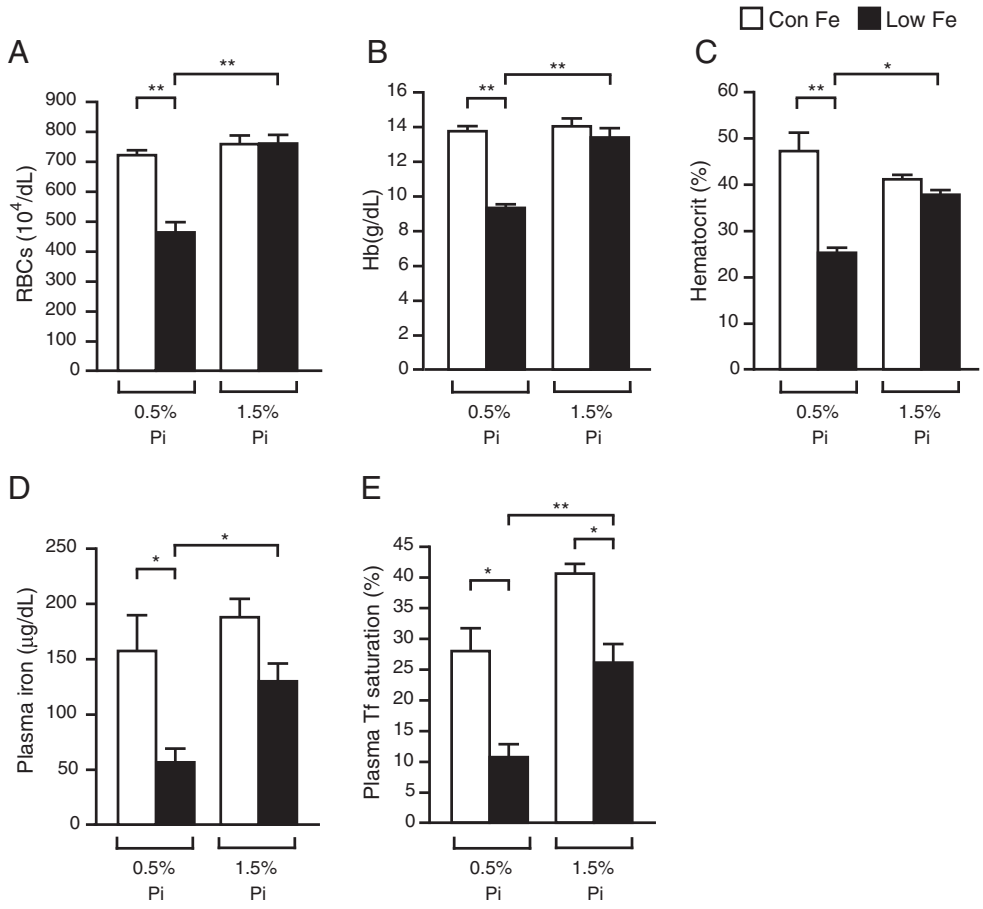

Fig. 2 – Effects of dietary Pi on RBCs, plasma iron, and Tf saturation in IDA rats after 14 days. Red blood cell analysis (A-C), plasma iron (D), and Tf saturation (E) in rats fed experimental diets for 14 days. Values are means ± SEM. The results are representative of 3 separate experiments. n = 5 rats per group. *P < .05; **P < .01.

3.6. *Effects of dietary Pi on hepatic hepcidin expression and hepatic iron in IDA*

In the rats fed a Low Fe diet, *hepcidin* mRNA levels in the liver were markedly decreased compared with rats fed the Con Fe diet (Fig. 6A). In contrast, the Con Fe + 1.5% Pi group exhibited a significantly increased hepatic *hepcidin* expression compared with rats in the Con Fe + 0.5% Pi group. To further elucidate the mechanism involved in the regulation of iron metabolism by dietary Pi, hepatic iron was measured. In contrast to rats fed the Con Fe diet, the rats fed the Low Fe diet showed a significantly reduced concentration of iron in liver tissues (Fig. 6B). Rats fed Con Fe + 1.5% Pi exhibited significantly increased concentrations of hepatic iron compared with rats that consumed the Con Fe + 0.5% Pi diet.

Table 4 – Erythrocyte parameters of whole blood in IDA rats after 14 days

Experimental diets	Con Fe + 0.5% Pi	Low Fe + 0.5% Pi	Con Fe + 1.5% Pi	Low Fe + 1.5% Pi
MCV (fl)	59.4 ± 0.2	55.2 ± 2.0	54.25 ± 1.0	49.8 ± 0.7 †,#
MCH (pg)	19.1 ± 0.1	20.7 ± 1.6	18.5 ± 0.1	17.6 ± 0.1
MCHC (%)	32.1 ± 0.2	35.8 ± 1.3	36.0 ± 1.8	35.4 ± 0.6

Values are expressed as means ± SEM. The results were representative of 3 separate experiments; n = 5 rats per group. Abbreviation: MCHC, mean corpuscular hemoglobin concentration.
† P < .05 vs Low Fe + 0.5% Pi.
# P < .05 vs Con Fe + 1.5% Pi.

4. Discussion

The present study demonstrated that dietary Pi supplementation delays the onset of IDA and modulates iron metabolism in iron-deficient rats during 14 days. Recent reports have

Table 5 – Effect of dietary Pi on RBCs in IDA rats after 28 days

Experimental diets	Con Fe + 0.5% Pi	Low Fe + 0.5% Pi	Con Fe + 1.5% Pi	Low Fe + 1.5% Pi
RBCs (10^4/dL)	794.0 ± 7.6	ND	755.2 ± 8.1	660.0 ± 21.4 #
Hb (g/dL)	14.6 ± 0.1	ND	13.8 ± 0.2	10.4 ± 0.3 #
Hematocrit (%)	44.3 ± 0.4	ND	41.8 ± 0.5	28.8 ± 0.9 #

Values are expressed as means ± SEM; n = 5 rats per group. Abbreviation: ND, not detected.
# P < .05 vs Con Fe + 1.5% Pi.

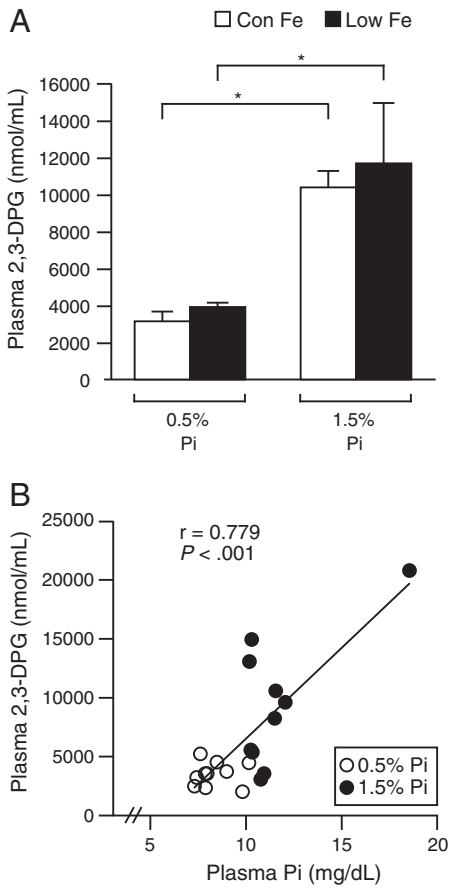

Fig. 3 – Correlation between Pi and 2,3-DPG in rat plasma. Plasma 2,3-DPG (A) and correlation between plasma Pi and 2,3-DPG (B) in rats. The white circles indicate 0.5% Pi groups, and the black circles indicate 1.5% Pi groups. Values are expressed as means ± SEM. n = 5 rats per group. *P < .05; **P < .01; *r* = Pearson correlation coefficient.

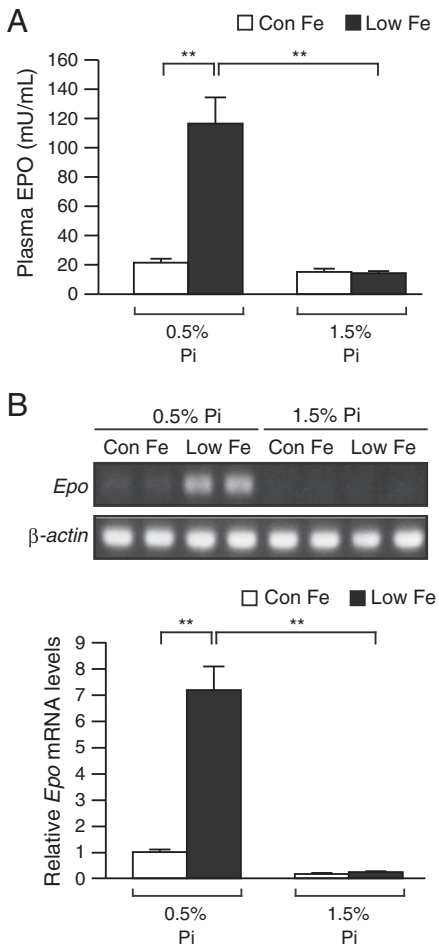

Fig. 4 – Suppression of rat plasma concentrations and renal expression of EPO by dietary Pi despite administration of low-iron diet for 14 days. Plasma EPO analysis (A) and quantitative PCR analysis measuring Epo in RNA from the kidney (B). Expression was normalized to ß-actin. Values are expressed as means ± SEM. The results are representative of 3 separate experiments. n = 5 rats per group. *P < .05; **P < .01.

shown that a low-iron diet can induce hypophosphatemia and increase the serum concentration of iFGF23 in neonatal mice [23], but not in mature mice [21]. We also examined the effect of iron deficiency on Pi metabolism and found that the plasma Pi, iPTH, and iFGF23 levels were not significantly different in 4-week-old rats fed a Low Fe diet for 14 days. Although an effect of iron deficiency on Pi metabolism was not observed clearly in the current study, we evaluated the effects of dietary Pi in a rodent model of IDA.

As we expected, rats fed the 1.5% Pi diets for 14 days retained normal RBCs, Hb, and hematocrit, even under conditions of low iron concentration in plasma. We also evaluated rats fed the Low Fe + 1.5% Pi diet for 28 days and confirmed that they had anemia. We found that compensation through phosphate is short term and that dietary Pi can play an important role in the development of the early stages of IDA. Mature human erythrocytes are dependent on anaerobic glycolysis, as it is the only pathway for net ATP synthesis and is essential for cellular integrity. Therefore, the glycolytic rate and glycolytic intermediates are intimately linked to erythrocyte survival and to the function of Hb [37]. It has been reported that Pi deficiency decreases the life span of erythrocytes, presumably resulting from energy depletion, as the concentration of Pi in serum directly stimulates the rate of erythrocyte glycolysis [38]. These reports raise the possibility that supplementation of Pi in the diet may provide energy to erythrocytes until the onset of systemic iron deficiency.

We noted that the concentration of 2,3-DPG in erythrocytes has an important function: it controls the amount of oxygen supplied to peripheral tissues. Plasma concentrations of 2,3-DPG were elevated by addition of 1.5% Pi to the diet and were positively correlated with plasma Pi concentration. Elevated concentrations of 2,3-DPG have been shown to shift the Hb-oxygen dissociation curve to the right and decrease the oxygen-binding affinity of Hb, thereby inducing oxygen release from Hb [26-28]. Zhang et al [39,40] proposed that 2,3-DPG was enhanced by adenosine, promoted oxygen release, and prevented acute ischemic tissue injury. In our study, plasma and renal expression of EPO were not increased in the groups of rats fed the Low Fe + 1.5% Pi diet. We suggest that

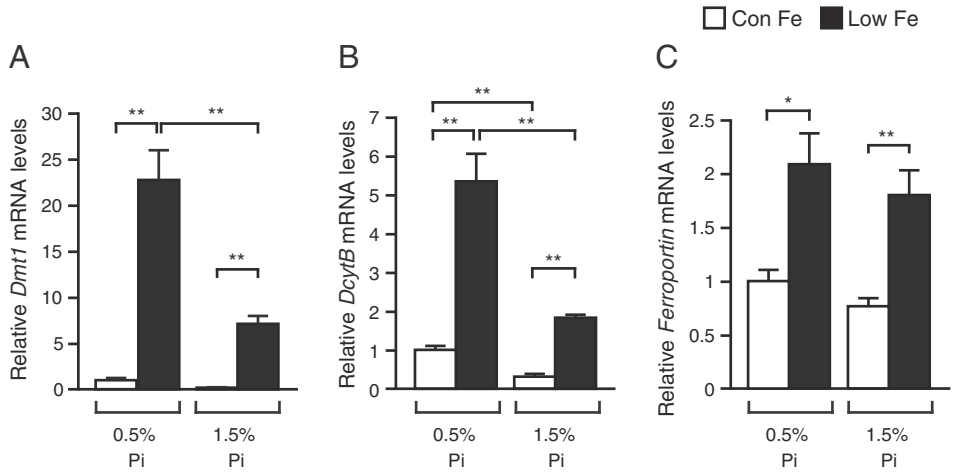

Fig. 5 – Effects of dietary Pi on expression of iron-regulated genes in IDA rats after 14 days. Quantitative PCR analysis measuring Dmt1 (A), DcytB (B), and ferroportin (C) expression in RNA from duodenal epithelium. Expression was normalized to ß-actin. Values are expressed as means ± SEM. The results are representative of 3 separate experiments. n = 4-5 rats per group. *P < .05; **P < .01.

the increase in the concentration of 2,3-DPG in the 1.5% Pi groups was implicated in an increased oxygen release from Hb. It has been shown that a 40% dietary calorie restriction caused microcytic and short-lived erythrocytes without anemia in mice [41]. However, despite the decreased glycolytic activity in calorie-restricted mice, it was also shown that erythrocyte function providing 2,3-DPG may be maintained [42]. In the present study, the MCV was significantly lower in the rats fed the Low Fe + 1.5% Pi diet than in rats fed the Low Fe + 0.5% Pi diet or the Con Fe + 1.5% Pi diet. Our results indicated that, in rats with IDA, Pi supplementation resulted in microcytic erythrocytes without the presence of anemia and with increased plasma 2,3-DPG levels. Based on these findings, it is plausible that increased 2,3-DPG concentrations support erythrocyte function and thereby maintain erythrocyte numbers. However, it remains to be determined whether dietary Pi increases the life span of erythrocytes or increases erythropoiesis. Further studies are needed to determine the mechanisms by which dietary Pi can avert IDA.

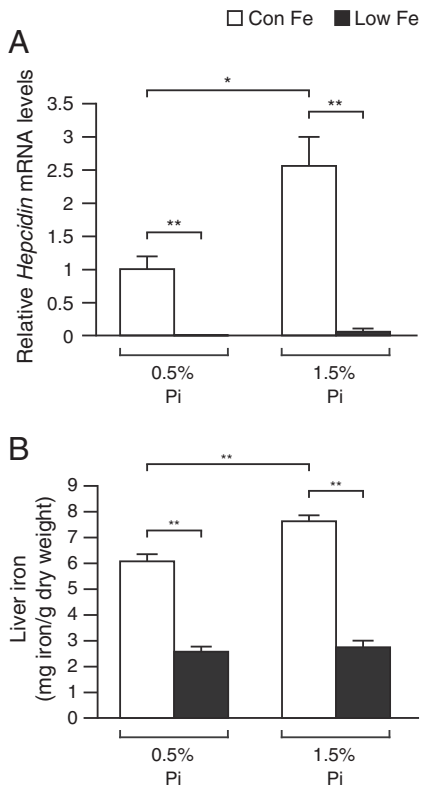

Fig. 6 – Effects of dietary Pi on the expression of hepcidin and hepatic iron in IDA rats after 14 days. Quantitative PCR analysis measuring hepatic hepcidin RNA expression normalized to ß-actin (A) and nonheme iron content of the liver (B). Values are expressed as means ± SEM. The results are representative of 3 separate experiments. n = 4-5 rats per group. *P < .05; **P < .01.

Importantly, a significant increase in hepatic *hepcidin* mRNA expression and a decrease in *Dmt1* and *DcytB* mRNA expression in the duodenum were evident in the rats fed the Con Fe + 1.5% Pi diet compared with those fed the Con Fe + 0.5% Pi diet. Furthermore, plasma concentrations of hepcidin were significantly increased after consumption of the 1.5% Pi diet (data not shown). There is a possibility that hepcidin modulates *Dmt1* expression in addition to *ferroportin* [43]. Our data suggest that dietary Pi induces hepcidin production and is implicated in iron-regulated gene expression. Hepcidin expression in hepatocytes can be induced by excess iron [19] and inflammation [44]. In the Con Fe + 1.5% Pi dietary group, hepatic iron concentrations were significantly increased compared with those in rats fed Con Fe + 0.5% Pi, indicating that dietary Pi may induce iron overload in the liver. In addition, it has been reported that Pi overload directly induces inflammation [45]. We also propose that Pi supplementation induces systemic inflammation and thereby increases the expression of *hepcidin* in the liver. A high Pi intake has been shown to cause hypocalcemia, and it can influence calcium homeostasis via many factors, such as FGF23, PTH, and vitamin D [7]. Dietary Pi may also be associated with iron

homeostasis, through by many factors such as anemia and hepcidin regulation.

Inorganic phosphate is well recognized as a negative regulator of iron absorption in humans and animals [46,47]. However, our findings provide additional insight into the regulation of iron metabolism in rats. Recently, sodium phosphate has gained recognition as an ergogenic substance that can enhance exercise performance. Several mechanisms have been proposed to explain the benefit provided after sodium phosphate ingestion, which includes enhancement of 2,3-DPG concentration [48,49]. Based on our study, we propose that Pi may also have potential as a novel nutritional ergogenic factor in such conditions as sports anemia. However, we analyzed only anemia and iron status in rats. Furthermore, as previously reported [50], body weights were significantly decreased in the 1.5% Pi diet groups. A high Pi intake causes hypocalcemia, hyperphosphatemia, and secondary hyperparathyroidism with bone resorption and bone loss [51,52], making it imperative to further elucidate the relationship between dietary Pi supplementation and erythropoiesis in clinical studies, while also considering possible side effects.

In conclusion, dietary Pi supplementation delays the onset of IDA in rats, possibly owing to an increase in the concentration of 2,3-DPG in blood. Furthermore, we found that dietary Pi supplementation induced iron accumulation in the liver and thereafter modulated the expression of iron-regulated genes.

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