# The Effect of Iron Dextran on Vitamin $D_3$ Metabolism in SD Rats

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**Research Article**

**Keywords:** Iron, 25-(OH)D3, 1,25-(OH)2D3, CYP2R1, CYP27A1, CYP27B1, CYP24A1

**Posted Date:** November 8th, 2021

**DOI:** https://doi.org/10.21203/rs.3.rs-968573/v1

# Abstract

**Background:** The status of iron and vitamin D (VD) is essential to health. Previous studies have shown that iron homeostasis has a potential effect on VD metabolism, but the mechanism is not fully understood.

**Objectives:** To explore the relationship between VD metabolism and iron metabolism, as well as the regulatory mechanism of iron on VD metabolism.

**Methods:** Methods: 40 male rats were fed adaptively for 7 days and randomly divided into control (C, n=6 normal diet) group and model (M, n=24 iron deficient diet) group according to body weight, the latter was used to establish iron deficiency anemia (IDA) model. After 6 weeks of feeding, the M group was randomly divided into: deficiency iron group (DFe), low iron group (LFe), medium iron group (MFe) and high iron group (HFe) according to the level of hemoglobin (Hb). Different doses of iron dextran (based on iron content (100g·bw·d)): 0, 1.1, 3.3 and 9.9mg were given respectively. After 4 weeks, the rats were anesthetized with 8% chloral hydrate, blood was collected from the abdominal aorta, liver and kidney tissues were collected. The serum and tissues were separately packed and frozen at -80℃ for testing.

**Results:** The results showed that the levels of Hb, RBC, serum iron (SI), liver iron, and kidney iron DFe group were lower than those in the other four groups, while the levels of total iron-binding capacity (TIBC), transferrin (TF) and transferrin receptor (Tfr) in DFe group were higher than those in other groups; The serum levels of 25-(OH)$D_3$ and 1,25-$(OH)_2D_3$ in DFe group were significantly lower than those in C group ($P < 0.05$). The correlation analysis showed that the levels of 25-(OH)$D_3$ and 1,25-$(OH)_2D_3$ were negatively correlated with TIBC, TF and Tfr no correlation with SI. Western blot, immunofluorescence, and q-PCR results showed that compared with C group, the protein and gene expressions of CYP2R1, CYP27A1, and CYP24A1 in DFe group were down-regulated, and the expression of CYP27B1 protein and gene was up-regulated in DFe group.

**Conclusion:** Therefore, iron may be involved in the metabolism of $VD_3$ by regulating the expression of $VD_3$ hydroxylase, suggesting that appropriate iron supplementation can promote the activation of $VD_3$.


# 1. Introduction

Vitamin D (VD) is an essential micronutrient for the body, which is vital to health. In addition to regulating calcium and phosphorus metabolism, VD with hormone properties can induce cell differentiation and apoptosis, inhibit cell proliferation and other cell signal transduction processes, participate in the regulation process of multiple genes, and involve in the physiological and pathological mechanism of various diseases[1–2]. VD deficiency is related to many diseases, including immune dysfunction, metabolic syndrome, insulin resistance, infection, cancer, and cardiovascular abnormalities[3–5]. According to statistics, about 30% and 60% of children and adults in the world are deficient in VD[6]. There is also a widespread VD deficiency in Chinese population. Ling[7] surveyed elderly people over 60 years

old in different latitudes in our country, and showed that 92.28% of the elderly suffer from VD deficiency and insufficient; Jinjian[8] said 62.40% of males and 52.39% of young people in East China of women had VD deficiency. VD is generally lacking in people all over the world, even in high sunshine areas such as Algeria, Libya, and Brazil [9–11].

VD is a fat-soluble vitamins, including five types of $VD_1$-$VD_5$, among which $VD_3$ (cholecalciferol) and $VD_2$ (ergosterol) have the greatest impact on human health[16]. Endogenous synthesis of $VD_3$ through ultraviolet rays exposure to cholesterol precursors in the skin. The liver 25-hydroxylase (CYP2R1 and CYP27A1 enzymes) catalyzes the formation of 25-(OH)$D_3$, and the circulating 25-(OH)$D_3$ is further hydroxylated in the kidneys, depending on 1α-hydroxylase (CYP27B1 enzyme). The active form of 1,25-$(OH)_2D_3$ combines with VDR to play a biological role in the body, and CYP24A1 participates in the catabolism of VD[17]. The activated VD can promote cell differentiation and proliferation, bone resorption, and improve immunity[18]. The search for continuous and stable 25-(OH)$D_3$ and highly active 1,25-$(OH)_2D_3$ has always been the focus of research.

Iron is another essential micronutrient and a cofactor of many enzymes, which participates in many physiological processes, especially in hemoglobin production, heme synthesis, electron transfer, and so on. More and more observational studies have shown that there is an association between VD deficiency and iron deficiency or iron load. The third national health and nutrition survey found that VD was related to anemia[21]. Atkinson[22] pointed out that 25-(OH)$D_3$ deficiency was associated with an increased risk of anemia. Katsumata[23] have shown that iron deficiency leads to VD deficiency, and iron plays an important role in VD synthesis. In iron overload diseases, VD deficiency is related to iron accumulation in the liver[24]. Based on these findings, iron homeostasis may affect the metabolic level of VD. But so far, the exact mechanism of the regulation of iron on VD metabolism has not been reported. Therefore, it is of great significance to explore the regulatory mechanism of iron on VD metabolism for VD deficiency caused by iron homeostasis imbalance.

In our study, iron deficiency anemia (IDA) rat model was used to determine the relationship between VD and iron metabolism; we hypothesized that iron deficiency would reduce the metabolism level of VD, and iron loading would inhibit the metabolism level of VD; iron might affect the metabolism level of VD through the regulation of hydroxylase.

# 2. Methods

# 2.1 Experimental designs

The 40 male newly weaned Specific Pathogen Free (SPF) SD rats, weighing 66.46 ± 4.22g, were purchased from Beijing Changyang Xishan animal farm, animal Certificate No: scxk (Beijing): 2019-0010. After 1 week of adaptive feeding, they were randomly divided into control (C, n=8) group and model (M, n=32) group. C group was fed with normal diet and normal drinking water; M group was fed with iron

deficiency diet (iron content was 21.33mg/kg) and drinking deionized water to establish IDA model. After 6 weeks, they were randomly divided into four groups: deficiency iron group (DFe) [0mg/(100g·bw·d)], low iron group (LFe) [1.1mg/ (100g·bw·d)], medium iron group (MFe) [3.3mg/(100g·bw·d)], high iron group (HFe) [9.9mg/(100g·bw·d)] according to hemoglobin (Hb) level, 2ml for each animal for 4 weeks, C and DFe groups were perfused with 2ml normal saline. After 4 weeks of intervention, the rats were anesthetized with 8% chloral hydrate. Blood samples were collected from the abdominal aorta for 8-10ml. The blood was placed in a centrifugal tube for 30 min at room temperature and then centrifuged at 3000 r/min for 10 min to separate the serum. After washing the liver and kidney with normal saline, some of them were fixed by immersion in 4% paraformaldehyde, and the others were put into nuclease free tube and frozen at -80℃. In addition to the different iron content, the other nutritional components of the two diets were the same. Both of them added 1500IU of $VD_3$, and used shading cloth to prevent the rats from sunlight to synthesize $VD_3$ through the skin. The temperature and relative humidity were 21±1℃ and 46% - 60% respectively. In order to avoid iron pollution, SD rats were housed in stainless steel cages, with stainless steel food containers and plastic drinking bottles.

## 2.2 Main reagents and instruments

Iron Dextran (Yuanye Biology, S51662); $VD_3$ (Beijing Solarbio, V8070); Tissue Iron Determination Kit (Nanjing Jiancheng, A039-2-1); Hb Kit (Nanjing Jiancheng, C021-1-1 ); 25-(OH)$D_3$ kit (Jianglai Biological, JL20734); 1,25-(OH)$D_3$ kit (Jianglai Biological, JL27246); TRF (Jianglai Biotechnology, JL-31079); sTrf (Jianglai Biotechnology, JL-30566); SI (Nanjing Jiancheng, A039-1-1); TIBC (Nanjing Jiancheng, A040-1-1); CYP2R1 primary antibody (ABclonal, A10470 ); CYP27A1 primary antibody (ABclonal, A1982); CYP27B1 primary antibody (ABclonal, A1716); CYP24A1 primary antibody (ABclonal, A1805); Cy3-goat anti-rabbit IgG (Beyotime, A0516). Microplate reader (Bio-Rad, USA); DAPI (Aladdin, D106471); Paraffin embedding machine (Wuhan Junjie Electronics Co, Ltd, JT-12S); Microtome (Lycra Microsystems Co, Ltd, RM2016); Electron microscope (Nikon Instruments Co, Ltd, Eclipse Ci-L); PCR instrument (Hangzhou Bioet Company, Line Gene9640PCR).

## 2.3 Detection of hematological and serological indexes

ELISA kit was used to detect 25-(OH)$D_3$, 1,25-$(OH)_2D_3$, TF, and Tfr; Biochemical kit was used to detect Hb, RBC,TIBC, SI, liver iron and kidney iron, and the operation was carried out strictly according to the operation procedures of the kit.

## 2.4 Western blot was used to detect the expression of CYP2R1, CYP27A1, CYP27B1 and CYP24A1 protein

The expression of CYP2R1 and CYP27A1 protein in liver, and the expression of CYP27B1 and CYP24A1 protein in kidney was detected, each sample was taken 20mg, and Radio Immunoprecipitation Assay (RIPA) lysate was added, The total protein was extracted, protein was prescribed by BCA Assay Kit, the total protein was sampled with 20μg protein in 96-well plate, gel electrophoresis, transfer membrane, block, 4℃ primary antibody CYP2R1, CYP27A1, CYP27B1, CYP24A1 (1:500), β-actin (1:5000) were

incubated overnight at room temperature, The secondary antibody (1:5000) was incubated for 2h, CYP2R1, CYP27A1, CYP27B1, and CYP24A1 were detected by the luminescent solution, and the gray value was analyzed by Image J software.

## 2.5 Immunofluorescence staining of CYP2R1, CYP27A1, CYP27B1 and CYP24A1 protein

The liver and kidney tissue specimens fixed with 4% paraformaldehyde, and then dehydrate after fixation, wax embedding, sectioning, dewaxing, hydration, gradient ethanol elution, antigen retrieval, CYP2R1, CYP27A1, CYP27B1, and CYP24A1 (diluted 1:100) primary antibody incubation, incubated with secondary antibody (diluted at 1:200) and counter-stain with diamidino-2-pheny1 indole (DAPI). The immunofluorescence intensity in the liver tissue was observed and photographed under a fluorescence microscope. The CYP2R1, CYP27A1, CYP27B1 and CYP24A1 protein staining were positive in red. The fluorescence intensity of CYP2R1, CYP27A1, CYP27B1 and CYP24A1 protein was detected by Image Pro Plus image analysis software, and the integrated optical density (IOD) was calculated as the final average immunofluorescence intensity.

## 2.6 The mRNA expressions of CYP2R1, CYP27A1, CYP27B1 and CYP24A1 were detected by q-PCR

Total RNA was extracted from 20mg liver and kidney tissues of rats. The cDNA was reverse transcribed and amplified by fluorescent quantitative PCR with β-actin as an internal reference. The upstream primer sequence of β-actin was 5´-TGTGATGGTGGGAATGGGTCAG-3´, downstream primer sequence 5´-TTTGATGTCACGCACGATTTCC -3´. CYP2R1 upstream primer sequence: 5´-TGCTACTACTCGTGCTGGTGGTC-3´, downstream primer sequence 5´-AGGGCCAGGGA GCAGATGTTG-3´. CYP27A1 upstream primer sequence: 5´-TCGCACCAATGTGAATCTGGC TAG-3´, downstream primer 5´-CTTCCACTGCTCCATGCTGTCTC-3´. CYP27B1 upstream primer sequence: 5´-GCACATAAACGGCAAGGCAAGTC-3´, downstream primer sequence 5´-AGCCAAGCCTCTCACCTCCTATG-3´. CYP24A1 upstream primer sequence 5´-GGAACTGT A CGCCGCTGTCAC-3´, downstream primer 5´-GCACGCTCTGAACTTCCTGAAGG-3´. PCR conditions: pre-denaturation 95℃, 30s; denaturation: 95℃, 5s, 60℃, 30s (40 cycles); annealing: 95℃, 5s, 60℃, 30s, 95℃, 1min; extension: 50℃, 30s. The relative quantitative values were calculated according to the Ct values of the original q-PCR data.

## 2.7 Statistical analysis

Spss 25.0 software was used for statistical analysis of the data. The HB value and body weight of group C and group M were tested by t test; $VD_3$ metabolism index, iron metabolism index, tissue iron and other indicators were compared by one-way ANOVA, LSD-t test or Dunnett's T3 test were used for pairwise comparison, $P < 0.05$ means the difference was statistically significant.

# 3. Results

### 3.1 General condition of SD rats

During the modeling period, the animals appeared some phenomena, such as fluffy hair, falling off, dull eyes, loose stool, fear of cold, white teeth, and slow growth and development and so on. After 6w of modeling, Hb in group M was detected to be lower than that in group C (63.61±5.39g/L) vs (125.58±2.90g/L) to judge the modeling situation. Except for DFe group, the condition of rats in each group recovered after intervention with the corresponding dose of iron dextran. The higher the intervention concentration, the faster the weight gain (Fig. 1).

### 3.2 Tissue iron content

The contents of liver iron and kidney iron in DFe group were much lower than those in other four groups ($P$< 0.05), and those in LFe group were significantly lower than those in HFe group ($P$< 0.05)(Table. 1).

### 3.3 Hematological indicators

Compared with the C group, the Hb and RBC contents were the lowest in the DFe group. The Hb content in the DFe group was significantly lower than that in the C, LFe, MFe and HFe groups, and the RBC content was significantly lower than that in the HFe group, and the differences were statistically significant ($P$<0.05)(Table. 2).

### 3.4 Serum levels of 25-(OH)$D_3$ and 1,25-$(OH)_2D_3$

The levels of 25-(OH)$D_3$ and 1,25-$(OH)_2D_3$ in serum of DFe group were much lower than those in C, MFe and HFe groups, and the differences were statistically significant ($P$<0.05). The content of 25-(OH)$D_3$ and 1,25-$(OH)_2D_3$ gradually increased in the LFe, MFe and HFe groups, and the difference between the LFe group and the HFe group was statistically significant ($P$<0.05); The content of 25-(OH)$D_3$ in MFe group was significantly different from that in HFe group ($P$<0.05) (Table. 3).

### 3.5 Serum iron metabolism indicators

The levels of TIBC, TF, and Tfr were the highest in the DFe group. Compared with the C group, the levels of TIBC and Tfr were significantly increased in the DFe group, and the differences were statistically significant ($P$<0.05); The level of SI was the lowest in the DFe group, which was significantly lower than that in the C, LFe, MFe and HFe groups ($P$<0.05); compared with the HFe group, the level of Tfr was significantly increased in the DFe and LFe groups ($P$<0.05) (Table. 4).

### 3.6 Correlation analysis of iron metabolism and VD metabolism

Correlation analysis showed that 25-(OH)$D_3$ level was significantly negatively correlated with TIBC, TF and Tfr indicators; 1,25-$(OH)_2D_3$ level was significantly negatively correlated with TIBC, TF and Tfr indicators; However, 25-(OH)$D_3$ and 1,25-$(OH)_2D_3$ levels had no correlation with SI (Table. 5).

### 3.7 Western blot was used to detect the expression of CYP2R1, CYP27A1, CYP27B1, CYP24A1 protein.

Compared with the C and HFe groups, the CYP2R1 protein expression level in the DFe group was significantly down-regulated ($P$<0.05). With the increase of iron intervention concentration, the CYP2R1 protein expression level gradually increased (Fig. 2A); the CYP27A1 protein expression in the DFe group was the lowest in DFe group, compared with the MFe group and the HFe group, the difference was statistically significant ($P$<0.05)(Fig. 2B); CYP27B1 was the highest in DFe group, and as the iron intervention concentration increased, the CYP27B1 protein expression level gradually decreased (Fig. 2C); compared with the C, MFe and HFe groups, the CYP24A1 expression was significantly down-regulated ($P$ 0.05)(Fig. 2D).

### 3.8 Immunofluorescence staining of CYP2R1、CYP27A1、CYP27B1、CYP24A1 protein

The immunofluorescence results showed that compared with C group, CYP2R1 protein expression in DFe group was significantly lower ($P$<0.05); CYP2R1 protein expression in DFe group was significantly lower than that of MFe and HFe groups ($P$<0.05). Compared with C group, CYP27A1 protein expression did not change significantly in DFe group, and the difference was not statistically significant; compared with HFe group, CYP27A1 protein expression was significantly lower ($P$<0.05); CYP27B1 protein expression was highest in DFe Group, which was statistically significant compared with C, MFe and HFe groups ($P$<0.05); compared with C group, CYP24A1 protein expression was significantly down-regulated ($P$<0.05), CYP24A1 protein expression in MFe and HFe groups were Higher than the DFe group ($P$<0.05) (Table. 6, Fig. 3).

### 3.9 The mRNA expressions of CYP2R1、CYP27A1、CYP27B1、CYP24A1 were detected by q-PCR

Compared with C group, the expression of CYP2R1mRNA was down- regulated in DFe group, but the difference was not statistically significant; the content of CYP2R1mRNA in DFe and LFe groups were significantly lower than that in HFe group ($P$ 0.05); the content of CYP27A1mRNA was the lowest in DFe group, which in DFe and LFe groups were significantly lower than that in C group ($P$<0.05); the content of CYP27B1mRNA was the highest in DFe group, and the content of CYP24A1mRNA was the lowest in DFe group, there was no statistically significant difference between the groups (Table. 7).

## 4. Discussion

Iron is an essential trace element that is required for the growth and survival of the body, and plays an important role in the whole life process of the body. The liver is the master storage place of iron and the master regulator of iron homeostasis. When the body is lack of iron, the development of the liver is slowed down, the structure of mitochondria and microsomes is abnormal, and the content of cytochrome P450 (CYP450) enzymes is reduced, which may even affect the biological metabolism process[25-26]. Any degree of iron deficiency can have an effect on health. Similarly, iron load can also damage health. Excessive iron can catalyze the production of reactive oxygen species through Fenton / Haber Weiss reaction[27]. The accumulation of reactive oxygen species may lead to DNA damage, cell cycle arrest and

mitochondrial dysfunction[28-29]. Increasing numbers of observational studies have shown that there is an association between VD and iron, and the specific mechanism has not been fully elaborated.

VD is mainly synthesized by ultraviolet rays, and environmental factors affect the seasonal fluctuation of VD concentration. The most classic role of VD is to regulate calcium and phosphorus metabolism and promote bone development. As research progresses, iron homeostasis also has a potential impact on bone formation, bone resorption and bone development[30]. The serum 1,25-$(OH)_2D_3$ concentration reduced after treatment the dietary iron deficiency in rat, based on the effect of iron deficiency on bone metabolism[31]. SD rats were injected with iron dextran (250mg/kg of body weight) every other day for 5 weeks, and iron loading caused bone injury[32]. These studies revealed that iron deficiency or iron loading caused reduced VD activity levels, and this chain reaction could further exacerbate bone diseases. In addition, dietary red meat intake is considered to be an important factor in serum 25-$(OH)D_3$. Portela[33] stated that the red meat intake group had higher serum 25-$(OH)D_3$ levels than the fish intake group. Because red meat with heme iron, dietary intake may promote the level of VD. Iron deficiency interferes with the transformation of VD in skin and intestine and metabolism in the body[34]. These studies indicate that there is a certain correlation between iron and VD.

The liver and kidney are the main sites for the hydroxylation of VD, and the enzymes of VD hydroxylation are mediated by CYP450. CYP450 enzyme is a member of the heme-containing monooxygenase family. Mitochondrial P450 is located in the inner membrane and participated in the biosynthesis of bile acids, from cholesterol in the liver, steroid hormones in the adrenal and gonads, and metabolic activation of $VD_3$ into the active form of 1,25-$(OH)_2D_3$ in the liver and kidney[35]. The selective hydroxylation of $VD_3$ by $VD_3$ hydroxylase requires the cofactor NADH or NADPH as an electron donor. NADH or NADPH provides electrons to ferredoxin reductase, then donates it to ferredoxin, and then delivers them to CYP450 enzyme completes the selective hydroxylation of the substrate $VD_3$ through a cascade of redox processes[36]. Iron is the essential element of CYP450 function[37-38]. Iron deficiency may affect the function of these enzymes and may lead to VD deficiency. IDA has been demonstrated to reduce the activity of iron-containing enzymes in liver mitochondria, leading to impaired oxygen metabolism[39]. In addition, the mitochondria of iron-deficient cells exhibit reduced cytochrome concentrations[40]. These studies indicate that iron may play an important role in the hydroxylation process of VD, but the precise mechanism is not clear. This study proposed that iron may affect the hydroxylation level of VD through the influence of hydroxylase. In this study, an IDA model of SD rats was established with iron-deficient feed, focusing on the effects of different doses of iron on the serum 25-$(OH)D_3$ and 1,25-$(OH)_2D_3$ levels in SD rats. Katsumata[41] found that iron deficiency reduced the iron concentration in the kidney, further reducing the activity of 1α-hydroxylase, resulting in a decrease in serum 1,25-$(OH)_2D_3$ concentration in an iron-deficiency animal model. Heldenberg's[42] study on infants and young children with IDA, although supplemented with adequate $VD_3$, the serum 25-$(OH)D_3$ concentration of the children was still very low, and the serum 25-$(OH)D_3$ concentration returned to normal after iron supplementation. In our experiment, iron deficiency led to the iron content of liver and kidney of SD rats decreased. The levels of serum 25-

$(OH)D_3$ and 1,25-$(OH)_2D_3$ in DFe group were significantly lower than those in C group. The levels of serum 25-$(OH)D_3$ and 1,25-$(OH)_2D_3$ gradually increased with the increase in iron concentration. It is suggested that iron can promote the metabolic level of $VD_3$ and convert it into an active form of 1,25-$(OH)_2D_3$ in the body. In this process, iron is used by the body and the level of $VD_3$ activity increases.

Other studies have shown that iron loading also affects the levels of 25-$(OH)D_3$ and 1,25-$(OH)_2D_3$. Napoli[43] found that adults suffering from thalassemia had lower serum 25-(OH)D levels. Otto-Duessel[44] observed that the lack of VD in iron overload disease was related to the accumulation of liver iron. Excessive iron accumulation in the kidney would affect VDR, which would aggravate iron deposition and cause damage to the proximal tubules of the kidney[45]. In our study, the serum 25-$(OH)D_3$ and 1,25-$(OH)_2D_3$ levels in the HFe group were not found to be inhibited. It possibly because of the IDA model built in the early stage that the iron consumption in the rat body took too long. Iron only satisfied the needs of pre-consumption and growth and development requirements, and could not accumulate too much in the body, and thus did not inhibit the serum 25-$(OH)D_3$ and 1,25-$(OH)_2D_3$ levels. We will extend the intervention period in future experiments to observe the effect of iron overload on VD.

25-$(OH)D_3$ is the major circulating form of VD, and the concentration of 25-$(OH)D_3$ in serum is usually used to assess the status of VD. However, Worf[46] stated that iron intervention in women with IDA did not significantly improve the serum 25-$(OH)D_3$ status. After intravenous injection of iron, there was no effect on the level of 1,25-$(OH)_2D_3$ [47].On the one hand, it could be explained as the severity of IDA. On the other hand, this could be explained by the severe lack of VD in IDA women, and the level of hydroxylation is not sufficient to maintain the body's utilization, which leads to unsatisfactory improvement of VD status after iron intervention. Pandey[48] showed in a small-scale population study that the serum 25-$(OH)D_3$ level of the iron and VD supplementation group was preferable to that of the iron supplementation group alone. The level of serum 25-$(OH)D_3$ increased after the intervention of iron by Min and Panpan in our country[49-50]. The research results are controversial, the specific mechanism of iron's effect on VD is still unclear, and further experiments are needed to verify it.

Our results show that compared with C group, iron-deficient rats weighed less after 10 weeks, and the growth rate of rats in the DFe group was slower than that of other groups. Our study found that iron deficiency reduced the iron concentration in the liver and kidney, and the SI concentration was increased accordingly. The SI concentration was decreased with increasing the intervention concentration of iron. The concentrations of TIBC, TF and Tfr increased in the DFe group. The down-regulation of CYP2R1 and CYP27A1 expression at the protein and gene levels can be explained by iron deficiency reducing the activity of CYP2R1 and CYP27A1. VD with hormonal properties is to maintain the stability of the body's hormone levels. The protein and gene expression levels of CYP27B1 were up-regulated, and the expression of VD catabolic enzyme CYP24A1 was down-regulated accordingly. In our study, the gene expression levels of CYP2R1 and CYP27A1 were the lowest in the DFe group, and the HFe group was 3 times higher than the DFe group. It is illustrated that the decrease of iron reserve may be a necessary

condition for the control of these hydroxylase enzymes. Even in the case of mild iron deficiency, the enzyme function may change. This phenomenon suggested that under the condition of sufficient iron, the hydroxylation of VD is the use of this metal enzyme, which can effectively carry out hydroxylation reaction; when iron deficiency, hydroxylase can not rely on iron, which leads to the down-regulation of liver hydroxylase expression and the up-regulation of kidney hydroxylase expression. It is suggested that iron may play a role in 25 hydroxylation by regulating CYP2R1 and CYP27A1 enzymes, and 1α-hydroxylation by regulating CYP27B1 and CYP 24A1 enzymes, thus affecting the levels of 25-(OH)$D_3$ and 1,25-$(OH)_2D_3$. Iron supplementation may improve VD deficiency. The mechanism of iron hydroxylation in VD is based on the regulation of VD hydroxylase.

The results showed that different doses of iron had different effects on serum 25-(OH)$D_3$ and 1,25-$(OH)_2D_3$ in SD rats. Correlation analysis showed that the serum levels of 25-(OH)$D_3$ and 1,25-$(OH)_2D_3$ were negatively correlated with TIBC, TF and Tfr indicators; there was no correlation with SI. We think that reasonable iron supplementation to increase the level of VD activity is a simple, safe, cheap and effective method. At the same time, accurate identification of VD hydroxylation cofactors improves the bioavailability of VD, and the participation of iron in the formation of CYP450 enzymes plays a key role in VD metabolism. In our study, iron-deficient feed was used to establish an IDA model, the method reduced the levels of SI and Hb in rats at a stable rate, with no trauma and reliable effects.

Our study takes the lead in investigate the systematic mechanism of iron on VD metabolism. When the body is deficient in iron, the activity of VD hydroxylase may be damaged, thereby reducing the level of VD metabolism. However, our research also had certain limitations, we only based on animal experiments, the digestibility and absorptivity of iron and VD metabolism in group C and group M were not studied. It is necessary to further explore the effect of iron on VD metabolism in the population, so that proper iron supplementation can promote the expression of VD active products in the VD deficiency patient population. To understand the potential benefits of VD deficiency, provide new ideas for VD deficiency diseases.

# 5.conclusions

Iron deficiency reduces iron reserves in liver and kidney, which may affect hydroxylase activity; Iron may reduce the levels of 25- (OH) $D_3$ and 1,25 -(OH) $_2D_3$ by regulating hydroxylase at the translation and transcription levels; It is illustrated that the decrease of iron reserve may be a necessary condition for the control of these hydroxylase enzymes, even in the case of mild iron deficiency, the enzyme function may change.

# Abbreviations

VD: vitamin D; Hb: hemoglobin；BRC: red blood cell; SI: serum iron; TIBC: iron binding capacity; TF:Transferrin；Tfr: transferrin receptor; IDA : iron deficiency anemia; SPF: specific pathogen free; RIPA:

Immunoprecipitation Assay; DAPI: diamidino-2-pheny1 indole; CYP450: cytochrome P450; IOD: integrated optical density; C: control; DFe: deficiency iron; LFe: low iron; MFe: medium iron; HFe: high iron.

# Declarations

### 1. Ethics approval and consent to participate

The study was conducted according to the guidelines of Declaration of Helsinki, and approved by Ethics Committee of Shanxi Medical University (NO. SYDL: 2020005).

### 2. Consent for publication

Not applicable.

### 3. Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

### 4. Competing interests

The authors declare no potential conflict of interest.


### 5. Funding

The work was supported by the National Natural Science Foundation of China (no. 81573156) and Animal Committee of Shanxi Medical University.


### 6. Authors' contributions

Conceptualization and Methodology-F.B.Q., M.D.; Validation, Formal analysis, Writing-Original Draft-R.L., M.M; Resources, Writing-Review & Editing-S.Y.G. M.M.; Data Curation-Y.M.Z. M.M.; Software and SD rats breeding-L. X.Y. M.M.


### 7. Acknowledgements

Thanks to Shanxi Medical University for supporting this project.


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# Tables

**Table 1 The level of tissue iron (± s, n=8)**

| Groups | Liver iron(μmol/g) | Kidney iron(μmol/g) |
|---|---|---|
| C | 0.60±0.18 | 0.44±0.05 |
| DFe | 0.32±0.03[a] | 0.33±0.03[a] |
| LFe | 0.44±0.13 | 0.39±0.06 |
| MFe | 0.66±0.16 [b] | 0.44±0.05 [b] |
| HFe | 0.77±0.11[bc] | 0.50±0.06 [bc] |

[a]*P*0.05 vs C group; [b]*P*<0.05 vs DFe group; [c]*P*<0.05 vs LFe group

**Table 2 Hematological indexes (μmol/g) (± s, n=8)**

| Groups | RBC($\times10^{10}$/L) | Hb(g/L) |
|---|---|---|
| C | 685.00±166.22 | 113.99±12.97[*] |
| DFe | 465.00±226.21 | 52.04±10.00 |
| LFe | 690.00±406.10 | 106.42±13.79[*] |
| MFe | 965±374.28 | 115.64±18.72[*] |
| HFe | 1570.00±749.48[*] | 121.33±13.63[*] |

[*]*P*0.05 vs DFe group

**Table 3 Serum levels of 25-(OH)$D_3$ and 1,25-$(OH)_2D_3$ in SD rats (± s, n=8)**

| Groups | 25(OH)$D_3$(ng/mL) | 1,25$(OH)_2D_3$(ng/mL) |
|---|---|---|
| C | 44.61±8.63 | 29.81±9.50 |
| DFe | 25.89±3.96[a] | 12.19±6.06[a] |
| LFe | 32.65±3.87 [ab] | 15.47±4.56[a] |
| MFe | 35.36±5.72[b] | 22.98±6.84[b] |
| HFe | 49.31±5.68[bcd] | 29.75±8.68[bc] |

[a]*P*<0.05 vs C group; [b]*P*<0.05 vs DFe group; [c]*P*<0.05 vs LFe group; [d]*P* <0.05 vs MFe group

**Table 4 The serum iron metabolism indexes of SD rats (± s, n=8)**

| Groups | TNBI (*m*mol/L) | SI (*m*mol/L) | TF (g/L) | Tfr (nmol/L) |
|---|---|---|---|---|
| C | 148.72±30.35 | 99.85±16.64 | 5.74±1.27 | 32.99±13.83 |
| DFe | 222.28±26.43[a] | 36.15±7.28[a] | 6.73±0.94 | 56.23±14.39[a] |
| LFe | 132.09±19.24[ab] | 119.48±42.73[b] | 6.71±1.11 | 48.84±9.07 |
| MFe | 123.45±27.48[b] | 93.66±26.17[b] | 6.03±0.93 | 35.52±12.11 |
| HFe | 86.67±16.03[abc] | 73.34±19.75 [b] | 5.59±0.89 | 32.7±10.00[bc] |

[a]*P*0.05 vs C group; [b]*P*<0.05 vs DFe group; [c]*P*<0.05 vs LFe group

**Table 5 Correlation among 25-(OH)$D_3$,1,25$(OH)_2D_3$ and each variable (± s, n=8)**

| variables | 25-(OH)$D_3$ | 1,25-$(OH)_2D_3$ |
|---|---|---|
| TNBI | -0.667[**] | -0.548[**] |
| SI | — | — |
| TF | -0.366[*] | -0.469[**] |
| Tfr | -0.491[**] | -0.500[**] |

[*]*P*0.05, [**]*P*0.05,"—"No correlation

**Table 6 The Immunofluorescence IOD values (± s, n=3)**

| Groups | CYP2R1 | CYP27A1 | CYP27B1 | CYP24A1 |
|---|---|---|---|---|
| C | 207.43±13.34 | 221.11±32.88 | 76.76±2.68 | 172.19±12.81 |
| DFe | 59.28±5.92[a] | 128.94±6.19 | 141.34±5.40[a] | 46.24±7.75[a] |
| LFe | 101.04±5.81[ab] | 192.43±21.20 | 110.56±9.71 | 88.59±18.54[a] |
| MFe | 278.9±20.79[bc] | 208.27±37.14 | 55.24±3.71[abc] | 167.93±27.06[b] |
| HFe | 323.7±16.37[abc] | 279.64±7.21[b] | 33.52±2.11[abcd] | 191.43±8.48[bc] |

[a]*P*0.05 vs C group; [b]*P*<0.05 vs DFe group; [c]*P*<0.05 vs LFe group

**Table 7 The mRNA expressions of CYP2R1、CYP27A1、CYP27B1、CYP24A1 (± s, n=4)**

| Groups | CYP2R1 | CYP27A1 | CYP27B1 | CYP24A1 |
|---|---|---|---|---|
| C | 1±0.19 | 1±0.09 | 1±0.72 | 1±0.10 |
| DFe | 0.44±0.65 | 0.17±0.01[a] | 3.54±1.42 | 0.59±0.030 |
| LFe | 0.61±0.07 | 0.32±0.18[a] | 2.25±0.70 | 0.71±0.260 |
| MFe | 0.96±0.18 | 0.56±0.16 | 1.26±0.53 | 0.76±0.110 |
| HFe | 1.80±0.17[bc] | 0.69±0.31 | 1.15±0.54 | 1.10±0.180 |

[a]*P*0.05 vs C group; [b]*P*<0.05 vs DFe group; [c]*P*<0.05 vs LFe group

# Figures

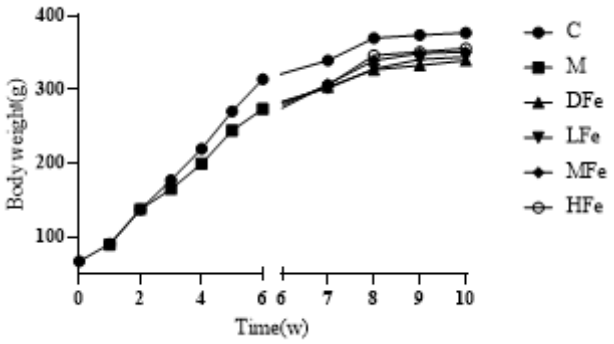



**Figure 1**

Weight changes of SD rats in each group

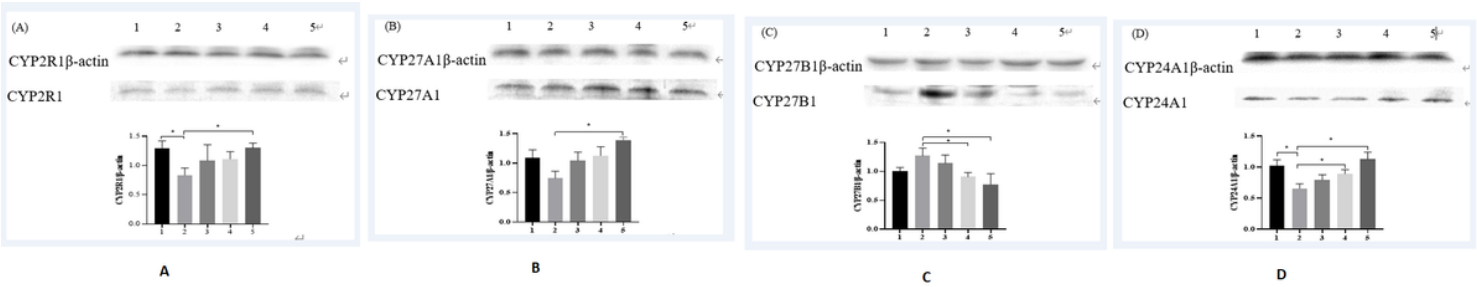
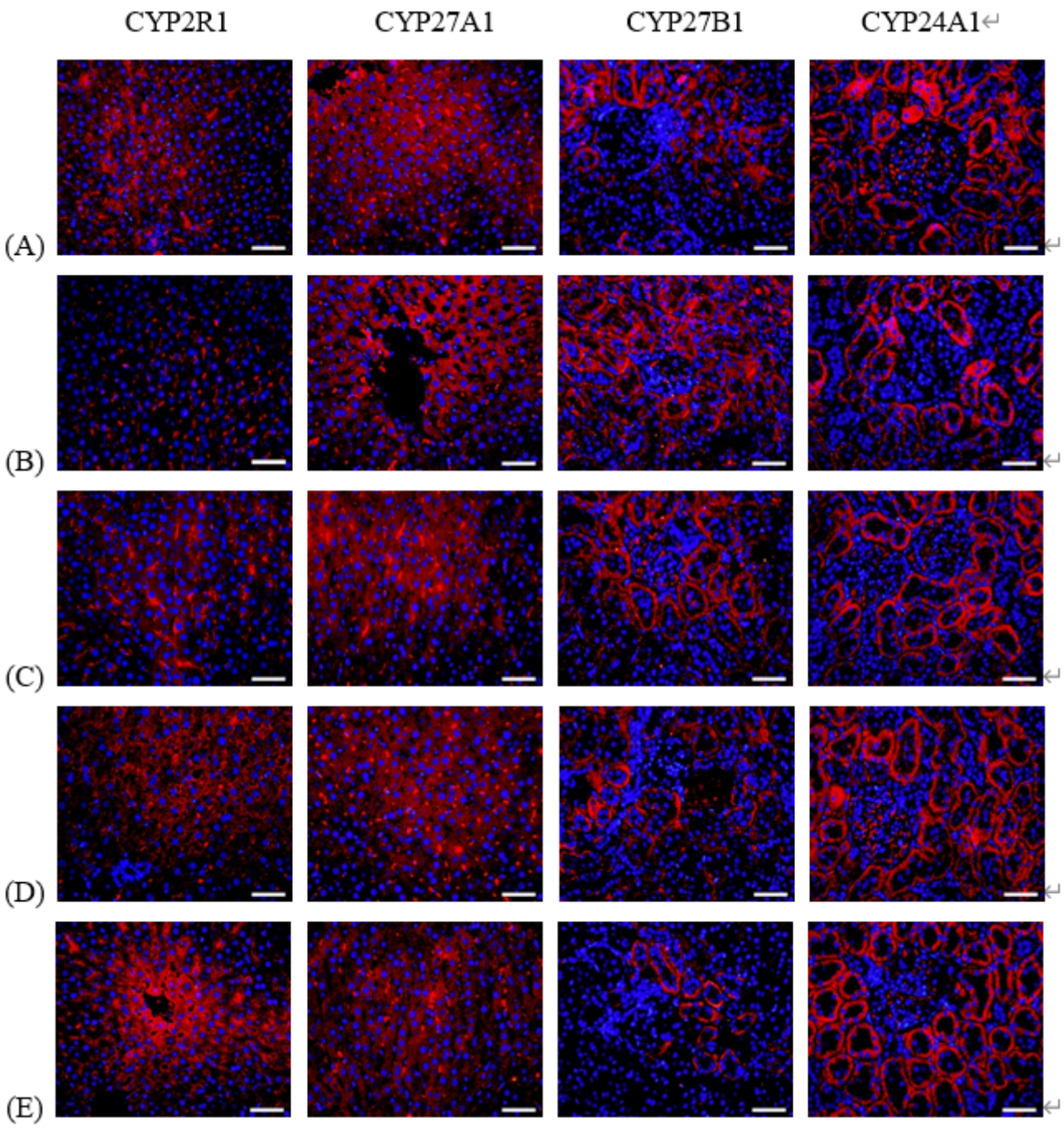



**Figure 2**

The protein expressions of CYP2R1、CYP27A1、CYP27B1、CYP24A1 n=4, 1:C, 2:DFe, 3:LFe, 4:MFe, 5:HFe, *P0.05




## Figure 3

The Immunofluorescence staining of CYP2R1、CYP27A1、CYP27B1、CYP24A1(400X) n=3, (A):C, (B):DFe, (C):LFe, (D): MFe, (E): HFe, Scale:50μm