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Farrah Deeba
Department of Clinical Medicine and Surgery, University of Agriculture Faisalabad, Pakistan

Anas Sarwar Qureshi Qaiser Bashir
Department of Anatomy, University of Agriculture Faisalabad, Pakistan

Correspondence
Anas Sarwar Qureshi Qaiser Bashir
Department of Anatomy, University of Agriculture Faisalabad, Pakistan

Investigations on copper and phosphorus deficiency associated post-parturient hemoglobinuria in dairy animals and clinical management with antioxidants

Farrah Deeba and Anas Sarwar Qureshi Qaiser Bashir

Abstract
Post-parturient hemoglobinuria (PPH), is reported as one of the important metabolic condition in high yielding cattle and buffaloes cA total of 40 dairy animals which were affected with hemoglobinuria were selected from field cases. Clinical examination and color of urine before and after treatment in animals were used as evaluation criteria for recovery in affected dairy animals. In group A, sodium acid phosphate (Inj.Alphos-40™) 200-250 and 120g of dicalcium phosphate were given i/v and orally b.i.d, respectively, for seven days. In group B, N-acetyl cysteine (Mucolator®) was given IV @ 8 mg bid and vitamin C (Tab C-Cone 10g) were given intravenously and orally, respectively. The body temperature was subnormal to normal in post parturient hemoglobinuria affected animals in the present study. There were significant differences in TPR values in group A and Group B after treatment. Serum Ca level (7.41±0.99) increased with a significant difference after the treatment (11.0280±0.44081) in group A. In group B, a significant rise was found in mean serum P level before (2.90±0.73) and after (5.67±0.06) the treatment. Serum Ca level (5.6740±0.06142) increased with a significant difference after (9.84±0.17) the treatment in group B. Mean serum P level displayed significant difference (6.19±0.19) in group A and group B (5.67±0.06).Mean serum cu concentration in positive cases (0.584±0.114)µg/dl showed significant deficiency of the trace mineral in the study area. In group A, RBCs, platelet count, Hb and PCV showed a significant improvement (*P*<0.05) after treatment. Similarly, a significant difference (*P*<0.05) was found in hematological values after the treatment in group B. Hypophosphatemia and hypocupremia was a constant feature in all cases of hemoglobinuria in the present study. It is worth mentioning that both treatment protocols corrected hypophosphatemia adequately in PPH affected animals, but treatment with Sodium acid phosphate and DCP was successful in restoring serum phosphorus level and hematological values in haemoglobinuria cases followed by treatment with Mucolator combined with Tab. C-Cone.

Keywords: Copper, phosphorus, post-parturient hemoglobinuria (PPH), antioxidants, cattle, buffaloes

Introduction
Post-parturient hemoglobinuria (PPH), is reported as one of the economically important nutritional deficiency associated metabolic condition in dairy animals in Pakistan and other countries (Akhtar *et al*. 2006; Ghenum and El-Deeb, 2010) [12, 13]. It is characterized by intravascular hemolysis leading to anemia, hemoglobinemia and haemoglobinuria (Akhtar *et al*. 2007; Gahlawat *et al*. 2007) [1, 2, 11]. This periparturient metabolic condition affects high yielding dairy animals in the first 30 days of lactation Phosphorus depletion or hypophosphatemia have been considered as contributing or causative factors for PPH in high yielding cattle and buffaloes. The etiopathogenesis of this metabolic condition is still controversial. The proposed mechanism is that hypophosphatemia results in reduced glycolysis and a marked reduction in ATP and 2.3-DPG (2, 3-diphosphoglycerate) synthesis in erythrocytes in bovine suffering hypophosphatemia. Erythrocytes need ATP to control cell volume and deformability by active Na extrusion. A decrease in ATP concentration in phosphorus deficient erythrocytes to 15% of normal values resulted in reduced osmotic resistance of erythrocytes and ultimately intravascular hemolysis (Grunberg *et al*. 2015) [14]. Furthermore, Low ATP production is related to reduced antioxidant system comprising of enzymes and some biological antioxidants vitamin c (ascorbic acid) etc. Other associated risk factors include ingestion of hemolytic or oxidative plant toxins including cruciferous plants, berseem (saponins), sugar beets and green forage.

In New Zealand bovine haemoglobinuria was associated with copper eficiency and oxidative stress. As activity of cu containing enzyme superoxide dismutase, part of erythrocyte protection system against oxidative stress is reduced. Excessive molybdenum in soil and fodder reduces phosphorus absorption from GIT and increasing its excretion through urine leading to hypophosphatemia in bovine (Soren *et al*. 2014; Resum *et al*. 2015). andard treatment of PPH is based on parenteral administration of sodium acid phosphate (Na2HPO4) together with oral dosing of Dicalcium phosphate (DCP) (Radostits *et al*. 2007) [28]. Its cure rate almost never exceeds 50%. Another limitation of the sodium acid phosphate therapy is that treated animals become off feed with a considerable loss in body weight and reduced milk production. It follows that an alternative effective treatment without these side effects is warranted. Under field conditions, farmers are following traditional practices and feeding their animals with no or less amount of concentrates and mineral supplements along with green fodder during 3rd to 6th lactation resulting in severe mineral imbalance. In advanced pregnancy the situation is intensified with ingestion of cruciferous plants resulting in development of PPH in dairy animals (Heuer and Bode, 1998; Akhtar, 2006) [15, 16]

The current study planned firstly, to investigate serum P and cu deficiency associated with Post-parturient hemoglobinuria (PPH) in dairy animals and, secondly to compare therapeutic efficacy of sodium acid phosphate along with Dicalcium phosphate (DCP) and with Mucolator® (N-acetyl cysteine) and Tab. C-Cone (Vitamin C) in PPH affected dairy animals.

Materials and Methods

Experimental layout

A cross-sectional study was designed to evaluate the prevalence of post parturient hemoglobinuria and the risk factors associated with this disease in cattle and buffaloes. A total of 40 dairy animals affected with hemoglobinuria were selected from Veterinary Teaching Hospital, Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Civil Veterinary Hospitals and private dairy farms of Punjab. The disease was identified clinically on the basis of particular signs and symptoms i.e. presence of blood in urine resulting dark red to coffee color of the urine, straining during defecation in post-parturient hemoglobinuric affected buffaloes and cows in last trimester of pregnancy or in early lactation (Radostitis *et al.* 2007) [28], pale to yellow mucous membrane of vulva, subnormal rectal temperature, loss of appetite, and constipation.

All animals were screened for haemoparasites through Giemsa stain by using standard laboratory techniques to exclude positive cases. (Soren *et al*. 2014). Information regarding management, clinical signs and treatment of the affected animals were entered in a “data capture form”

Biochemical Analysis

10 ml blood samples were collected from jugular vein of affected and healthy cattle and buffaloes (OIE, 2010). Serum was separated by centrifugation at 3500 rpm for 10 minutes and was stored at-20 ^{0}C until analysis (Tajik and Nazaifi, 2013) [33]. The serum samples were analyzed for the determination of cu by using atomic absorption spectrophotometer (Hitachi Polarized Zeeman AAS, Z-8200, Japan) as described by Nazifi *et al*. (2003) The serum calcium and serum phosphorus levels were measured by using a diagnostic kit. The samples of serum were checked for the evaluation of inorganic P levels by the help of evaluation kit (Cat. No 1520099314, DiaSys Diagnostic System GmbH Alte Strabe 9 65558 Holzheim Germany).

Hematology

Blood samples were collected from post parturient haemoglobinuria affected cattle and buffaloes according to office of international epizootic method (OIE, 2010) and transferred in to EDTA vaccutainers. These samples were preserved at 4 °C until used for the evaluation of RBCs, WBCs, HB, MCV, MCH and MCHC.

Treatments

In group A, sodium acid phosphate (Inj.Alphos-40™) 200-250 and 120g of dicalcium phosphate were given intravenously and orally bid, respectively, for seven days (Radostitis *et al*. 2007) [28].

In group B, N-acetylcysteine (Mucolator®) was given IV @ 8 mg bid and vitamin C (Tab C-Cone 10g) were given *PO*, respectively

Clinical examination and color of urine before and after the treatment were used as evaluation criteria for recovery in PPH affected animals.

Data Analysis

The data thus obtained presented in tabulated form with Mean ± SD. Comparison of copper level between the diseased and healthy buffaloes was made by x^2 test, the value was considered significant at $P < 0.05$. The data was analyzed by using paired sample T-test and independent T-test for the determination of post parturient hemoglobinuria.

Results

In the present study feed intake was recorded as i.e. normal feed intake (n=20), 1/2 feed intake, (n=7), 1/4 feed intake (n=8) and off feed (n=5). Ruminal motility (2.29±0.19) was significantly lower in affected animals as compared to healthy animals (3.46±0.14) as shown in Table A and Fig A. Ten animals were in the pre-parturient (approaching to term in the next 40 days) phase of their reproductive cycle whereas the remaining 30 animals developed nutritional hemoglobinuria in the post calving period. All the affected animals were anemic (100%). The color of urine in PPH affected animals ranged from dark red oxide (36%) to red oxide (64%) depending upon the severity of disease. Mucous membranes were pale in all the diseased animals. Nutritional hemoglobinuria affected animals showed symptoms as dullness (100%), dehydration, reduction in milk production (70%) and one case of agalactia. The mean serum copper levels of nutritional hemoglobinuria affected animals was compared with that of twenty apparent normal animals. Mean serum cu concentration in diseased animals recorded as 0.584 ± 0.114µg/dl. The comparison of the mean values with normal animals having serum copper level 0.650 ± 0.071 ug/dl showed that the deficiency of serum copper was significant (Table A) in affected animals.

The body temperature was subnormal to normal in post parturient hemoglobinuria affected animals in the present study. Statistically, there was significant difference in animal’s body temperature (F°) before (99.39±1.59) and after (101.87±0.20) treatment in group A (Table 1). Pulse rate was significantly higher after treatment (64.2000±1.54) in group A (Table 1). Respiration rate was non-significant before and after treatment in group A as shown in Table 1. Statistically, there was significant difference in animal's body temperature (F°) before (99.41±1.68) and after (100.99±0.15) treatment group B (Table 2). Pulse and respiration rate show non-significant difference before and after treatment in group B (Table2). There was significant difference in TPR values in group A and Group B after treatment as shown in Table 3. In group A, a significant difference found in mean serum P level before (3.25±0.928) and after (6.19±0.19) the treatment (Table.4). Serum Ca level (7.41±0.99) increased with a significant difference after the treatment (11.03±0.44) in group A as displayed in Table4. In group B, a significant difference found in mean serum P level before (2.9030±0.73) and after (5.67±0.06142) the treatment (Table.4). Serum Ca level (5.67.06) increased with a significant difference after (9.84±0.17) the treatment in group B as displayed in Table4. Mean serum P level displayed significant difference (6.19±0.19) in group A and group B (5.67±0.06) as shown in Table 5.

In group A, red blood cell count, hemoglobin and hematocrit showed a significant difference (*P*<0.05) after treatment (Table 6). There was also a significant increase noted in platelet count after the treatment in group A. Similarly, a significant difference (*P*<0.05) was found in red blood cells count, hemoglobin and hematocrit after the treatment (Table 7). A significant difference (*P*<0.05) displayed in red blood cell count, hemoglobin, hematocrit and platelet count in group A and group B (Table7).

Table A: Comparison of serum copper level and ruminal motility (mean ±SD) in affected and healthy dairy animals

Group	Copper level	Ruminal motility
Affected	0.584±0.114	2.29±0.19
Healthy	0.650±0.071	3.46±0.14

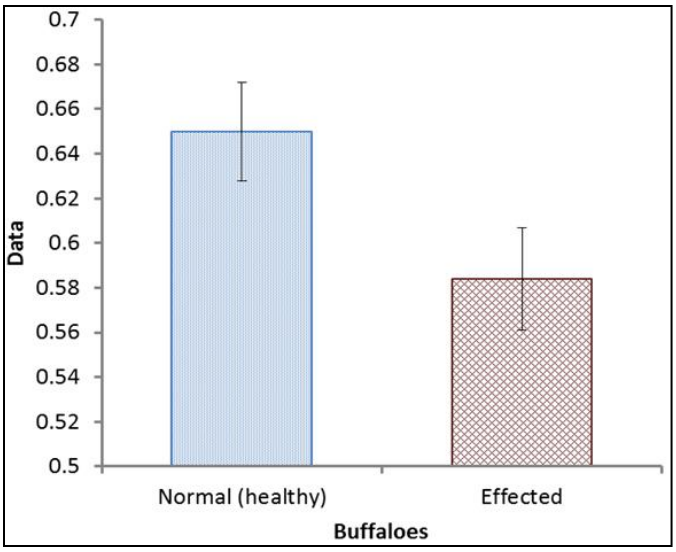

Fig A: Graphical comparison of copper level of affected and healthy dairy animals

Table 1: TPR profile of dairy animals suffering from post-parturient hemoglobinuria before and after treatment with Sodium Acid Phosphate and DCP.

Parameter	Before Treatment	After Treatment
Temperature °F	99.39±1.59^{a}	101.87±0.198^{b}
Pulse Per Minute	61.45±4.25^{a}	64.20±1.54^{b}
Respiration Per Minute	20.65±2.81^{a}	20.40±2.21^{a}

Different superscripts with in row indicates significant difference (*P*<0.05).

Table 2: TPR profile of dairy animals suffering from post-parturient hemoglobinuria before and after treatment with Mucolator and Cecon.

Parameter	Before Treatment	After Treatment
Temperature °F	99.4100±1.67894^{a}	100.9900±0.15183^{b}
Pulse Per Minute	60.5000±2.54434^{a}	59.4500±1.23438^{a}
Respiration Per Minute	20.4000±2.21003^{a}	19.6000±1.18766^{a}

Different superscripts with in row showed significant difference (*P*<0.05).

Table 3: Comparison of TPR profile of dairy animals suffering from post-parturient hemoglobinuria after treatment with Sodium Acid Phosphate + DCP and Mucolator + Cecon.

Parameter	Group A	Group B
Temperature ^{0}F	101.3700±0.19762^{a}	100.9900±0.15183^{b}
Pulse Per Minute	64.2000±1.54238^{a}	59.4500±1.23438^{b}
Respiration Per Minute	20.40±2.21a	19.6000±1.18766^{b}

Different superscripts with in row showed significant difference (*P*<0.05).

Table 4: Serum phosphorus profile of dairy animals suffering from post-parturient hemoglobinuria before and after treatment with Sodium Acid Phosphate and DCP

Parameter	Before Treatment	After Treatment
Phosphorus	3.2490±0.92796^{a}	6.1955±0.19481^{b}
Calcium	7.4150±0.99609^{a}	11.0280±0.44081^{b}

Different superscripts with in row showed significant difference (*P*<0.05).

Table 5: Serum phosphorus profile of dairy animals suffering from post-parturient hemoglobinuria before and after treatment with Mucolator and Cecon.

Parameter	Before Treatment	After Treatment
Phosphorus	2.9030±0.73346^{a}	5.6740±0.06142^{b}
Calcium	7.8155±0.62603^{a}	9.8400±0.16995^{b}

Different superscripts with in row showed significant difference (*P*<0.05).

Table 6: Comparison of serum phosphorus profile of dairy animals suffering from post-parturient hemoglobinuria after treatment with Sodium Acid Phosphate+ DCP and Mucolator + Cecon.

Parameter	Group A	Group B
Phosphorus	6.19481±0.19481^{a}	5.6740±0.06142^{b}
Calcium	11.0280±0.44081^{a}	9.9400±0.16995^{b}

Different superscripts with in row showed significant difference (*P*<0.05).

Table 7: CBC profile of dairy animals suffering from post-parturient hemoglobinuria before and after treatment with Sodium Acid Phosphate and DCP.

Parameter	Before Treatment	After Treatment
RBC	2.7435±1.03806^{a}	9.6185±0.98752^{b}
MCV	63.9085±5.81827^{a}	61.8685±4.422403^{b}
HCT	15.2385±9.97257^{a}	35.8465±2.63467^{b}
PLT	82.6420±8.27858^{a}	191.0500±10.97040^{b}
MPV	8.4430±0.93047^{a}	6.4640±0.21273^{b}
WBC	6.6155±2.44357^{a}	9.5770±1.63793^{b}
HGB	5.3005±1.34151^{a}	12.6410±0.88629^{b}
MCH	17.0740±5.15794^{a}	17.8850±1.83381^{a}
MCHC	26.9715±7.02192^{a}	36.2380±3.18083^{b}
LYM	2.6195±1.12935^{a}	49.9360±3.14898^{b}
GRAN	2.1775±0.97746^{a}	25.7980±3.74484^{b}

Different superscripts with in row showed significant difference (*P*<0.05).

Table 7: CBC profile of dairy animals suffering from post-parturient hemoglobinuria before and after treatment with Mucolater and Cecon.

Parameter	Group A	Group B
RBC	2.5275±0.65133[a]	7.3760±0.48857[b]
MCV	62.4825±4.67503[a]	55.7700±3.75192[b]
HCT	16.8420±8.13471[a]	29.2140±2.21662[b]
PLT	88.6910±7.51770[a]	117.3650±9.157697[b]
MPV	7.9625±1.05347[a]	4.3580±0.45664[b]
WBC	5.7785±2.16588[a]	6.1410±0.72378[a]
HGB	4.9365±1.14264[a]	10.1090±0.20501[b]
MCH	18.6855±5.14091[a]	11.1515±0.81426[a]
MCHC	28.5715±8.35527[a]	30.2925±0.84655[a]
LYM	2.6265±1.06692[a]	45.1790±0.44607[b]
GRAN	3.3485±2.50875[a]	18.5045±1.93678[b]

Different superscripts with in row showed significant difference (P<0.05).

Table 8: Comparison of CBC profile of dairy animals suffering from post-parturient hemoglobinuria after treatment them with Sodium Acid Phosphate + DCP with Mucolator + Cecon.

Parameter	Group A	Group B
RBC	9.6185±0.98752[a]	7.3760±0.48857[b]
MCV	61.8685±4.22403[a]	55.7700±3.75192[b]
HCT	35.8465±2.63467[a]	29.2140±2.21662[b]
PLT	191.0500±14.75040[a]	117.3650±12.57697[b]
MPV	6.4640±0.95137[a]	4.3580±0.45664[b]
WBC	9.5770±1.63793[a]	6.1410±0.72378[b]
HGB	12.6410±0.88629[a]	10.1090±0.20501[b]
MCH	17.8850±1.83381[a]	11.1515±0.81426[a]
MCHC	36.2380±3.18083[a]	30.2925±0.84655[b]
LYM	49.9360±3.14898[a]	45.1790±0.44607[b]
GRAN	25.7980±3.74484[a]	18.5045±1.93678[b]

Different superscripts with in row showed significant difference (P<0.05).

Discussion

In the present study, all cases of nutritional haemoglobinuria PPH in dairy animals were reported during winter (November to February) and spring (March to April) seasons. All diseased animals were stall-fed. Berseem (*Trifolium alexandrinum)* was the major fodder (68.33%) given to all the animals. In addition, all animals were fed 2-5 kg of concentrates daily. Similar findings were reported in epidemiological analysis by Soren *et al*. (2015) where higher incidence of PPH (87.50%) was reported during winter in comparison to sumer (12.50.%). In Punjab, similar findings were reported by Muhammad *et al*. (2000) [25]. Present study showed higher occurrence of the condition in post parturition phase (75% cases) than in advance pregnancy (25%) as stated by Gahlawat *et al*. (2007) [11]. Urine color in PPH affected animals varied from red (28%), dark red (20%) and coffee colored (52%) as observed in the present study depending upon the severity of the condition as mentioned by Khan and Akhtar (2007) [1, 2]. Hematologically, PPH has the features of an acute intravascular hemolytic anemia. The packed cell volume falls rapidly to its lowest level four to nine days after the onset of hemoglobinuria. In the present study hematological finding i.e. mean erythrocyte count, hemoglobin concentration, and hematocrit were lower similar findings were also reported in the literature (Durrani *et al*. 2010; Soren *et al*. 2014; Kumar *et al*. 2014) [9, 17].

In the present study, marked anorexia with ruminal stasis and severe straining in affected animals was also reported by Akhtar *et al*. (2006). According to Digraskar *et al*. (1991) [8] excessive formation of haemosiderin and its deposition in gastro-intestinal mucosae in affected dairy animals could be responsible for gastro-intestinal disturbances i.e. ruminal stasis, constipation and straining. In PPH affected animals mucous membranes were icteric (pale) with progress in anemia, shallow and rapid breathing, tachycardia and reduced milk production same clinical signs were observed by Ghanem and El-Deeb (2010) [12, 13]. The body temperature was below the normal range in affected animals before the treatment (Table1). In the present study 42% of animals were in their 4th stage of lactation. According to researchers (Macwillims *et al.* 1982; Gahlawat *et al*., 2007) [20, 11] risk of hemoglobinuria is high around parturition mostly cases of hemoglobinuria occurred within 60 days of parturition and during second to sixth lactation.

Pulse rate per minute was significantly lower (47.52±4.15) in affected buffaloes as compared to healthy buffaloes (57.20±5.14) in contrast to the findings reported by Shahid (2011) [30]. The comparison of mean values of cu in healthy and affected dairy animals showed significant deficiency of trace mineral in affected dairy animals (Table1). Significantly decreased cu levels could be attributed to a 3-way interaction between cu, molybdenum, and sulphur as reported by Suttle (1991) [32]. This interaction can occur with concentrations of molybdenum and sulphur naturally present in feed stuffs, and involved in formation of thiomolybdates in the rumen. It binds with cu and form a highly insoluble complex, that does not release copper even under acidic conditions and renders it unavailable to the animals for utilization resulting in hypocupraemia. In the present study there was significant difference in P level of dairy animals before and after treatment in group A and group B. Moreover, a significant difference was observed in P level between two treatment groups. Digraskar *et al.* (1991) [8] reported that in advanced gestation, more P and Ca are required for the developing fetus if supplementary P is not provided, thereby leading to hypophosphatemia. Moreover, high Ca to P ratio results in decreased absorption from the intestinal tract and ultimately leads to hypophosphatemia. Akhtar *et al.* (2007) [1, 2] found that fodders grown on phosphorus-deficient soils are consequently low in phosphorus content, and thereby prolonged feeding on such fodders can lead to hypophosphatemia. Kumar *et al.* (2014) [17] reported that diet deficient in any cereals or concentrate resulted in dietary P deficiency leading to hemoglobinuria. The transition between late pregnancy and early lactation, from calving until a 3 to 4 week postpartum, is a high-risk period for the occurrence of the disease in the dairy cow (Mahmut *et al.* 2009). Fibrinolytic agents have also been suggested in the treatment of PPH (Chug *et al.*, 1987) [7]. In the present study Mucolator® N-acetyl cysteine (NAC) was used for treatment of PPH affected animals.). N-acetyl cysteine stimulates glutathione biosynthesis, promotes detoxification, and acts directly as a scavenger of free radicals. It is a powerful antioxidant and a potential treatment option for diseases characterized by the generation of free oxygen radicals). Studies have shown no maternal or fetal harmful effects of NAC treatmen (Mokhtari *et al*. 2017) [24] Vitamin c was used to have better recovery rate in affected animals. Use of ascorbic acid in PPH affected buffaloes has been previously recorded by Bhikane and Kawitkar (2000) [5]. Ascorbic acid ameliorate the condition by reducing oxidative stress of RBCs intravascular hemolysis and Fe to stay in reduced state (Benerjee, 1998) [4]

Conclusion

Hypophosphatemia and hypocupremia was a constant feature in all cases of hemoglobinuria in the present study. It is worth mentioning that both treatment protocols corrected hypophosphatemia adequately in hemoglobinuria animals, but treatment with Sodium acid phosphate and DCP was successful in restoring serum phosphorus level and hematological values in affected animals followed by treatment with Mucolator combined with Tab. C-Cone.

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