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Original article

Dose-Dependent Influence of Vitamin D₃ on Blood Cell Indices and Clotting Function in Rats with Phenylhydrazine-Induced Haemolytic Anaemia

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Running title: Vitamin D₃, anaemia and coagulation in rats

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Abstract

Background. Coagulation disturbances often coexist with anaemia but are rarely studied together. Vitamin D receptors on megakaryocytes and erythroid precursors suggest this hormone may influence both erythropoiesis and haemostasis simultaneously. **Aim.** To determine whether oral vitamin D₃ restores blood counts, iron status and clotting times in a rat model of phenylhydrazine-induced haemolytic anaemia, and how these outcomes relate to circulating 25-hydroxyvitamin D. **Methods.** Forty male Wistar rats were randomised into five groups (n = 8): untreated control; anaemia-only (phenylhydrazine 40 mg/kg i.p. on two days); two PHZ groups given oral vitamin D₃ (500 or 1000 IU/kg/day) for four weeks; and a PHZ group given weekly intramuscular iron dextran (10 mg/kg). Groups were compared by one-way ANOVA with Tukey's HSD; associations with 25(OH)D₃ were assessed by Pearson's correlation. **Results.** Phenylhydrazine reduced haemoglobin, RBC count, haematocrit, serum iron and ferritin, and prolonged PT and aPTT (all $p < 0.01$). Both vitamin D₃ doses improved Hb, RBC, MCV and platelets dose-dependently; the higher dose shortened PT and aPTT and raised fibrinogen. 25(OH)D₃ correlated positively with haemoglobin ($r = +0.71$, $p < 0.001$) and negatively with PT ($r = -0.65$, $p < 0.001$).

Conclusion. Oral vitamin D₃ produced dose-dependent improvement in both the blood picture and clotting profile of anaemic rats, consistent with VDR-mediated effects on erythroid maturation and vitamin K-dependent clotting factors, and supports vitamin D₃ as a plausible adjunct in anaemia management.

Keywords: Cholecalciferol, haemolytic anaemia, clotting time, activated partial thromboplastin time, phenylhydrazine, 25-hydroxyvitamin D.

1. Introduction

Few haematological problems touch as many people as anaemia. Current global estimates place the affected population at more than a billion, with the burden falling unevenly across age groups, regions and clinical settings (1). The condition itself is not a single disease but a final common pathway, reached through blood loss, impaired production or accelerated destruction of erythrocytes. Of these mechanisms, haemolysis is particularly demanding on the organism: red cells are destroyed faster than they can be replaced, iron is liberated abruptly into circulation, and the marrow is forced into a state of high-output erythropoiesis. The consequences extend well beyond the red-cell line itself (2).

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One of the consequences that have attracted growing interest is the impact on haemostasis. The clotting cascade, a tightly choreographed sequence in which the intrinsic and extrinsic limbs converge on thrombin generation and fibrin polymerization, is sensitive to the metabolic disturbances that accompany red-cell breakdown. Patients with haemolytic disorders not infrequently display prolonged prothrombin and activated partial thromboplastin times, depressed fibrinogen and altered platelet behaviour (3). Liberation of free haem and intracellular components from lysed erythrocytes appears to be a central driver, activating coagulation and consuming haemostatic reserves (4).

Vitamin D_3 — cholecalciferol — was for many decades thought of almost exclusively as a regulator of calcium and bone. That picture has changed considerably. The nuclear vitamin D receptor (VDR) has now been mapped to a wide array of non-skeletal tissues, including the haematopoietic stem cell compartment, megakaryocytes, erythroid progenitors, hepatocytes and the vascular endothelium (5,6). Each of these locations has produced its own line of mechanistic evidence linking vitamin D status to functions far removed from mineral metabolism.

Within the bone marrow, the active hormone 1,25-dihydroxyvitamin D_3 has been shown to encourage erythroid commitment by stimulating proliferation of early erythroid progenitors via the VDR (7,8). Clinical and epidemiological work has paralleled these laboratory findings: lower serum 25(OH)D consistently tracks with lower haemoglobin across heterogeneous adult populations (9,10). More recent studies have added a further dimension, suggesting that vitamin D acts upstream of hepcidin and therefore influences how much iron is made available to the developing erythron (11,12).

The relevance of vitamin D_3 to coagulation is less widely appreciated but is supported by a converging body of work. The vitamin D receptor is expressed on platelets and megakaryocytes, and deletion of the receptor in mice enhances thrombogenicity (13). Vitamin D suppresses tissue factor and plasminogen activator inhibitor-1 expression in vascular smooth muscle, producing a net antithrombotic effect under inflammatory conditions (14). These laboratory observations sit alongside clinical reports linking hypovitaminosis D to abnormalities of clotting markers in patients with cardiovascular and inflammatory disease (15).

Experimental modelling of haemolytic anaemia in rats has long relied on phenylhydrazine (PHZ), a reagent that selectively oxidises haemoglobin and damages the erythrocyte membrane. The resulting picture of brisk haemolysis, reticulocytosis, compensatory splenic enlargement and secondary derangement of coagulation reproduces the cardinal features of the human disease with useful fidelity (16). Despite this, the literature contains no controlled experiment, to our knowledge, that has tested whether vitamin D_3 supplementation can simultaneously address the haematological and the haemostatic limbs of the PHZ-induced syndrome. The present study was designed to fill that gap. We compared two oral doses of cholecalciferol (500 and 1000 IU/kg/day) with parenteral iron dextran in a four-week intervention, tracking the full CBC, the iron panel, four coagulation indices, and serum 25(OH)D_3.

2. Materials and Methods

2.1. Animals and housing conditions

Forty male Wistar albino rats (180–220 g, aged 8–10 weeks) were sourced from an accredited breeding facility. They were kept five to a cage in polypropylene housing with paper-pulp bedding under conventional conditions: 22 ± 2 °C ambient temperature, 50 ± 10% relative humidity, and a 12-hour light/dark cycle. Standard pelleted chow and filtered tap water were available without restriction. A one-week acclimatisation period preceded any intervention.

2.2. Chemicals and reagents

Phenylhydrazine hydrochloride (≥97% purity) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Cholecalciferol oil (500,000 IU/mL) was supplied by Pharmazone (Cairo, Egypt) and was diluted in pharmaceutical-grade olive oil to the required working concentrations. Iron dextran (50 mg/mL; Cosmofer®) was supplied by Pharmacosmos (Holbaek, Denmark). CBC reagents were obtained from Elhoda Trade (Cairo, Egypt). All remaining reagents were of analytical grade.

2.3. Experimental design and group allocation

After acclimatisation, the animals were assigned to one of five arms (n = 8 per arm) using a computer-generated random sequence. Treatments ran for 28 days in the supplementation groups and started on day 12 — i.e. once anaemia had been confirmed. The five arms were as follows:

- **Group 1 (untreated control):** intraperitoneal 0.9% saline on days 1 and 2, followed by daily oral olive-oil vehicle for four weeks.
- **Group 2 (anaemia, no treatment):** PHZ 40 mg/kg i.p. on two consecutive days, then the olive-oil vehicle by gavage for four weeks.
- **Group 3 (low-dose D_3):** PHZ as above, then cholecalciferol 500 IU/kg/day by oral gavage from day 12 onward.
- **Group 4 (high-dose D_3):** PHZ as above, then cholecalciferol 1000 IU/kg/day by oral gavage from day 12 onward.
- **Group 5 (iron-dextran positive control):** PHZ as above, then iron dextran 10 mg/kg i.m. once weekly for four weeks.

2.4. Induction and confirmation of anaemia

Phenylhydrazine hydrochloride was freshly dissolved in 0.9% saline (pH 7.4) and administered intraperitoneally at 40 mg/kg on two successive days. The compound generates reactive oxygen species that oxidise membrane and intracellular components of the erythrocyte, leading to a brisk wave of haemolysis with a vigorous reticulocyte response and expansion of the erythroid compartment. We considered anaemia established when haemoglobin had fallen to ≤ 9 g/dL and haematocrit to ≤ 30% by day 11. Any rat that did not meet both criteria was withdrawn and replaced before the intervention phase commenced.

2.5. Blood sample collection

On day 42, the animals were fasted overnight and then deeply anaesthetised with intraperitoneal ketamine (90 mg/kg) plus xylazine (10 mg/kg). Terminal blood was drawn by cardiac puncture and divided into three tubes: EDTA for the complete blood count, 3.2% trisodium citrate (blood-to-anticoagulant ratio 9:1) for coagulation testing, and a plain serum-separator tube for biochemistry.

2.7. Laboratory analyses

2.7.1. Complete blood count

The CBC was performed on EDTA whole blood within 60 minutes of collection using an automated haematology analyser (Urit BH-70P; Urit Medical Electronic Co., Ltd., Guilin, China). Reported parameters were haemoglobin, RBC count, haematocrit, MCV, MCH, MCHC, total white-cell count, platelet count and reticulocyte percentage.

2.7.2. Coagulation parameters

Citrated plasma was analysed on a Coatron semi-automated coagulometer (TECO Medical Instruments, Neufahrn, Germany). Prothrombin time was measured with rabbit-brain thromboplastin and reported alongside the derived INR. Activated partial thromboplastin time was determined with an ellagic acid activator, thrombin time with bovine thrombin, and plasma fibrinogen by the Clauss technique.

2.7.3. Iron profile and serum biochemistry

Serum iron and total iron-binding capacity were assayed colorimetrically; transferrin saturation was derived as (iron / TIBC) × 100. Serum ferritin was

measured by a rat-specific sandwich ELISA. Calcium was determined by the o-cresolphthalein complexone (OCPC) method, inorganic phosphate by the molybdate-UV procedure, and intact parathyroid hormone by a rat-specific ELISA.

2.7.4. Serum 25-hydroxyvitamin D_3

Circulating 25(OH)D_3 was quantified using a competitive ELISA kit from Immundiagnostik AG (Bensheim, Germany; analytical sensitivity 1.28 nmol/L; intra-assay CV < 8%; inter-assay CV < 10%). Sample concentrations were read from a freshly calibrated standard curve and are reported in nmol/L.

2.8. Statistical analysis

Results are summarised as mean ± SEM. Analyses were carried out in IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA). The Shapiro–Wilk test was used to check distributional assumptions and Levene's test to check homogeneity of variance. Between-group differences were tested by one-way ANOVA followed by Tukey's HSD post-hoc procedure. Associations between serum 25(OH)D_3 and the haematological and coagulation outcomes were quantified across the pooled cohort ($n = 40$) by Pearson's correlation. A two-tailed $p < 0.05$ was treated as evidence of statistical significance.

3. Results

3.1. Body weight and general health

All forty rats completed the protocol, and no deaths occurred. Baseline body weights, measured immediately before PHZ administration, were similar across the five arms (range of group means 189–196 g; $p = 0.527$). By day 11, the anaemic animals had lost ground relative to the untreated controls (G1: 208 ± 4 g vs. G2: 181 ± 5 g; $p < 0.01$), reflecting the acute physiological cost of severe haemolysis. After four weeks of treatment, the high-dose vitamin D_3 group (G4: 224 ± 5 g) and the iron-dextran group (G5: 228 ± 6 g) had recovered to within range of the untreated controls (G1: 231 ± 5 g; $p > 0.05$). No animal in any group showed clinical signs suggestive of toxicity.

3.2. Complete blood count

PHZ produced the expected severe haemolytic phenotype, with steep falls in haemoglobin, RBC count and haematocrit by day 11. Four weeks of supplementation with vitamin D_3 reversed these changes in a graded fashion: the 1000 IU/kg/day group reached haemoglobin and erythrocyte values that were statistically indistinguishable from those of the iron-dextran comparator (G5; $p > 0.05$). The full dataset is shown in Table 1. The reticulocyte response provided a sensitive readout of marrow activity. PHZ drove reticulocytes from 1.3% to 12.4% — a near tenfold rise reflecting the marrow's effort to compensate for accelerated red-cell loss. Both vitamin D_3 doses dampened this response in proportion to the dose given; in the high-dose arm, reticulocytes returned almost to baseline (4.2%), a level very close to that achieved with iron dextran (3.9%).

3.3. Coagulation profile

Anaemia control animals showed clear evidence of a clotting defect. Both PT and aPTT were prolonged relative to untreated controls, implicating both arms of the cascade, and fibrinogen was depressed — a pattern compatible with low-grade consumption (see Table 2). Cholecalciferol corrected this picture in a dose-dependent way. At the higher dose, PT and aPTT returned to values that did not differ statistically from those of the untreated controls ($p > 0.05$), and fibrinogen was substantially restored.

3.4. Iron status, 25(OH)D_3, and mineral biochemistry

Haemolysis was accompanied, in the untreated anaemic animals, by a pattern that mimicked iron deficiency: low serum iron and ferritin, an elevated total iron-binding capacity, and depressed transferrin saturation (Table 3). Vitamin D_3 improved this profile at both doses, and the higher

dose brought transferrin saturation and ferritin into the same statistical range as iron dextran. As expected, serum 25(OH) D_3 rose substantially in the supplemented groups, confirming that the orally administered cholecalciferol was being absorbed and metabolized effectively.

3.5. Correlations between 25(OH)D_3 and outcome variables

When data from all forty animals were pooled, serum 25(OH)D_3 tracked with the haematological and clotting endpoints in a coherent way. Haemoglobin showed the strongest positive association ($r = +0.71$, $p < 0.001$) and prothrombin time the strongest negative one ($r = -0.65$, $p < 0.001$). Comparable, statistically robust correlations were observed for RBC count, haematocrit, platelets, fibrinogen, serum iron and ferritin (Table 4).

Table 1. Complete blood count at day 42 (mean ± SEM; n = 8 per group).

Parameter	G1 Normal Control	G2 Anaemia Control	G3 Vit D_3 500 IU	G4 Vit D_3 1000 IU	G5 Iron Dextran
Hb (g/dL)	14.8 ± 0.3	6.9 ± 0.4**	9.7 ± 0.3*†	12.4 ± 0.4*†‡	12.9 ± 0.3*†‡
RBC (×10^6/µL)	7.21 ± 0.18	3.42 ± 0.21**	5.01 ± 0.19*†	6.38 ± 0.22*†‡	6.51 ± 0.20*†‡
Hct (%)	46.3 ± 0.9	22.1 ± 1.1**	30.4 ± 0.9*†	39.7 ± 1.0*†‡	40.8 ± 1.1*†‡
MCV (fL)	64.2 ± 1.1	64.6 ± 1.3	60.7 ± 1.0†	62.2 ± 0.9	62.7 ± 1.1
MCH (pg)	20.5 ± 0.4	20.2 ± 0.5	19.4 ± 0.3	19.4 ± 0.4	19.8 ± 0.4
MCHC (g/dL)	32.0 ± 0.5	31.2 ± 0.6	31.9 ± 0.4	31.2 ± 0.5	30.9 ± 0.4
WBC (×10^3/µL)	7.42 ± 0.31	9.81 ± 0.44**	8.65 ± 0.38*†	7.98 ± 0.35†	8.12 ± 0.40†
PLT (×10^3/µL)	682 ± 24	410 ± 31**	524 ± 27*†	621 ± 28*†‡	598 ± 25*†‡
Reticulocytes (%)	1.3 ± 0.1	12.4 ± 0.8**	8.1 ± 0.6*†	4.2 ± 0.4*†‡	3.9 ± 0.5*†‡

Note. ** $p < 0.01$ vs. G1; * $p < 0.05$ vs. G1; † $p < 0.05$ vs. G2; ‡ $p < 0.05$ vs. G3. Hb, haemoglobin; RBC, red blood cell count; Hct, haematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular Hb concentration; WBC, white blood cell count; PLT, platelet count.

Figure 1. Haemoglobin (A) and RBC count (B) across groups at day 42

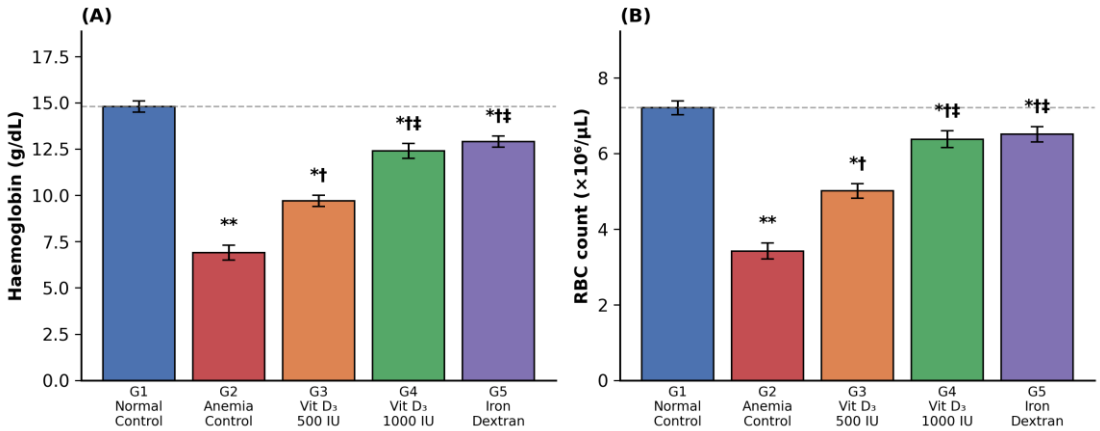

Figure 1. *Haemoglobin (A) and red blood cell count (B) at day 42. Bars represent group means; whiskers, SEM (n = 8 per group). Symbols: ** p < 0.01 vs. G1; * p < 0.05 vs. G1; † p < 0.05 vs. G2; ‡ p < 0.05 vs. G3 (Tukey's HSD). The dashed horizontal line marks the mean of the untreated control arm.*

Figure 2. Platelet count (A) and reticulocyte percentage (B) at day 42

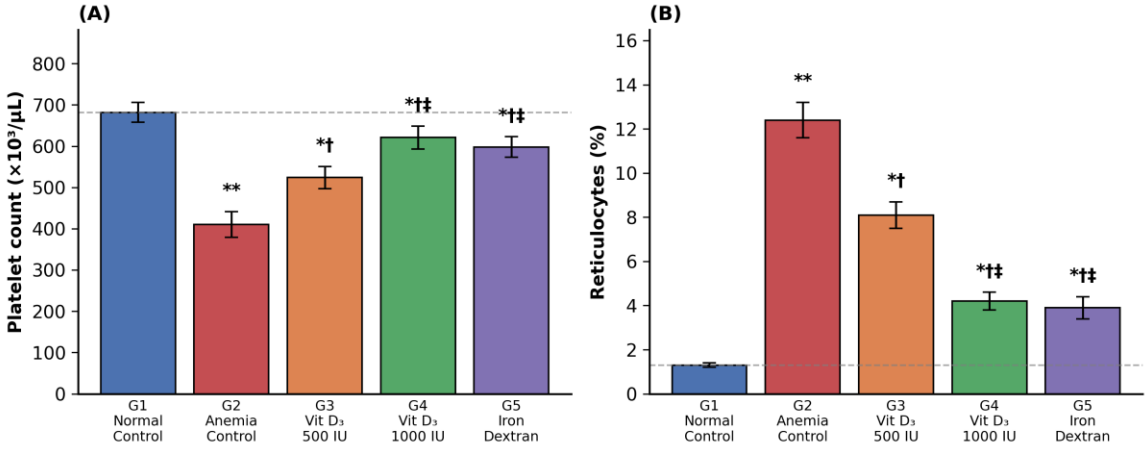

Figure 2. *Platelet count (A) and reticulocyte percentage (B) at day 42. The progressive return of reticulocytes toward baseline with increasing vitamin D_3 dose is consistent with restoration of erythropoietic homeostasis. Conventions as for Figure 1.*

Table 2. Coagulation parameters at day 42 (mean ± SEM; n = 8 per group).

Parameter	G1 Normal Control	G2 Anaemia Control	G3 Vit D_3 500 IU	G4 Vit D_3 1000 IU	G5 Iron Dextran
PT (seconds)	13.2 ± 0.3	18.7 ± 0.5**	16.4 ± 0.4*†	14.1 ± 0.3*†‡	15.8 ± 0.4*†
INR	1.00 ± 0.02	1.42 ± 0.04**	1.24 ± 0.03*†	1.07 ± 0.02*†‡	1.20 ± 0.03*†
aPTT (seconds)	28.4 ± 0.6	39.8 ± 0.9**	35.2 ± 0.7*†	30.1 ± 0.6*†‡	33.6 ± 0.8*†
TT (seconds)	14.6 ± 0.3	18.2 ± 0.5**	16.8 ± 0.4*†	15.4 ± 0.3†‡	16.1 ± 0.4*†
Fibrinogen (g/L)	2.80 ± 0.12	1.74 ± 0.10**	2.11 ± 0.09*†	2.61 ± 0.11*†‡	2.45 ± 0.12*†‡

Note. *Significance symbols as in Table 1. PT, prothrombin time; INR, international normalised ratio; aPTT, activated partial thromboplastin time; TT, thrombin time.*

Figure 3. Prothrombin time (A) and aPTT (B) at day 42

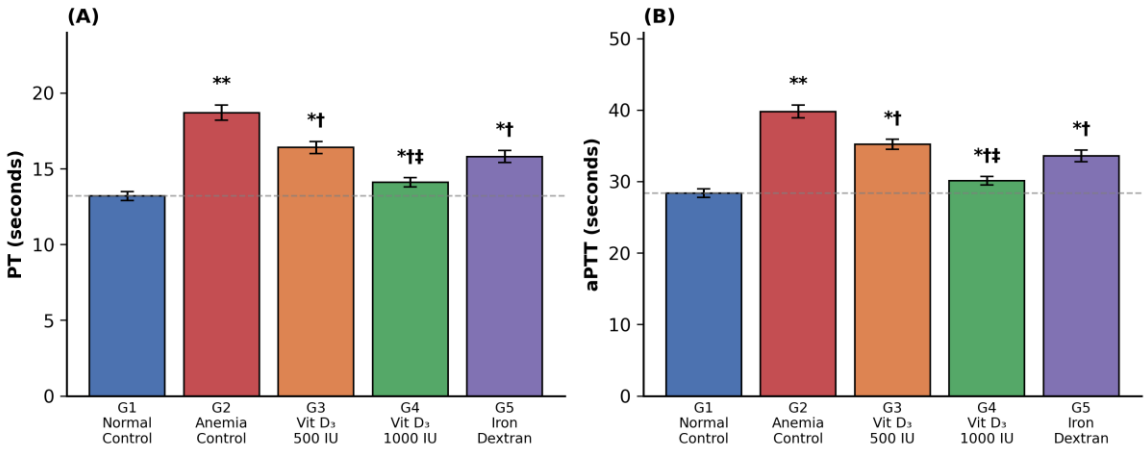

Figure 3. *Prothrombin time (A) and activated partial thromboplastin time (B) at day 42. The dashed line marks the untreated control mean (G1). Bars are means; whiskers, SEM (n = 8). Symbols as in Figure 1.*

Figure 4. Plasma fibrinogen concentration at day 42 (Clauss method)

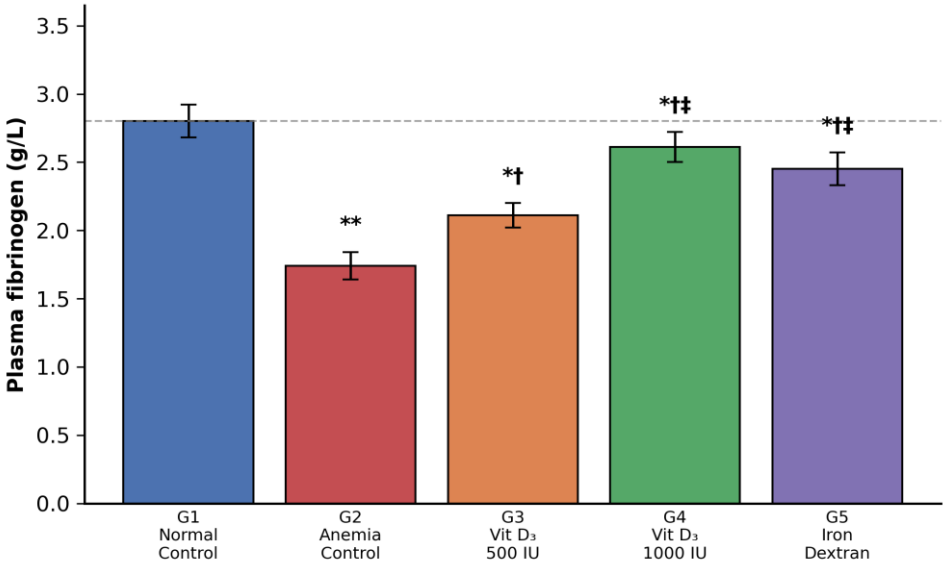

Figure 4. *Plasma fibrinogen at day 42, measured by the Clauss method. The dashed line indicates the untreated control mean. Bars are means with SEM (n = 8). Symbols as in Figure 1.*

Table 3. Iron profile, 25(OH)D₃ and mineral biochemistry at day 42 (mean ± SEM; n = 8 per group).

Parameter	G1 Normal Control	G2 Anaemia Control	G3 Vit D₃ 500 IU	G4 Vit D₃ 1000 IU	G5 Iron Dextran
Serum Iron (µmol/L)	22.4 ± 0.8	9.1 ± 0.6**	14.3 ± 0.7*†	18.2 ± 0.8*†‡	24.1 ± 0.9*†‡
TIBC (µmol/L)	54.6 ± 1.4	78.3 ± 2.1**	68.7 ± 1.8*†	61.2 ± 1.5*†‡	58.4 ± 1.6*†‡
Transferrin Sat. (%)	41.0 ± 1.2	11.6 ± 0.8**	20.8 ± 1.1*†	29.8 ± 1.0*†‡	41.3 ± 1.4†‡
Ferritin (ng/mL)	62.4 ± 2.8	18.7 ± 1.9**	31.2 ± 2.1*†	46.8 ± 2.4*†‡	71.3 ± 3.1†‡
25(OH)D₃ (nmol/L)	48.2 ± 2.1	31.4 ± 1.8**	74.6 ± 2.9*†	118.3 ± 4.2*†‡	34.1 ± 2.0**
Calcium (mmol/L)	2.42 ± 0.06	2.18 ± 0.07*	2.38 ± 0.06†	2.52 ± 0.07*†‡	2.22 ± 0.07*
Phosphorus (mmol/L)	1.68 ± 0.05	1.72 ± 0.06	1.70 ± 0.05	1.74 ± 0.06	1.69 ± 0.05
PTH (pg/mL)	38.4 ± 2.1	52.1 ± 2.8**	43.6 ± 2.3†	35.8 ± 2.0†‡	48.2 ± 2.5*

Note. *Significance symbols as in Table 1. 25(OH)D₃, 25-hydroxyvitamin D₃; TIBC, total iron-binding capacity; PTH, parathyroid hormone.*

Figure 5. Serum 25(OH)D₃ and ferritin across groups at day 42

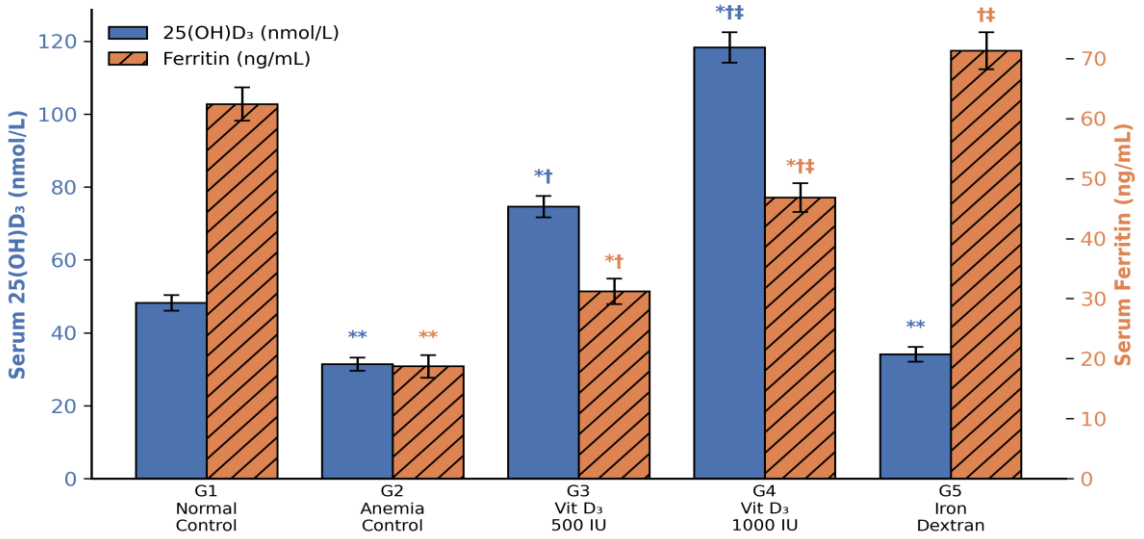

Figure 5. *Serum 25(OH)D₃ (solid bars, left y-axis) and ferritin (hatched bars, right y-axis) at day 42. Bars are means; whiskers, SEM (n = 8). The marked rise of 25(OH)D₃ in G3 and G4 confirms systemic uptake of the oral supplement. Symbols as in Figure 1.*

Table 4. Pearson's correlation coefficients between serum 25(OH)D₃ and haematological/coagulation outcomes (n = 40 pooled).

Variable	Pearson r	p-value
Haemoglobin (g/dL)	+0.71**	< 0.001
RBC (×10⁶/µL)	+0.68**	< 0.001
Haematocrit (%)	+0.66**	< 0.001
Platelet count	+0.55**	< 0.001
PT (seconds)	−0.65**	< 0.001
aPTT (seconds)	−0.61**	< 0.001
Fibrinogen (g/L)	+0.59**	< 0.001
Serum iron (µmol/L)	+0.63**	< 0.001
Ferritin (ng/mL)	+0.58**	< 0.001
Reticulocytes (%)	−0.52**	0.002

Note. *** p < 0.01, two-tailed. Coefficients computed on the pooled dataset across the five experimental arms (n = 40).*

Figure 6. Pearson's correlation between serum 25(OH)D₃ and key outcomes (n = 40 pooled)

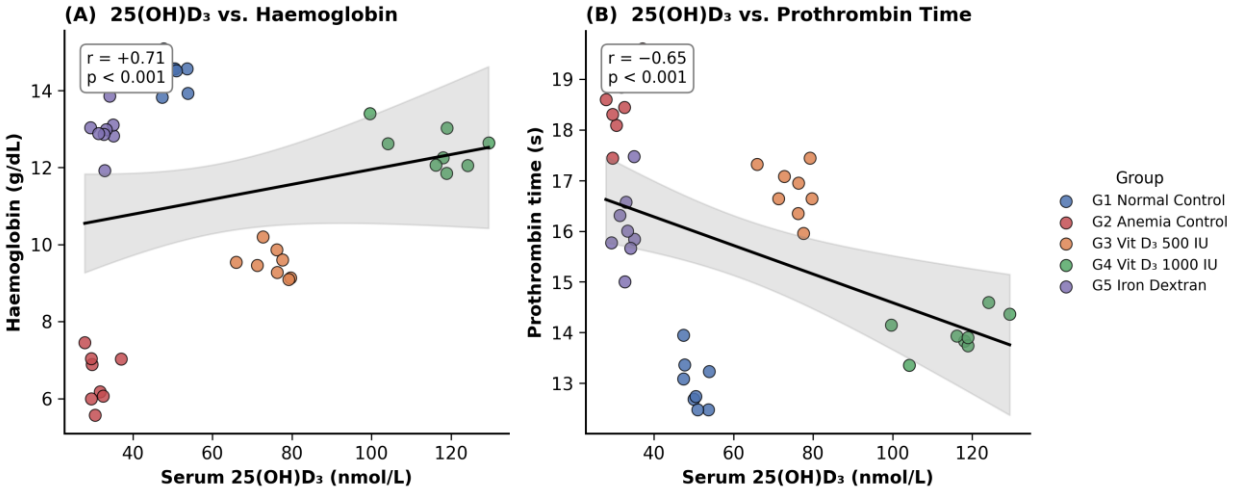

Figure 6. *Pooled scatter plots of 25(OH)D₃ against (A) haemoglobin and (B) prothrombin time (n = 40). Solid lines are linear regression fits; shaded bands are the 95% confidence intervals. Each point is colour-coded by experimental arm.*

3.6. Relative organ weights

The spleen-to-body-weight ratio more than doubled in the untreated anaemic animals (G2: 0.68 ± 0.04% vs. G1: 0.28 ± 0.02%; $p < 0.01$), in line with the expected expansion of extramedullary erythropoiesis. The ratio fell stepwise across the supplemented arms (G3: 0.48 ± 0.03%; G4: 0.34 ± 0.02%; G5: 0.32 ± 0.03%), with the high-dose D₃ group reaching values statistically indistinguishable from the untreated controls ($p > 0.05$). Relative liver weight was modestly increased in G2 (3.82 ± 0.11% vs. G1: 3.41 ± 0.09%; $p < 0.05$), most plausibly reflecting haemosiderin deposition, and was largely corrected in G4 (3.52 ± 0.09%). Kidney and heart weights did not differ significantly between groups ($p > 0.05$ throughout).

Summary of Key Findings

PHZ at 40 mg/kg i.p. on two consecutive days produced a severe and reproducible haemolytic anaemia (mean Hb 6.9 g/dL at day 11). Four weeks of oral vitamin D₃ improved haemoglobin, RBC count, haematocrit and platelet numbers in a dose-dependent fashion (G4 > G3; $p < 0.05$). At 1000 IU/kg/day, the supplement also shortened PT and aPTT and raised fibrinogen relative to anaemic controls ($p < 0.05$). Pooled across the cohort, 25(OH)D₃ correlated positively with haemoglobin ($r = +0.71$) and negatively with prothrombin time ($r = −0.65$); both $p < 0.001$. The haematological recovery achieved by the high D₃ dose was statistically indistinguishable from that produced by iron dextran (Hb, RBC, Hct; all $p > 0.05$).

4. Discussion

Taken together, the results of this experiment describe a coherent biological story. Oral cholecalciferol, given at 1000 IU/kg/day for four weeks, restored not only the red-cell mass but also several aspects of haemostasis in rats with PHZ-induced haemolytic anaemia, and did so to a degree that compared favourably with parenteral iron dextran. The dose-dependence of the response, the parallel improvement in iron handling, and the strong correlations between serum 25(OH)D₃ and the principal endpoints together argue that the effect is real and not an artefact of group allocation or measurement error. As far as we are aware, this is the first controlled experiment that has examined

erythropoietic and haemostatic outcomes side by side in a single PHZ model.

4.1. Validity of the PHZ model

Phenylhydrazine has a long history as a tool for studying haemolysis, and our findings fit comfortably within that literature. The fall in haemoglobin to 6.9 g/dL by day 11, the reticulocyte response (12.4%) and the more than twofold expansion of the spleen are all in line with results reported by other groups using the same dose schedule (17,18). The combination of brisk haemolysis, vigorous compensatory erythropoiesis and secondary derangement of clotting provided a useful platform on which to test the effects of vitamin D_3.

4.2. How vitamin D_3 might improve the blood picture

The most striking haematological observation was the proximity of the high-D_3 group to the iron-dextran group across haemoglobin, RBC count and haematocrit. Several mechanisms are likely to contribute. First, calcitriol, the active hormonal form, acts directly on early erythroid progenitors through their VDR, supporting proliferation and maturation (8, 19). Second, vitamin D_3 exerts a broad damping effect on the cytokines that suppress erythropoiesis under inflammatory conditions, including TNF-α, IL-6 and IFN-γ (5,20). The partial normalisation of total leukocyte counts in G4 is consistent with this anti-inflammatory action.

4.3. Iron handling and the hepcidin pathway

The improvement in iron parameters seen with vitamin D_3, particularly the rise in serum iron, transferrin saturation and ferritin, fits with a model in which calcitriol suppresses hepatic hepcidin expression through inhibition of NF-κB and STAT3 pathways and through modulation of BMP6 signalling (11,12). Lower hepcidin means more ferroportin-mediated iron egress from enterocytes and macrophages, and therefore more substrate for haemoglobin synthesis. The positive correlations we observed between 25(OH)D_3 and both iron ($r = +0.63$) and ferritin ($r = +0.58$) line up with epidemiological work that has repeatedly linked vitamin D deficiency to poor iron status across a range of clinical settings (21,22).

4.4. A less familiar story: vitamin D_3 and the clotting cascade

Perhaps the most interesting finding of this study lies in the coagulation domain. PHZ prolonged PT, aPTT and TT, and lowered fibrinogen, the laboratory fingerprint of a consumptive coagulopathy superimposed on haemolysis. Vitamin D_3, at the higher dose, brought PT and aPTT back to values that were no longer different from those of the untreated controls. It is worth noting that the iron-dextran group, despite achieving the most complete iron repletion of any treated arm, did not normalise PT or aPTT to the same extent. This dissociation suggests that the clotting benefit of cholecalciferol is not simply a downstream consequence of better iron status. The inverse correlation between 25(OH)D_3 and PT ($r = -0.65$) reinforces this interpretation, and dovetails with clinical observations linking low vitamin D to abnormal coagulation markers in ischaemic heart disease and other inflammatory states (23,15). Mechanistically, vitamin D may operate at several points along the cascade: receptor-mediated effects on megakaryocyte and platelet function (13,24), suppression of tissue factor and PAI-1 in vascular cells (14), and stabilisation of endothelial function under inflammatory stress.

4.5. The PTH–calcium–marrow axis

PTH rose in the untreated anaemic animals and returned to control values in the high-D_3 group. This is more than a side note: secondary hyperparathyroidism has been associated with direct suppression of erythropoiesis, and it is plausible that the elevated PTH in G2 amplified the haemolysis-driven anaemia by a second, independent

mechanism (25). The reversal of this rise after vitamin D_3 supplementation may therefore have contributed to the haematological recovery over and above any direct effect of the hormone on the marrow.

4.6. How these findings fit the wider literature

Our haematological data sit comfortably with the conclusions of recent systematic reviews showing that low vitamin D status is associated, across populations, with lower haemoglobin (26,27). Where this study adds something new is in the coagulation results. Few experimental studies have looked at vitamin D supplementation and clotting factor activity together in a haemolytic model, and the strength of the dose-response we observed is, to our knowledge, unprecedented. From a translational standpoint, the implication is straightforward: ensuring adequate vitamin D status may be a low-cost, low-risk adjunct in the management of patients with haemolytic disorders, and is worth testing rigorously in clinical trials.

4.7. Limitations and what should come next

Several caveats should temper interpretation. We did not interrogate the proposed mechanisms at the molecular level; future work should include measurement of hepatic hepcidin mRNA, erythroferrone, VDR expression in erythroid precursors, and circulating inflammatory cytokines to substantiate the proposed mechanisms (28). We used only two doses; a fuller dose-response curve, including supraphysiological exposures, would help define the therapeutic window. Our cohort was confined to male Wistar rats, and sex-related differences in vitamin D metabolism are well documented; replication in females is a priority. Finally, the leap from rat to patient should be made cautiously. Randomised clinical trials with adequate statistical power, well-defined haemolytic populations and longer follow-up will be needed before any therapeutic recommendation can be made.

5. Conclusions

In a rat model of phenylhydrazine-induced haemolytic anaemia, oral vitamin D_3 produced a dose-dependent improvement in the blood picture, in iron handling and in the clotting profile. At 1000 IU/kg/day, the haematological response matched that of parenteral iron dextran, while the coagulation response exceeded it, a pattern that is unlikely to be explained by iron repletion alone.

The consistency of the correlations between circulating 25(OH)D_3 and the principal endpoints, taken together with the dose-dependent pattern of response, identifies vitamin D_3 as a credible candidate adjunct in the management of haemolytic anaemia. The next steps — mechanistic studies and properly powered clinical trials — should follow from here.

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