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Original Paper

Vitamin D-Rich Diet in Mice Modulates Erythrocyte Survival

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Key Words
Vitamin D • Eryptosis • Hyperosmotic shock • Cell shrinkage • Energy depletion

Abstract
Background/Aims: Epidemiological evidence suggests that vitamin D deficiency is associated with anemia. The potent metabolite 1,25(OH)$_2$ vitamin D$_3$ [1,25(OH)$_2$D$_3$] activates various signaling cascades regulating a myriad of cellular functions including suicidal cell death or apoptosis. Suicidal death of erythrocytes or eryptosis is characterized by cell shrinkage and cell membrane scrambling leading to phosphatidylserine (PS) externalization. Stimulation of eryptosis may limit lifespan of circulating erythrocytes and thus cause anemia. In the present study, we explored the effect of a high vitamin D diet (10,000 I.U. vitamin D for 14 days) in mice on eryptosis. ***Methods:*** Plasma concentrations of erythropoietin were estimated using an immunoassay kit, blood count using an electronic hematology particle counter, relative reticulocyte numbers using Retic-COUNT® reagent, PS exposure at the cell surface from annexin V binding, cell volume from forward scatter, and cytosolic Ca^{2+} ($[Ca^{2+}]_i$) from Fluo3-fluorescence in FACS analysis. ***Results:*** Vitamin D treatment decreased mean corpuscular volume, reticulocyte count, and plasma erythropoietin levels. Vitamin D treatment slightly but significantly decreased forward scatter but did not significantly modify spontaneous PS exposure and $[Ca^{2+}]_i$ of freshly drawn erythrocytes. Vitamin D treatment augmented the stimulation of PS exposure and cell shrinkage following exposure to hyperosmotic shock (addition of 550 mM sucrose) or energy depletion (glucose removal) without significantly modifying $[Ca^{2+}]_i$. ***Conclusions:*** The present observations point to a subtle effect of exogenous vitamin D supplementation on erythrocyte survival.

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Introduction

The biologically active fat-soluble vitamin D is the precursor of the active hormone 1α,25-dihydroxyvitamin D [1,25$(OH)_2D_3$] that plays a pivotal role in mineral homeostasis and skeletal health [1, 2]. Metabolites of vitamin D further modify the physiology of nonskeletal tissues and, thus, regulate a wide array of biological functions [3, 4]. 1,25$(OH)_2D_3$ activates various signaling cascades via the vitamin D receptor which plays a decisive role in a myriad of cellular functions such as cell proliferation and differentiation, membrane transport, redox balance, adhesion, hemostasis and apoptosis [3-7]. Formation of 1,25$(OH)_2D_3$ is down-regulated by fibroblast growth factor 23 (FGF23) which, in turn, requires the membrane protein Klotho as a coreceptor [8-10]. As part of a negative feedback loop, both FGF23 and Klotho are stimulated by 1,25$(OH)_2D_3$ [10]. Restriction of dietary vitamin D reverses accelerated aging in mice deficient in either FGF23 or Klotho [11-13]. Mounting epidemiological evidence suggests that vitamin D deficiency is associated with anemia [14-20]. Previous clinical observations have shown that vitamin D deficiency is linked to decreased haemoglobin, enhanced reticulocytosis and a reduced response to erythropoiesis-stimulating agents [20, 21]. Intriguingly, 1,25$(OH)_2D_3$ levels in bone marrow are a magnitude higher in comparison to their levels in plasma [22]. Animal experiments have revealed that FGF23 deficiency leads to hyperphosphatemia paralleled with increased levels of vitamin D and enhanced prenatal and postnatal erythropoiesis [23]. However, enhanced erythropoiesis in those mice was shown to be independent of increased 1,25$(OH)_2D_3$ levels [23].

Similar to nucleated cells, erythrocytes may undergo suicidal death or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling [24, 25]. Triggers of eryptosis include activation of Ca^{2+}-permeable cation channels with Ca^{2+} entry, activation of Ca^{2+}-sensitive K^+ channels, exit of KCl with osmotically obliged water and, thus, cell shrinkage. Cytosolic Ca^{2+} further elicits scrambling of the erythrocyte membrane resulting in phosphatidylserine (PS) externalization [24-26]. The Ca^{2+} sensitivity of cell membrane scrambling is increased by ceramide. PS exposing erythrocytes are rapidly phagocytosed and, thus, cleared from circulating blood [24, 25]. Accordingly, accelerated eryptosis enhances the turnover of erythrocytes, which may lead to anemia, if the accelerated loss of erythrocytes is not compensated by a similar increase of erythrocyte formation, which is evident from reticulocytosis [27].

Both eryptosis and reticulocytosis were shown to be enhanced in Klotho-deficient mice and blunted in those mice by a vitamin D-depleted diet [28]. In conditions such as dehydration which is associated with decreased Klotho expression and enhanced 1,25$(OH)_2D_3$ levels, erythrocytes are particularly susceptible to eryptosis [29]. Furthermore, eryptosis could be enhanced by both, increase of extracellular phosphate concentration [30] and dietary phosphate depletion [31]. More recently, Klotho deficiency and increased vitamin D levels were shown to considerably impair erythropoiesis [32].

Whether excessive dietary intake of vitamin D influences erythrocyte lifespan, however, remained elusive. Thus, the present study explored whether a vitamin D-rich diet influences parameters of anemia and erythrocyte survival in mice.

Materials and methods

Animals and treatment with vitamin D

All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities. Experiments were performed in female C57Bl6 mice at the age of 10 weeks. The mice had free access to water and control food (Sniff, Soest, Germany). To analyze the effects of vitamin D, mice were fed either a control diet (containing 500 I.U./kg vitamin D) or a vitamin D-rich diet (containing 10,000 I.U./kg vitamin D; Altromin, Lage, Germany) for 14 days [33].

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Blood parameters

For all experiments except for the blood count, heparin blood was retrieved from the mice. The plasma concentrations of erythropoietin were determined using an immunoassay kit according to the manufacturer's instructions (R&D Systems, Wiesbaden, Germany). For the blood count, EDTA blood was analyzed using an electronic hematology particle counter (scil Vet abc, Weinheim, Germany). Murine erythrocytes were isolated by being washed two times with Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1 $MgSO_4$, and 32 HEPES/NaOH (pH 7.4), 5 glucose, and 1 $CaCl_2$. Relative reticulocyte numbers were determined using the Retic-COUNT® reagent (BD, Heidelberg, Germany) according to the manufacturer's instructions.

Incubations and solutions

For the *in vitro* experiments on suicidal death of erythrocytes, incubations were carried out at 37°C in Ringer solution at a hematocrit of 0.4% in a total volume of 500 µl. Where indicated, glucose was removed (energy depletion) or 550 mM sucrose was added (hyperosmotic shock).

Phosphatidylserine exposure and forward scatter

After incubation, erythrocytes were washed once in Ringer solution containing 5 mM $CaCl_2$. The cells were then stained with annexin V-Fluos (Roche, Mannheim, Germany) at a 1:500 dilution. After 15 min, samples were measured by flow cytometric analysis (FACS-Calibur; BD). Cells were analyzed by forward scatter, and annexin V fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of intracellular Ca^{2+}

After incubation, erythrocytes were washed in Ringer solution and then loaded with Fluo3/AM (Calbiochem) in Ringer solution containing 5 mM $CaCl_2$ and 2 µM Fluo3/AM. The cells were incubated at 37°C for 20 min and washed twice in Ringer solution containing 5 mM $CaCl_2$. The Fluo3/AM-loaded erythrocytes were resuspended in 50 µl Ringer. Then, Ca^{2+}-dependent fluorescence intensity was measured in fluorescence channel FL-1 in FACS analysis.

Statistics

Data are expressed as arithmetic mean ±SEM, and statistical analysis was made by paired or unpaired *t* test or ANOVA, as appropriate. Values of $P<0.05$ were considered statistically significant.

Results

Blood analysis of the mice revealed significantly reduced MCV in vitamin D-treated mice as compared to control mice (Fig. 1A). The hemoglobin concentration tended to be higher in vitamin D-treated mice, an effect, however, not reaching statistical significance (Fig. 1A). On the contrary, however, the percentage of reticulocytes and plasma erythropoietin levels were significantly lower in vitamin D-treated mice than in control mice (Fig. 1B and 1C).

Examination of erythrocyte survival by quantifying PS exposure using fluorescent annexin V binding revealed that the percentage of annexin V positive erythrocytes tended to be lower in freshly drawn erythrocytes from vitamin D-treated mice as compared to erythrocytes drawn from control mice (Fig. 2A and 2B). After 24 h incubation in Ringer solution, annexin V binding again tended to be lower in erythrocytes from vitamin D-treated mice than in erythrocytes from control mice (Fig. 2C). Changes in erythrocyte cell volume were studied using forward scatter in FACS analysis. Forward scatter was significantly smaller in freshly drawn erythrocytes from vitamin D-treated mice than in freshly drawn erythrocytes from control mice (Fig. 2D and 2E). Following incubation for 24 h in Ringer solution forward scatter was again significantly smaller in erythrocytes from vitamin D-treated mice than in erythrocytes from control mice (Fig. 2F).

Enhanced cytosolic Ca^{2+} activity mediates erythrocyte cell membrane scrambling and shrinkage via Ca^{2+}-activated K^+ channels. To this end, cytosolic Ca^{2+} activity was examined

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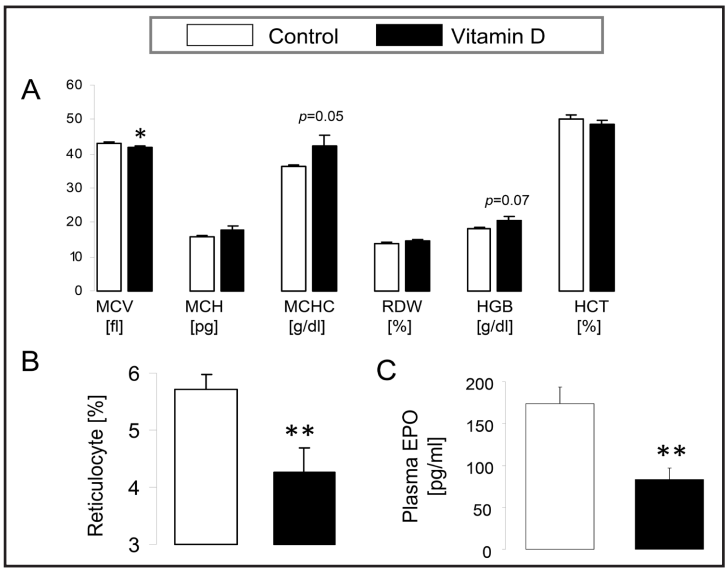


Fig. 1. Effect of vitamin D treatment on blood parameters, reticulocyte count and plasma erythropoietin. **A.** Means ± SEM (n=10) of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), hemoglobin concentration (HGB) and hematocrit (HCT) of mice were fed for 14 days either a control diet of 500 I.U./kg vitamin D (white bars), or a vitamin D-rich diet of 10,000 I.U./kg vitamin D (black bars). * significant ($p<0.05$) from Control. **B,C.** Means ± SEM (n=10) of the percentage of reticulocytes (**B,** n=10) and plasma erythropoietin levels (**C,** n=5) in control (white bars) and vitamin D-treated (black bars) mice. ** significant ($p<0.01$) from control.

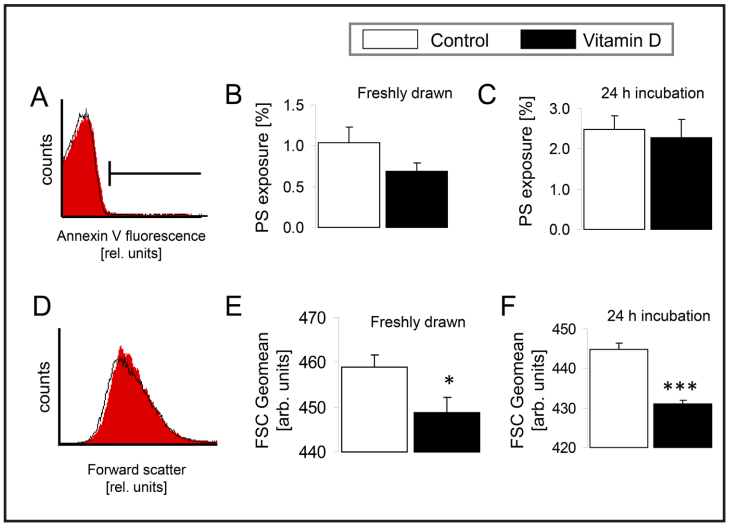


Fig. 2. Effect of vitamin D treatment on erythrocyte phosphatidylserine externalization and cell shrinkage. (**A**) Histogram of annexin V-binding in Control (*red shadow*) and vitamin D-treated mice (*black line*). **B,C.** Means ± SEM (n=5-10) of PS-exposing erythrocytes determined in fresh drawn (**B**) erythrocytes and in erythrocytes incubated for 24 h (**C**) drawn from control (white bars) and vitamin D-treated (black bars) mice. **D.** Histogram of forward scatter in Control (*red shadow*) and vitamin D-treated mice (*black line*). **E,F.** Means ± SEM (n=5-10) of forward scatter geomean determined in fresh drawn (**E**) erythrocytes and in erythrocytes incubated for 24 h (**F**) drawn from control (white bars) and vitamin D-treated (black bars) mice. *, *** ($p<0.05$, $p<0.001$) different from Control.

using Fluo3 fluorescence in FACS analysis. As a result, fluo3 fluorescence in freshly drawn erythrocytes was similar without (15.3 ± 0.3 a.u., n = 10) and with (15.4 ± 0.3 a.u., n = 10) prior vitamin D treatment. Following 24 hours incubation, fluo3 fluorescence tended to be lower in erythrocytes from vitamin D treated animals (15.5 ± 0.9 a.u., n = 5) than in erythrocytes from untreated animals (17.3 ± 0.5 a.u., n = 5), a difference, however, not reaching statistical significance.

Further experiments explored whether vitamin D treatment influenced the susceptibility of erythrocytes to known stimulators of eryptosis. In order to explore the impact of vitamin D treatment on susceptibility to eryptosis triggered by hyperosmotic shock, erythrocytes were exposed to hyperosmotic Ringer solution. As illustrated in Fig. 3A and 3B, osmotic shock

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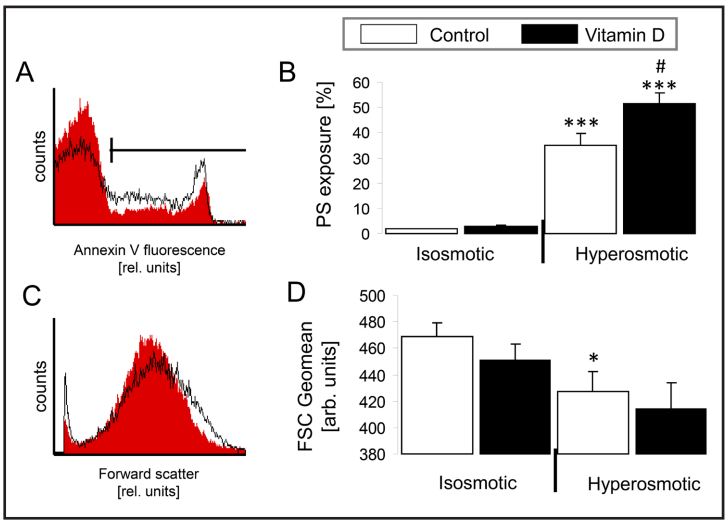


Fig. 3. Effect of vitamin D treatment on erythrocyte phosphatidylserine externalization and cell shrinkage following hyperosmotic shock. **A.** Histogram of annexin V-binding in Control (*red shadow*) and vitamin D-treated mice (*black line*) exposed to Hyperosmotic (+550 mM sucrose) Ringer and **B.** means ± SEM (n=10) of PS-exposure determined in erythrocytes incubated for 1 h in Isosmotic or Hyperosmotic (+550 mM sucrose) Ringer. *** significant (p<0.001) from Isosmotic. # significant (p<0.01) from Control. **C.** Histogram of forward scatter geomean in Control (*red shadow*) and vitamin D-treated mice (*black line*) exposed to Hyperosmotic (+550 mM sucrose) Ringer. **D.** Means ± SEM (n=10) of forward scatter geomean determined in erythrocytes incubated for 1 h in Isosmotic or Hyperosmotic (+550 mM sucrose) Ringer. * significant (p<0.001) from Isosmotic.

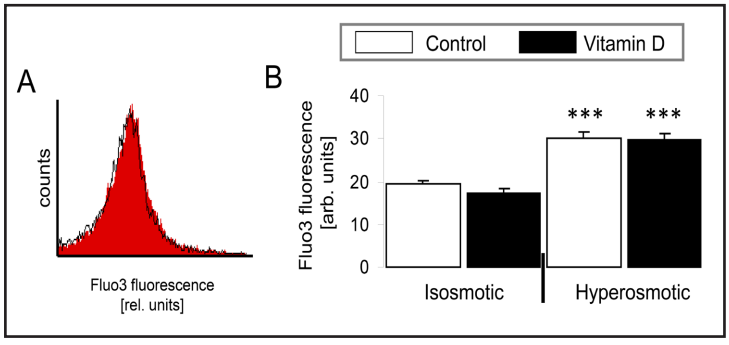


Fig. 4. Effect of vitamin D treatment on erythrocyte cytosolic Ca^{2+} activity following hyperosmotic shock. **A.** Histogram of Fluo3 fluorescence in Control (*red shadow*) and vitamin D-treated mice (*black line*) exposed to Hyperosmotic (+550 mM sucrose) Ringer and **B.** Means ± SEM (n=10) of Fluo3 geomean determined in erythrocytes incubated for 1 h in Isosmotic (left bars) or Hyperosmotic (+550 mM sucrose, right bars) Ringer. *** significant (p<0.001) from Isosmotic.

enhanced PS exposure, an effect significantly more pronounced in erythrocytes from vitamin D-treated mice than in erythrocytes drawn from control mice. Osmotic shock decreased the forward scatter to a similar extent in erythrocytes from vitamin D-treated and control mice (Fig. 3C and 3D).

A further series of experiments explored whether vitamin D treatment similarly augmented the effect of hyperosmotic shock on cytosolic Ca^{2+} activity. As illustrated in Fig. 4, hyperosmotic shock significantly increased Fluo3 fluorescence to similar values in erythrocytes from control and vitamin D-treated mice (Fig. 4).

As eryptosis is further stimulated by energy depletion, an additional series of experiments explored the effect of glucose removal from the extracellular medium. As a result, incubation of erythrocytes for 12 h in glucose-free Ringer solution increased PS exposure, an effect significantly more pronounced in erythrocytes from vitamin D-treated mice than in erythrocytes from control mice (Fig. 5).

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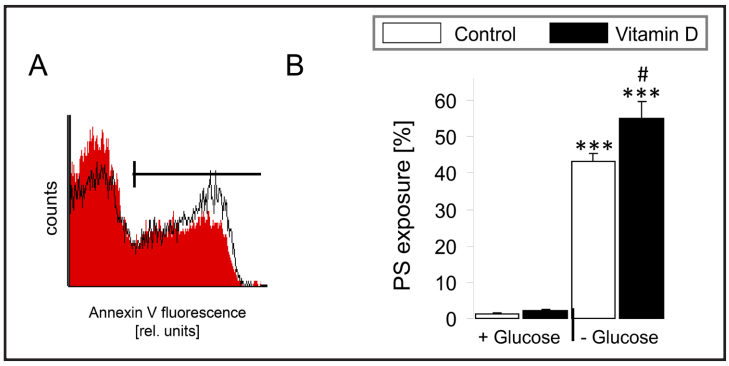


Fig. 5. Effect of vitamin D treatment on erythrocyte phosphatidylserine externalization following energy depletion. **A.** Histogram of annexin V-binding in Control (*red shadow*) and vitamin D-treated mice (*black line*) exposed to glucose-depleted Ringer and **B.** Means ± SEM (n=5) of PS-exposure determined in erythrocytes incubated for 12 h in Ringer solution (+Glucose) or glucose-depleted Ringer solution (-Glucose). *** significant ($p<0.001$) from energy repletion. # significant ($p<0.01$) from Control.

Discussion

According to the present observations, systemic administration of vitamin D suppresses the reticulocyte count, an effect paralleled by and possibly due to decreased plasma erythropoietin levels. Volume of freshly drawn erythrocytes was slightly but significantly smaller in Vitamin D treated animals than in control animals. Most importantly, erythrocytes from vitamin D treated animals were more susceptible to eryptosis than erythrocytes from control animals to eryptosis following *ex vivo* cell stress such as hyperosmotic shock and energy depletion.

Eryptosis is enhanced in Klotho-deficient mice [28], which suffer from excessive formation of $1,25(OH)_2$ vitamin D_3 [8-10]. The enhanced eryptosis in Klotho-deficient mice could be reversed by reduction in dietary vitamin D intake [28]. According to the present study, vitamin D treatment alone did not cause anemia and did not stimulate eryptosis of freshly drawn erythrocytes but sensitized the erythrocytes to the proeryptotic effect of hyperosmotic shock and energy depletion.

In a variety of nucleated cells $1,25(OH)_2D_3$ has been shown to foster Ca^{2+} entry [6, 34-36]. In erythrocytes the eryptosis following hyperosmotic shock is not paralleled by appreciable increase of cytosolic Ca^{2+} and vitamin D treatment is presumably not effective by stimulating Ca^{2+}-entry. Hyperosmotic shock elicits ceramide formation in eryptotic erythrocytes, which may trigger eryptosis even at constant cytosolic Ca^{2+} [37]. Vitamin D analogs have previously been shown to potentiate ceramide-dependent apoptosis of tumor cells [38]. Osmotic shock is further followed by phosphorylation of erythrocyte p38 MAPK [24], which participates in the signaling of $1,25(OH)_2D_3$-induced nucleated cell death [39, 40]. $1,25(OH)_2D_3$ further affects nitric oxide formation [41] and protein kinase C activity [42], which both participate in the regulation of eryptosis [43, 44]. Other signaling molecules participating in the signaling of eryptosis include AMPK [45], CK1α [46], Janus kinase 3 [24] and PAK2 [47]. Whether those kinases participate in the effects of vitamin D on erythrocytes, remains to be shown.

Eryptosis is enhanced by erythrocyte age [48], a wide variety of anemia-causing xenobiotics and endogeneous substances [29, 30, 49-68] as well as several clinical disorders, such as iron deficiency [27], hepatic failure [69], chronic kidney disease [70], sepsis [71], malignancy [72] and Wilson's disease [73]. Eryptosis may further influence erythrocytes stored for transfusion [74]. It is likely that excessive vitamin D may compound the susceptibility to the eryptotic effect of eryptosis-inducing xenobiotics, endogeneous mediators and clinical disorders.

Phosphatidylserine-exposing erythrocytes adhere to the vascular wall [75], and stimulate blood clotting [76]. Thus, excessive eryptosis may compromise microcirculation. Along those lines, enhanced eryptosis has been suggested to participate in the vascular injury of metabolic syndrome [77].

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Conclusion

Taken together, our data suggest a subtle effect of exogenous vitamin D supplementation on erythrocyte survival *in vivo* and in response to cell stress *ex vivo*.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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