

Haemato-Biochemical Aspects of Parturient Haemoglobinuria in Buffalo

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Abstract: This study aimed to determine the haematological and biochemical changes that occur in buffaloes with parturient haemoglobinuria (PHU). For this purpose, serum samples from 60 PHU-affected and 60 apparently healthy buffaloes were collected and analysed. Mean erythrocyte count ($3.6 \pm 1.0 \times 10^{12}/l$), haemoglobin concentration (5.8 ± 1.4 g/dl), and haematocrit (16.9 ± 2.8 %) of the PHU-affected buffaloes were lower ($P < 0.001$), while their erythrocyte sedimentation rate (104.1 ± 36.2 mm/h) was higher ($P < 0.001$) in comparison to the healthy buffaloes. Neutrophils ($43.0 \pm 4.5\%$), urea (49.7 ± 7.8 mg/dl) and creatinine (2.1 ± 0.4 mg/dl) concentrations were significantly higher in the PHU-affected buffaloes, while lymphocytes ($48.7 \pm 2.9\%$) and erythrocytic glucose-6-phosphate dehydrogenase (G6PD) (92.3 ± 13.2 mU/ 10^9 TECs) were lower than in the healthy buffaloes. Serum phosphorus (1.9 ± 0.6 mg/dl) and copper (65.4 ± 6.0 µg/dl) were lower ($P < 0.001$), while molybdenum (171.5 ± 56.7 µg/dl) was higher in the PHU-affected buffaloes as compared to those values in the healthy buffaloes. It was concluded that PHU-affected buffaloes usually suffer from severe anaemia and hypophosphataemia, and erythrocytes with significantly reduced G6PD are prone to haemolysis, leading to haemoglobinuria in buffaloes.

Key Words: Parturient haemoglobinuria, buffaloes, haematology, biochemistry

Introduction

Parturient haemoglobinuria (PHU) is a major disease of dairy animals with detrimental economic consequences (1,2). It is an acute disease of high yielding dairy animals characterised by hypophosphataemia, intravascular haemolysis, haemoglobinuria, and anaemia (3). The exact aetiology and pathogenesis of PHU are not known, as a variety of aetiological factors have been reported to be associated with the disease in different parts of the world.

Nonetheless, hypophosphataemia is documented consistently in affected animals (2,4). Dietary phosphorus deficiency and/or rations containing cruciferous plants are suspected causes of severe hypophosphataemia and have been associated with haemolytic anaemia in cows (1). Copper deficiency is also an aetiological factor of post-PHU, as its deficiency reduces the activity of the copper-containing enzyme, superoxide dismutase, which is part

of the erythrocyte protection mechanism against oxidative stress (5). According to Singari et al. (6), decreased erythrocytic glucose-6-phosphate dehydrogenase (G6PD) activity in haemoglobinuric buffaloes may be partially responsible for a decrease in reduced glutathione, thereby causing oxidative stress to erythrocytes, which results in haemolytic syndrome.

From the above discussion it is clear that the exact aetiology and pathogenesis of PHU is not known, although hypophosphataemia is documented consistently. It is hypothesised that hypophosphataemia results in decreased red blood cell glycolysis and ATP synthesis. Subnormal concentration of ATP predisposes red blood cells to altered structure and function, a loss of normal deformability, and an increase in fragility and haemolysis, with resultant haemoglobinaemia and haemoglobinuria. In view of the above speculations, the present investigation was undertaken to study the haematological

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and serum biochemical changes in buffaloes suffering from PHU so that the developed hypothesis could be tested. It is hoped that the present study will contribute to the understanding of the aetiopathology of PHU and to suggest measures for the prevention and control of haemoglobinuria in buffaloes.

Materials and Methods

Animals

The study included 60 buffaloes (*Bubalus bubalis*) suffering from PHU that were randomly selected from field cases that occurred in the Faisalabad, Toba Tek Singh, and Jhang districts of Punjab province. The controls were 60 clinically healthy buffaloes of similar description from the same localities. The study animals were stall fed, and seasonal green fodders, including *Trifolium alexandrinum* (Berseem), *Brassica campestris* (Sarson), *Zea mays* (Maize), *Sorghum vulgare* (Sorghum), and *Saccharum officinarum* (Sugarcane), were offered. The case fatality rate was 15%. The disease was clinically diagnosed on the basis of specific signs, such as haemoglobinuria and characteristic straining while defecating during early lactation or advanced pregnancy (7). Other diseases that cause a reddish discolouration of urine, like babesiosis, leptospirosis, and bacillary haemoglobinuria, were ruled out through laboratory tests.

Haematological and biochemical studies

Blood samples from were collected from each buffalo, with and without anticoagulant (Na_2EDTA : 1 mg/ml). Blood samples with anticoagulant were used for the determination of erythrocyte and leukocyte counts (haemocytometer method), haemoglobin concentration (cyanmethaemoglobin method), haematocrit (microhaematocrit method), and erythrocyte sedimentation rate (Westergren tube method), following the techniques described by Benjamin (8). Differential leukocyte counts were determined by staining the blood smears with Giemsa stain (8).

Serum was separated from blood samples collected without anticoagulant and preserved at $-20\text{ }^\circ\text{C}$ for further biochemical analysis. Serum urea (cat. no. CS 612; Crescent Diagnostics, Jeddah, Saudi Arabia), creatinine (cat. no. 448, Biocon Diagnostik, Germany), and erythrocytic G6PD (cat. no. PD 410, Randox

Laboratories Ltd., UK) were estimated spectrophotometrically using the diagnostic kits according to the manufacturers' instructions. Calcium, copper, and molybdenum concentrations were determined by using an atomic absorption spectrophotometer (Varian Spector AA-5), and phosphorus was analysed with a spectrophotometer (Philips, Model 1100).

Statistical analysis

The data were subjected to paired t-test. Pearson's correlations were calculated using the Minitab statistical package.

Results

In the PHU-affected buffaloes, mean erythrocyte count, haemoglobin concentration, and haematocrit were lower, while the erythrocyte sedimentation rate was higher than in the healthy buffaloes. Blood smears from PHU-affected buffaloes showed macrocytes and spherocytes. Total leukocyte count, monocytes, and eosinophils did not differ between the 2 groups of animals. Neutrophils were higher and lymphocytes were lower in the PHU-affected buffaloes (Table 1).

Urea and creatinine concentrations in PHU-affected buffaloes were higher, whereas erythrocytic G6PD was lower than in healthy buffaloes. Serum phosphorus and copper concentrations were significantly lower in PHU-affected buffaloes, while molybdenum was significantly higher (Table 2).

The mean total erythrocyte count was positively correlated with haemoglobin concentration ($r = 0.291$, $P < 0.05$) and haematocrit ($r = 0.392$, $P < 0.01$) in PHU-affected buffaloes. The mean total leukocyte count was positively correlated with neutrophils ($r = 0.694$, $P < 0.001$) and lymphocytes ($r = 0.712$, $P < 0.001$). Creatinine was positively correlated with molybdenum ($r = 0.273$, $P < 0.05$) and urea ($r = 0.873$, $P < 0.001$).

Discussion

Hypophosphataemia in PHU-affected animals is consistently documented (2,4). In the present study, significantly decreased serum phosphorus in PHU-affected buffaloes was recorded, as has been reported previously in PHU-affected buffaloes (9-11) and cattle (12). Heavy drainage of phosphorus through milk,

Table 1. Haematological variables (mean \pm SD) in healthy and PHU-affected buffaloes.

Parameters	Buffaloes		t value	P value
	Healthy	PHU affected		
Erythrocyte count ($10^{12}/l$)	6.2 \pm 0.6	3.6 \pm 1.0	-17.04	< 0.001
Haemoglobin concentration (g/dl)	11.0 \pm 1.2	5.8 \pm 1.4	-22.86	< 0.001
Haematocrit (%)	34.3 \pm 2.4	16.9 \pm 2.8	-38.68	< 0.001
Erythrocyte sedimentation rate (mm/1 h)	73.1 \pm 29.9	104.1 \pm 36.2	4.59	< 0.001
Total leukocyte counts ($\times 10^9/l$)	9.5 \pm 2.2	10.0 \pm 1.9	1.59	0.137
Neutrophils (%)	35.1 \pm 3.8	43.0 \pm 4.5	9.98	< 0.001
Lymphocytes (%)	56.5 \pm 4.3	48.7 \pm 2.9	-10.78	< 0.001
Monocytes (%)	5.2 \pm 0.9	5.1 \pm 1.0	-0.73	0.468
Eosinophils (%)	3.3 \pm 0.9	3.2 \pm 0.9	-0.77	0.442

Table 2. Biochemical variables (mean \pm SD) in healthy and PHU-affected buffaloes.

Parameters	Buffaloes		t value	P value
	Healthy	PHU affected		
Urea (mg/dl)	33.9 \pm 5.1	49.7 \pm 7.8	12.60	< 0.001
Creatinine (mg/dl)	1.3 \pm 0.3	2.1 \pm 0.4	12.68	< 0.001
Erythrocytic G6PD (mU/ 10^9 TECs)	108.9 \pm 19.6	92.3 \pm 13.2	-4.85	< 0.001
Calcium (mg/dl)	9.8 \pm 1.0	9.9 \pm 1.3	0.10	0.907
Phosphorus (mg/dl)	5.41 \pm 0.6	1.9 \pm 0.6	-30.56	< 0.001
Copper (μ g/dl)	118.4 \pm 5.2	65.4 \pm 6.0	-54.60	< 0.001
Molybdenum (μ g/dl)	54.8 \pm 13.6	171.5 \pm 56.7	15.69	< 0.001

particularly in high milk yielding animals, leads to hypophosphataemia (13). In advanced gestation, more phosphorus and calcium are required for the developing foetus if supplementary phosphorus is not provided, thereby leading to hypophosphataemia. Moreover, a high calcium to phosphorus ratio results in decreased phosphorus absorption from the intestinal tract and ultimately leads to hypophosphataemia (8).

Phosphorus deficient soils are common in dry tropical countries like Pakistan. Although many soils are naturally deficient in phosphorus, heavy leaching by rain and constant crop removal also contribute to phosphorus deficiency in soil. Fodders grown on such soils are consequently low in phosphorus content and, thereby, prolonged feeding on such fodders can lead to

hypophosphataemia (3,14). According to Dhillon et al. (15), soils in the Indian Punjab have high molybdenum content. The fodders, in particular Berseem (*Trifolium alexandrinum*), grown on such soils have high molybdenum content. The excess of this element reduces phosphorus content of the body by interfering with its absorption from the gastro-intestinal tract and by increasing phosphorus elimination through urine.

A significant decrease in erythrocyte count, haemoglobin concentration, and haematocrit in PHU-affected buffaloes indicates severe anaemia. This could be attributed to intravascular haemolysis (7,8,14,16) due to an impaired glycolytic pathway and depletion of ATP in erythrocytes, which results from phosphorus deficiency. Subnormal concentration of ATP predisposes red blood

cells to alter functions and structure, a loss of normal formability, and an increase in fragility, ultimately leading to haemolysis (17,18).

In the present study, total erythrocyte count was positively correlated with hemoglobin concentration ($r = 0.291$, $P < 0.05$) and hematocrit ($r = 0.392$, $P < 0.01$) in PHU-affected buffaloes, which were also anaemic. In anaemic cases, total erythrocyte count, haemoglobin concentration, and haematocrit were reported to decrease simultaneously (8), indicating a possible positive correlation between total erythrocyte count and both haemoglobin concentration and haematocrit.

Serum molybdenum and copper were significantly high and low, respectively, in PHU-affected buffaloes. Significantly decreased copper levels could be attributed to a 3-way interaction between copper, molybdenum, and sulphur. As reported by Suttle (19), this interaction can occur with concentrations of molybdenum and sulphur that are naturally present in feed stuffs, and is involved in the formation of thiomolybdates in the rumen (20). Sulphides are produced by micro-organisms in the rumen via the reduction of sulphate and degradation of sulphur amino acids. These sulphides react with molybdate to form thiomolybdates, which bind with copper and form a highly insoluble complex that does not release copper, even under acidic conditions, and renders it unavailable to the animal for utilisation, resulting in copper deficiency (21).

The erythrocytic G6PD activity in PHU-affected buffaloes was significantly lower than that in healthy buffaloes (Table 2). Singari et al. (6) suggested that decreased erythrocytic G6PD activity in haemoglobinuric buffaloes may be partially responsible for the decrease in reduced glutathione, thereby causing oxidative stress to erythrocytes, which leads to haemolytic syndrome. Among the 2 major pathways of glucose metabolism in red blood cells, the pentose phosphate pathway (PPP) is of critical significance for normal red cell survival. The first reaction in PPP is the catalytic action of the enzyme G6PD in oxidising glucose-6-phosphate. NADPH generated by the cell's PPP has a reducing potential on glutathione, and glutathione maintained in a reduced state protects red cells from oxidative stress; thus, a deficiency of G6PD will result in haemolytic anaemia (22). Deficiency of G6PD, owing to mutation, is the most common enzymatic abnormality in humans and has a high incidence rate, and over 300 genetic variants of the

enzyme have been identified; at least 100 million people are deficient in this enzyme owing to these variants (23). G6PD may exist in haemoglobinuric buffaloes, but this needs further exploration.

The increasing and decreasing trend in neutrophil and lymphocyte counts, respectively, in PHU-affected buffaloes could be attributed to the endogenous release of corticosteroids. Increased stress due to PHU (a metabolic disorder) is the source of the release of corticosteroids (6) that results in increased neutrophils and depressed lymphocytes. Neutrophils are short-lived and normally the entire neutrophilic population in circulation is replaced 2.5 times daily (8); therefore, these have to leave circulation rapidly (about 9-10 h), but under disease conditions these are retained in circulation. Moreover, marginal neutrophils are pooled in the main circulation and increased release of neutrophils from the maturation pool (8,22) seems to be the main source of neutrophilia in PHU-affected buffaloes. According to Latimer et al. (22), recirculating lymphocytes under the influence of corticosteroids remain transiently sequestered in the lymphoid tissues or bone marrow rather than entering efferent lymph and blood, resulting in lymphopenia. Furthermore, lysis of lymphocytes in all tissues and a decline of lymphoid mitosis in lymph nodes, due to corticosteroids, can also lead to lymphopenia.

Increased blood urea levels in PHU-affected buffaloes could be attributed to the endogenous release of corticosteroids, starvation, and tubular epithelial necrosis (7,9). Additionally, dehydration usually occurs with PHU, which is a source of decreased renal perfusion, resulting in a reduced glomerular filtration rate and increased blood urea level (8,22,24,25). Alternatively, increased blood urea could be due to the failure of the urea recycling process through salivary glands and its non-utilisation by microbes in the rumen during digestive disorders. Most of the urea formed by the liver circulates in the circulatory system and remains unutilised (26). In the present study, creatinine was significantly increased in PHU buffaloes (2.1 ± 0.4 mg/dl). In this regard, Benjamin (8) considered that concentrations over 2 mg/dl lead to a reduced glomerular filtration rate, which affects creatinine in a manner similar to that of blood urea (22).

Both urea and creatinine levels were elevated and positively correlated to each other in PHU-affected buffaloes. Urea and creatinine are waste products that

the kidneys normally filter from the blood, and these are interrelated. If the kidneys are not working properly (7,9), these substances build up in the body, and elevated blood levels of urea and creatinine are indications of pathological kidney function (22).

It was concluded from the present study that phosphorous deficiency plays a key role in causing haemoglobinuria in buffaloes, although PHU-affected buffaloes showed hypocupraemia and reduced G6PD in erythrocytes.

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