

Red Blood Cell Phosphate Concentration and Osmotic Resistance During Dietary Phosphate Depletion in Dairy Cows

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Background: Hypophosphatemia in early lactating dairy cows has been implicated as primary cause for postparturient hemoglobinuria in cattle. Decreased availability of phosphorus has been proposed to reduce adenosine triphosphate synthesis of erythrocytes and thereby reduce osmotic resistance of these cells.

Hypothesis/Objectives: To study the effect of phosphorus depletion on the phosphate concentration ([Pi]) in plasma and erythrocytes and the osmotic resistance of erythrocytes and to determine the association between plasma [Pi] and erythrocyte [Pi].

Animals: Ten healthy midlactating dairy cows in their 3rd to 5th lactation.

Methods: Prospective study. Dietary phosphorus depletion for 5 weeks followed by phosphorus supplementation. Plasma and erythrocyte [Pi] and erythrocyte osmotic resistance were measured. Four cows underwent continuous dextrose infusion at the end of phosphate depletion to exacerbate hypophosphatemia.

Results: Dietary P depletion resulted in a marked decline of the plasma [Pi] from 4.1 ± 1.0 mg/dL to a nadir of 1.7 ± 0.5 mg/dL, but did not alter erythrocyte [Pi] or osmotic resistance. Similarly, dextrose infusion induced a decline of the plasma [Pi] from 2.4 ± 0.5 mg/dL to 1.5 ± 0.5 mg/dL, but had no effect on erythrocyte [Pi] or osmotic resistance.

Conclusions and Clinical Importance: In cattle, marked hypophosphatemia induced by dietary P depletion was neither associated with a decline in erythrocyte [Pi] nor with decreased osmotic resistance of erythrocytes. Phosphorus depletion alone is therefore unlikely to cause intravascular hemolysis and the plasma [Pi] is an unreliable index for the intracellular [Pi] of erythrocytes.

Key words: Hemolysis; Hypophosphatemia; Osmotic resistance; Postparturient hemoglobinuria.

Hypophosphatemia is a common finding in early lactating and anorectic dairy cows.¹ The clinical relevance of hypophosphatemia in sick and periparturient cows is still under contentious debate, but hypophosphatemia and phosphorus (P) depletion have empirically been implicated as causative or contributing factors for typical periparturient diseases of dairy cattle such as the downer cow syndrome and postparturient hemoglobinuria (PPH). Postparturient hemoglobinuria is a condition of early lactating dairy cattle that is characterized by acute intravascular hemolysis and hemoglobinuria with a sporadic occurrence, but with a considerable fatality rate.^{2,3} The disease occurs in many countries and predominantly affects mature high-yielding dairy cows in the first 4–8 weeks of lactation.⁴ Although PPH is widely believed to be the result of acute hypophosphatemia occurring at the onset of lactation in highly productive milking cows, the etiology and pathophysiology of the condition are not unequivocally elucidated. The proposed mechanism is a marked drop in red blood cell (RBC) adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG) synthesis in bovine RBCs in states of P depletion presumably because of insufficient availabil-

Abbreviations:

[Pi]	inorganic phosphate concentration
2,3-DPG	2,3-diphosphoglycerate
ATP	adenosine triphosphate
Pi	inorganic phosphate
P	phosphorus
PPH	postparturient hemoglobinuria
RBC	red blood cells
TMR	total mixed ration

ity of intracellular phosphate (Pi).⁵ Red blood cells require ATP among other things to control the cell volume and deformability through active extrusion of sodium.⁶ A decrease in the ATP concentration in P-depleted human RBCs to 15% of normal values resulted in decreased osmotic resistance of RBCs, spherocytosis, and intravascular hemolysis.⁷ Notwithstanding spherocytosis and increased osmotic fragility are not pathognomonic for intracellular ATP or P depletion of RBCs, but have also been documented in patients of other species suffering from other conditions such as autoimmune-mediated hemolytic anemia or poisoning with aniline or naphthalene. Studies investigating osmotic resistance of RBCs in cattle with clinical PPH yielded conflicting results.^{8,9} Furthermore, hypophosphatemia has been reported in many but not all cases of PPH and response to treatment with phosphate salts is variable in affected animals.^{3,6} A number of alternative etiologies of PPH have been proposed, including the ingestion of beet pulp products or cruciferous plants containing saponins, copper-molybdenum imbalances as reported in cases of PPH in New Zealand or oxidative stress causing increased RBC membrane permeability and hemolysis.^{3,4,6}

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The objectives of the present study were to study the effect of dietary P depletion on the Pi concentration ([Pi]) in bovine RBCs and the osmotic resistance of RBCs and to determine the association between plasma [Pi] and RBC [Pi].

Materials and Methods

Animals, Housing, and Feeding

The national and institutional guidelines for the care and use of experimental animals were followed and all experimental procedures were approved by the Utrecht University Institutional Animal Care and Use Committee (DEC; permit no 2013.iii.03.033).

A total of 10 healthy, lactating, nonpregnant Holstein-Friesian cows were used for this study. Cows were between 5 and 7 years old and between 100 and 200 days in lactation. The mean body weight was 604 ± 40 kg (mean \pm SD) and the mean 305 day milk yield of the previous lactation was $9,920 \pm 1,360$ kg. All cows were healthy based on complete physical examination, and hematologic and blood biochemical examination. Cows were housed in individual tie stalls with rubber bedding, covered with sawdust, in a temperature-controlled facility.

After an acclimatization period of 2 weeks, animals on study underwent dietary P depletion over a period of 5 weeks. The P depletion phase was followed by a 2-week period during which P was supplemented in excess of requirements. The cows received the same base ration offered as total mixed ration that was based on corn silage, grass seed straw, and beet pulp, and was formulated to meet the current dietary recommendations for lactating cattle, except for the P content.¹⁰ During the acclimatization period, the diet was supplemented with NaH_2PO_4 to obtain a dietary P content meeting current recommendations for lactating cattle (0.36% of dietary P in dry matter).¹⁰ The unsupplemented ration with a dietary P content of 0.2% in dry matter was fed during the entire P depletion and was at least 40% below current recommendations.¹⁰ During the P supplementation phase, the ration was again supplemented with NaH_2PO_4 to obtain a dietary P content of 0.42% in dry matter. Feed was offered twice daily ad libitum between 06.00 and 07.00 hours and between 18.00 and 19.00 hours andorts were weighed back to determine feed intake. Cows were milked twice daily between 06.00 and 07.00 hours and between 18.00 and 19.00 hours.

In an attempt to induce the so called refeeding syndrome, a subset of 4 cows were administered a continuous intravenous infusion of dextrose through a previously placed 14G jugular catheter^a to which an extension set^b was attached. Dextrose was administered as a 20% solution at a dose rate of 300 mg/kg/h over a period of 6 hours. The administration was started at 8.00 hours of the treatment day and discontinued at 14.00 hours.

Blood Sample Collection

Blood samples were collected by venipuncture of a jugular vein between 7.30 hours and 8.00 hours of each sampling day. Sampling days were at the end of the acclimatization period (T0), after 1, 3, and 5 weeks of dietary P depletion (T1, T3, and T5 respectively) as well as after 2 weeks of P repletion (T7). In the subset of 4 cows treated with intravenous dextrose, blood samples were also obtained as described above from the jugular vein not used for dextrose infusion immediately before starting and immediately before discontinuing dextrose infusion ($T_{\text{pre-dextrose}}$ and $T_{\text{post-dextrose}}$ respectively).

Blood samples were collected into 2 blood tubes containing lithium-heparin and 1 tube containing EDTA as anticoagulant and were processed within 1 hour as described below.

Biochemical Analysis

EDTA tubes were used for erythrocyte counts and determination of the hemoglobin (Hb, cyanization) concentration, and hematocrit with an automated hematology system.^c

Li-heparin tubes were kept at room temperature and centrifuged within 1 hour of sample collection at $1,600 \times g$ for 10 minutes. Harvested plasma was assayed immediately as described below. The remaining packed cells were washed 3 times by adding one part of isotonic NaCl to one part of packed cell volume. One mL of washed packed cells was then added to 5 mL of deionized water to induce hemolysis. The lysate was then centrifuged for 10 minutes at $1,600 \times g$ and the supernatant was collected for biochemical analysis. The inorganic phosphate (Pi) concentration ([Pi]) was determined on an automated analyzer^d by determining the change in absorbance at 340 nm after the addition of ammonium molybdate at acidic pH. The sensitivity of the assay is 0.3 mmol/L. Addition of a fixed amount of phosphate to RBC lysates yielded a recovery rate of 99.5%.

Osmotic Resistance

Osmotic resistance was determined as described elsewhere.¹¹ Briefly, 14 NaCl solutions covering a range from 0.1 to 0.9% were prepared and 5 mL of each solution was mixed with 50 μL of heparinized blood. Vials containing the NaCl solutions mixed with blood were then incubated at room temperature for 30 minutes before centrifuging for 10 minutes at $1,600 \times g$. The degree of hemolysis was determined spectrophotometrically by measuring the absorbance at a wavelength of 540 nm in the supernatant. Spectrophotometric absorbance of blank isotonic NaCl solution (0.9%) was used as equivalent to 0% hemolysis and blood mixed with 0.1% salt solution was used as equivalent to 100% hemolysis. The concentrations of the salt solutions were then plotted against the degree of hemolysis (in percent) and the concentration of the NaCl solution at which 10% and 90% hemolysis occurred were obtained from the plotted curve. Osmotic resistance of each sample was therefore characterized by the concentration of the NaCl solution at which 10% and 90% of hemolysis occurred.

Statistical Analysis

Results are expressed as mean and standard deviation. Normality of distribution was tested by Shapiro-Wilk's test for normality and logarithmic transformations were used where appropriate to achieve normal distribution. Repeated measures analysis of variance (ANOVA) was used to determine time effects; a paired *t*-test was used to determine differences in parameters determined before and after dextrose infusion in the subset of cows treated with intravenous dextrose. Significance was assumed at $P < .05$. Bonferroni corrected *P* values were used. A statistical software package was used for the statistical analysis.^e

Results

All cows remained healthy and completed the entire study. None of the clinical signs commonly associated with acute P depletion such as decreased milk production, feed intake depression, hemolysis, clinically apparent muscle weakness or recumbency were observed in any of the cows on study.¹

The concentration time curves for plasma [Pi], RBC [Pi], and concentrations of NaCl solution at which 10% and 90% hemolysis occurred are presented in Figure 1. Dietary P depletion resulted in a significant decline of the plasma [Pi] to 40% of the baseline value within 1 week. Thereafter, a continuous increase in the plasma [Pi] was observed until the end of the depletion period despite unchanged dietary P content and feed intake. Dietary P supplementation in excess of requirements in the 2 weeks after the P depletion phase resulted in a significant increase in the plasma [Pi] to over 50% above concentrations measured at the end of the depletion phase (Fig 1).

In contrast to the plasma [Pi], the RBC [Pi] was not significantly altered by dietary P depletion throughout the depletion phase. Supplementation of P at the end of the study resulted in a significant increase in the RBC [Pi] of over 20% (Fig 1).

No significant changes in osmotic resistance of RBCs, as characterized by the concentration of NaCl at which 10% and 90% of hemolysis occurred were observed throughout the study (Fig 1).

Erythrocyte indices including RBC count, hematocrit, and hemoglobin concentrations are presented in Table 1. None of these variables showed a significant change over time.

Intravenous infusion of dextrose over a period of 6 hours in a subset of 4 cows on study at the end of the P depletion phase resulted in a significant decline of the plasma [Pi] by over 35%. This decline in plasma [Pi] was neither accompanied by a change in RBC [Pi] nor a change in osmotic resistance of RBCs (Fig 2).

Discussion

The main findings of this study were that although dietary P depletion resulted temporarily in a marked decline of the plasma [Pi], this was not associated with a decline in RBC [Pi], altered osmotic resistance of RBCs or altered erythrocyte parameters such as RBC counts, hemoglobin concentration, or hematocrit. Feeding a ration that was at least 40% below the daily P requirements of lactating cattle over 5 weeks resulted in a rapid decline of the plasma [Pi] reaching its nadir within 1 week of P depletion. The following continuous increase in the plasma [Pi] after this nadir despite ongoing dietary P depletion suggests that counter regulatory mechanisms became effective within the first week of the depletion phase. In an attempt to rapidly exacerbate hypophosphatemia in P-depleted animals, a subset of 4 animals was treated with dextrose IV at the end of the dietary depletion phase. Parenteral dextrose administration is known to induce a compartmental shift of Pi from the extracellular to the intracellular space of insulin responsive tissue in cattle and other species thereby causing marked but transient hypophosphatemia.^{12,13} We hypothesized that dextrose infusion would further impair Pi uptake by RBCs that are insensitive to insulin and thereby cause or exacerbate intracellular Pi depletion. As expected, this treatment induced a further significant decline of the plasma [Pi],

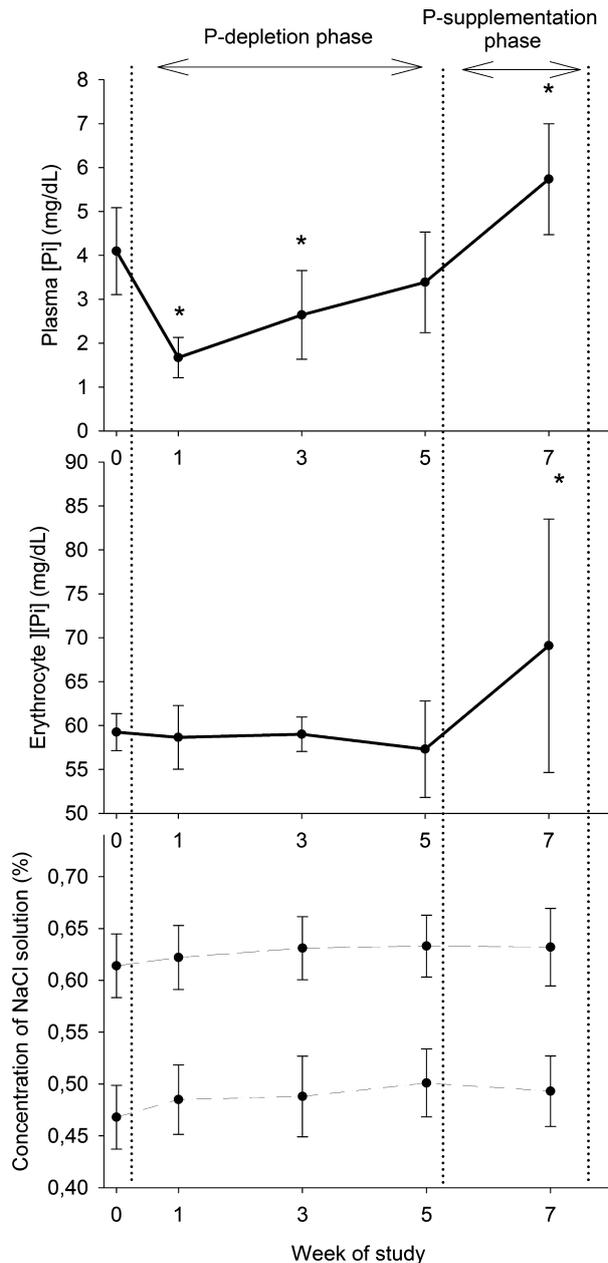


Fig 1. [Pi] in plasma (upper panel) and red blood cells (RBCs, middle panel) as well as concentrations of NaCl solutions at which 10% (dashed line, lower panel) and 90% (dash-dotted line, lower panel) of hemolysis occurred at the different blood sampling times. The vertical dotted lines mark the beginning and end of the phosphate depletion and repletion phase of the study. Time points marked with *differ significantly from the baseline value (0, $P < .05$, Bonferroni corrected) (mean \pm SD).

but did neither alter the [Pi] of RBCs nor their osmotic resistance.

The stability of the intracellular [Pi] of RBCs despite marked dietary P depletion and hypophosphatemia is remarkable as RBCs obtain intracellular Pi through translocation of plasma Pi independently of insulin. The constant RBC [Pi] with declining P supply and hypophosphatemia indicates that the maintenance of

Table 1. Erythrocyte counts (RBC count), hemoglobin concentration (Hb), and hematocrit as mean \pm SD at the different blood sampling times. Blood samples were collected before P depletion (T0), after 1, 3, and 5 weeks of P depletion (T1, T3, T5 respectively) and after a 2-week P repletion phase (T7). Significant time effects were not identified for any of the studied variables.

Sampling Time	RBC Count ($\times 10^6$ cells/ μ L)	Hb (mmol/L)	Hematocrit (%)
T0	5.3 \pm 0.8	6.3 \pm 0.8	27 \pm 3
T1	6.2 \pm 0.7	6.6 \pm 0.6	27 \pm 3
T3	6.2 \pm 0.6	6.4 \pm 0.4	27 \pm 2
T5	6.1 \pm 0.5	6.3 \pm 0.4	27 \pm 2
T7	6.2 \pm 0.6	6.4 \pm 0.5	27 \pm 2

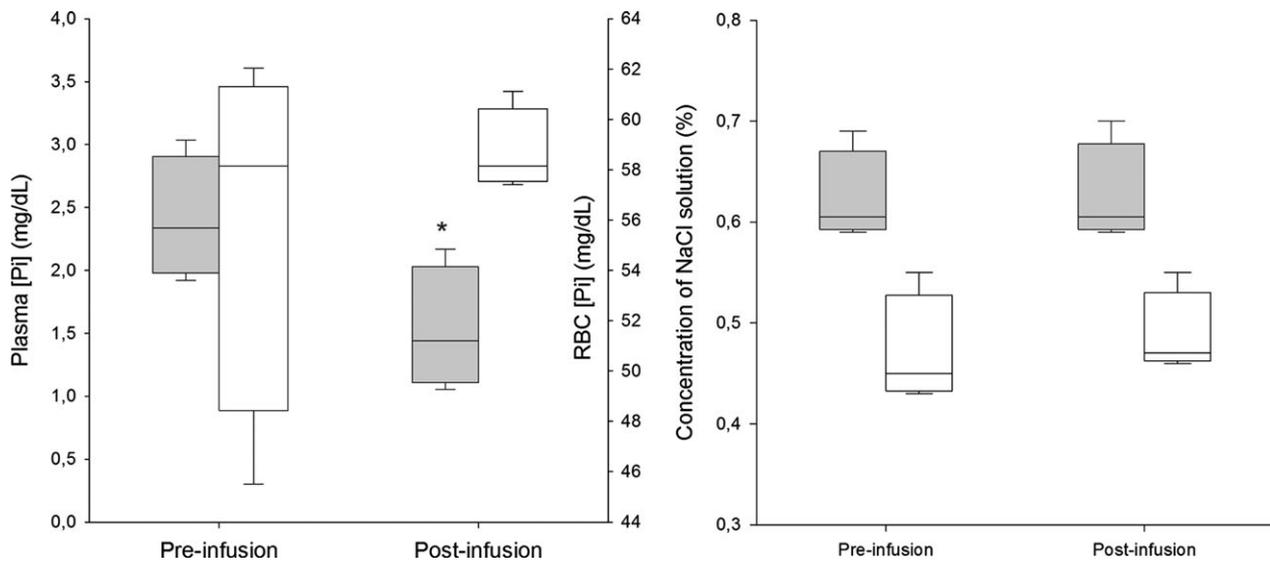


Fig 2. [Pi] in plasma (left panel, gray boxes) and red blood cells (RBCs, left panel, white boxes) before (preinfusion) and at the end (postinfusion) of intravenous infusion of dextrose in 4 cows at the end of the P depletion phase. Concentrations of NaCl solutions at which 10% (right panel, gray boxes) and 90% (right panel, white boxes) of hemolysis occurred at the different blood sampling times. Time points marked with * differ significantly from preinfusion values (0, $P < .05$) (mean \pm SD).

physiologic [Pi] at least in RBCs is highly effective and prioritized over the maintenance of physiologic plasma [Pi]. The significant increase in RBC [Pi] at the time of dietary P supplementation that was associated with a concomitant increase in the plasma [Pi] above baseline values suggests that mechanisms preventing intracellular P depletion in states of negative P balance are more effective than the prevention of excessive intracellular [Pi] with excessive P supply. The [Pi] in RBCs reported in this study are in good agreement with previously reported values in lactating dairy cows.¹⁴

Assuming that the underlying mechanism of decreased osmotic resistance and intravascular hemolysis observed in cattle with PPH is the decrease in the intracellular ATP concentration resulting from insufficient availability of intracellular Pi, it is not surprising that the osmotic resistance of RBCs remained unaffected by dietary P depletion in the present study as RBCs were able to maintain a normal intracellular [Pi].

Studies conducted on RBCs of cattle suffering from intravascular hemolysis induced experimentally through dietary P depletion reported markedly decreased ATP concentration in combination with pro-

nounced hypophosphatemia.¹⁵ The authors of this study also reported that only a small number of experimentally P-depleted cows developed intravascular hemolysis, whereas most P-depleted animals remained clinically healthy despite markedly decreased plasma [Pi]. The same observation has repeatedly been made under field conditions where PPH only affects individual animals, whereas herd mates with similarly severe hypophosphatemia remain healthy.^{4,6} The results of our study indicate that healthy dairy cows are able to maintain the [Pi] of RBCs within physiologic limits over a prolonged period of time despite marked dietary P depletion and hypophosphatemia. It appears that dietary P depletion alone over a course of several weeks is not sufficient to decrease osmotic resistance of RBCs or induce intravascular hemolysis. It is nonetheless conceivable that counter regulation to dietary P depletion is impaired in certain animals presenting a thus far unidentified contributing factor. Such a scenario could also explain the discrepancy between the considerable incidence of periparturient hypophosphatemia and the very low incidence of PPH in dairy cattle.¹

Intravascular hemolysis is not typically recognized in chronically P-depleted cows, but occurs in early lactating cows suffering from hypophosphatemia even while being fed a diet with adequate P content.⁶ Hypophosphatemia in early lactation has a complex etiology which presumably includes feed intake depression in the periparturient period, increased P loss through the mammary gland and compartmental shifts of P from the extracellular to the intracellular space.¹

Assuming that decreased availability of intracellular Pi is the underlying cause of ATP depletion and decreased osmotic resistance of RBCs, then the determining factor for the occurrence of intravascular hemolysis would be the intracellular rather than the plasma [Pi]. Compartmental shifts of P between intra- and extracellular space could result in intracellular [Pi] depletion of RBCs without hypophosphatemia as it may occur with acidosis or acidemia.¹ As observed in the present study, hypophosphatemia may also occur without concomitant intracellular P depletion.¹ In any case, the results of the present study indicate that the plasma [Pi] is a poor surrogate parameter for the intracellular [Pi] of RBCs and that hypophosphatemia is not generally associated with decreased osmotic resistance of RBCs in cattle.

It was the objective of the present study to elucidate the specific role of P depletion in the etiopathogenesis of PPH. We therefore opted to induce P depletion in cows in mid to late lactation to avoid interference of other electrolyte, metabolic, or hormonal deregulations commonly occurring in the periparturient period that would have complicated the interpretation of the results. However, the results of this study do not rule out the possibility of phosphorus depletion contributing to the development of intravascular hemolysis in combination with other predisposing factors in the early periparturient period.

In conclusion, the results presented here indicate that in cattle, marked hypophosphatemia induced by dietary P depletion over a course of 5 weeks was neither associated with a decline of RBC [Pi] nor with decreased osmotic resistance of RBCs. P depletion alone is therefore unlikely to cause intravascular hemolysis and the plasma [Pi] is an unreliable surrogate parameter for the intracellular [Pi] of RBCs.

Footnotes

^a Angiocath, Becton-Dickinson, Heidelberg, Germany

^b Discifix C-3, 10 cm, Braun Melsungen AG, Melsungen, Germany

^c Advia 120, Siemens Healthcare Diagnostics GmbH, Eschborn, Germany

^d DxC-600, Beckman Coulter Inc, Brea, CA

^e SAS 9.4, SAS Institute Inc, Cary, NC

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Conflict of Interest Declaration: The authors disclose no conflict of interest.

Off-label Antimicrobial Declaration: The authors declare no off-label use of antimicrobials.

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