Bulgarian Journal of Veterinary Medicine, 2023 ONLINE FIRST
ISSN 1311-1477; DOI: 10.15547/bjvm.2023-0100

Original article

CYTOMORPHOLOGY, OSMOTIC FRAGILITY, GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND OXIDANT/ANTIOXIDANT STATUS IN POSTPARTURIENT HAEMOGLOBINURIA IN DAIRY CATTLE AND BUFFALOES

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Summary

Hassanin, A. I., M. H. Karam & N.-E. M. Aref, 2023. Cytomorphology, osmotic fragility, glucose-6-phosphate dehydrogenase and oxidant/antioxidant status in postparturient haemoglobinuria in dairy cattle and buffaloes. *Bulg. J. Vet. Med.* (online first).

The present study was carried out to elucidate the role of blood phosphorus (P), erythrocytic glucose-6-phosphate dehydrogenase (G6PD), glutathione peroxidase (GSH-Px), malonaldehyde (MDA), and nitric oxide (NO) on the integrity of the cell membranes of red blood cells (RBCs) and the development of postparturient haemoglobinuria (PPH) in dairy cattle and buffaloes. The study also aimed to demonstrate the association between these parameters and erythrocyte osmotic fragility. A total of 58 cows and buffaloes were included and categorised into control group (n=20) and diseased group (n=38). Complete history and clinical examination were conducted. Blood samples were collected and prepared accordingly to assess complete blood count (CBC), reticulocyte count, cytomorphology of RBCs, osmotic fragility, erythrocytic G6PD, GSH-Px, blood phosphorus (P), calcium (Ca), parathormone (PTH), MDA and NO. The hallmark sign of diseased animals was the passage of red to coffee colored urine. CBC data revealed significant reduction ($P<0.05$) in the RBCs count, haemoglobin (Hb) concentration and packed cell volume (PCV) in the diseased group. Cytomorphological examination revealed strong evidence of regenerative anaemia including reticulocytosis and presence of nucleated RBCs in peripheral blood. The study demonstrated a marked increase in the osmotic fragility of RBCs in PPH. Biochemical data revealed a significant decrease ($P<0.05$) in the blood level of P, G6PD, GSH-Px, PTH and significantly increased ($P<0.05$) MDA in PPH. Ca:P ratio showed a marked disturbance in diseased buffaloes. The study also established a strong positive association between the osmotic fragility index (OFI) and the blood MDA level. On the other hand, there was a strong negative association between OFI and blood levels of P and G6PD suggesting the important role of P, G6PD and oxidative stress in PPH etiopathogenesis. The study validated the hypothesis that phosphorus deficiency affected the blood G6PD level with subsequent alteration of glycolysis and generation of GSH-Px predisposing RBCs to oxidative damage and intravascular haemolysis.

Key words: antioxidants, haemoglobinuria, hypophosphataemia, G6PD, osmotic fragility

INTRODUCTION

In high yielding cows and buffaloes, maintenance of normal metabolic rate is important for partitioning of energy and various nutritional metabolities to meet the demand for adequate production of milk (Piantoni & VandeHaar, 2023). Postparturient haemoglobinuria (PPH) is among major metabolic disorders and represents a great threat to dairy cows and buffaloes during advanced pregnancy and early lactation in Egypt and worldwide (Resum *et al.,* 2017; Gruenberg, 2020). The disease is characterised by intravascular haemolysis, haemoglobinemia, haemoglobinuria and anaemia (Soren *et al.,* 2014; Abramowicz *et al.,* 2022). It causes a significant drop in milk production, high treatment expenses and can pose a serious risk of mortality in severe cases. Therefore, the condition is considered a disease of significant economic interest (Gruenberg, 2020).

Previous studies reported 12% to 15% mortalitiy due to PPH (Khan & Akhtar, 2007; Kumar *et al.,* 2019), however, exceptionally high mortality of 53.5–63.4% was recorded in Pakistan (Raz *et al.,* 1988). The fatality is dependent upon the time of initiation of therapy from the onset of disease (Gruenberg, 2020). Several risk factors have been documented in PPH including age, lactation number, stage of pregnancy, postpartum period, previous history of haemoglobinuria and ingestion of cruciferous and/or toxic plants (Khan & Akhtar, 2007).

The etiopathology of PPH is still unclear, however phosphorus deficiency has been incriminated as a primary cause (Stockdale *et al.,* 2005; Albayati *et al.,* 2020; Abramowicz *et al.,* 2022). It is hypothesised that hypophosphataemia results in decreased red blood cells (RBCs) glycolysis with resultant intravascular haemolysis. This assumption came from the importance of phosphorus in the formation of erythrocytic glucose 6-phosphate dehydrogenase (G6PD), which plays a pivotal role in glycolysis process, adenosine triphosphate (ATP) synthesis, and regeneration of nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione.

An early study suggested a role of oxidative stress in the etiology of dairy cattle disorders since the supplementation with certain antioxidants ameliorated the severity of a variety of metabolic and infectious diseases (Miller *et al.,* 1993). Additionally, several studies supported the concept that oxidative stress is a significant underlying factor to dysfunctional host immune and inflammatory responses (Bernabucci *et al.,* 2005; Sordillo 2005; Wilde 2006; Aref *et al.,* 2017).

Therefore, the present study aimed to elucidate the role of blood phosphorus, erythrocytic G6PD, and oxidative stress on the integrity of cell membranes of RBCs and development of PPH in dairy cattle and buffaloes. The study also aimed to demonstrate the association between these blood biomarkers and erythrocyte osmotic fragility.

MATERIALS AND METHODS

Animals

A total 58 dairy cow cattle (n=30) and buffaloes (n=28) were included in the present study and categorised into 2 groups: control (n=20) and diseased (n=38). The diseased group was subdivided based on the severity of intravascular haemolysis, according to PCV cutoff value 20% into mild to moderate (PCV

value >20%, n=16) and severe (PCV value <20%, n=22). Categorisation of the investigated animals and their data about age, parity body weight and time point of sample collection after parturition were presented in Tables 1 and 2. They were fed on *Trifolium alexandrium* (Barseem) as a main source of nutrients during winter and spring seasons (November to May) and cruciferous plants. All animals were investigated to exclude haematuria and blood protozoan parasites (*Babesia*, *Theileria*, *Anaplasma* and *Trypansoma* spp.) infections.

Clinical examination

Thorough clinical examination of diseased animals was performed according to the method described by Jackson & Cockcroft (2008). Body temperature, respiratory rate and heart rate were recorded. Status of the visible mucous membranes (oral, nasal, conjunctival) and general condition of the animal were thoroughly evaluated.

Blood samples

Whole blood samples (WBS). Ten mL blood samples were collected from all animals by jugular venipuncture using 20G needles. Blood samples were mixed with EDTA, allotted, and used immediately for complete blood count (CBC) and determination of osmotic fragility and G6PD activity.

Blood film. Blood smears were prepared from WBS according to Coles (1986) and stained by Leishman and Brilliant cresyl blue stains for determination of differential leukocyte counts (DLC) and reticulocyte counts, respectively. The blood film was also used for cytomorphological evaluation of RBCs and blood parasites.

Plasma samples. A portion of WBS was then centrifuged for plasma collection. Plasma samples were collected and stored at −20 ^{o}C for assay of malondialdehyde (MDA).

Table 1. Categorisation of investigated animals

Group/ animal	Healthy controls (n=20)	Diseased (n=38)		Total
		Mild-moderate PPH (PCV>20%)	Severe PPH (PCV <20%)	
Cattle	10	5	13	28
Buffaloes	10	11	9	30
Total	20	16	22	58

Table 2. Age, body weight, parity and time of sampling (mean value ± SD) in control and diseased animals

Parameter	Animal	Control	PPH
Age (years)	Cattle	6 ± 1.5	6.28± 1.6
	Buffaloes	6.1±1.5	7.15±2.1
BW (kg)	Cattle	349 ± 6.8	377.2±19.8
	Buffaloes	377.78±18.84	387.89±14.92
Parity	Cattle	3.9	4.1
	Buffaloes	4.2	5
Sampling day after parturition	Cattle	44.2±23	26.39±11.4
	Buffaloes	43.8 ± 13.12	35.7±12.9

Erythrocyte lysate. It was prepared for determination of glutathione peroxidase (GSH-Px). Briefly, whole blood with EDTA was centrifuged at 4000 rpm for 10 min at 4 °C. Plasma and buffy coat layers were drawn off and RBCs were left at the bottom of the centrifuge tube. RBCs were then washed once by 10 volumes of cold buffer (50 mM TRIS-HCL, pH 7.5, containing 5 mM EDTA and 1 mM dithiothreitol). The RBCs were then lysed by adding 4 volumes of cold deionised water. RBCs stroma was removed by centrifugation at 4000 rpm for 10 min at 4 °C and erythrocyte lysate supernatants were collected and stored at −70 °C till use.

Serum samples. Two mL blood without anticoagulant was used for serum collection and determination of inorganic phosphorus (Pi), calcium (Ca), parathormone (PTH) and nitric oxide (NO).

Blood analyses

Haematological analysis. EDTA blood samples were used for estimation of red blood cell count (RBCs, $\times 10^{12}$/L), white blood cells count (WBCs $\times 10^{9}$/L), haemoglobin concentration (Hb, g/L), packed cell volume (PCV, %), mean corpuscular volume (MCV, fL), mean corpuscular haemoglobin (MCH, pg) and mean corpuscular haemoglobin concentration (MCHC, g/L) using an automated CBC analyzer (Hemacount30, Human, Germany).

Blood films were prepared for cytomorphological examination of RBCs, DLC (%) and detection of blood parasites. Any abnormalities of RBC shape and size including poikilocytosis and anisocytosis, reticulocytosis, intracellular parasites were recorded. The percentage of individual WBCs was also recorded.

Osmotic fragility test (OFT)

It is based on the measurement of RBCs lysis as a function of osmotic stress and measures the amount of released haemoglobin in a blood sample placed in a series of 16 tubes containing different concentrations of aqueous NaCl solutions (0.00, 0.10, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50, 0.55, 0.60, 0.65, 0.70, 0.75, 0.80 and 0.85%) (Pagana *et al.,* 2019). The amount of released haemoglobin, which is proportional to the lysed cell number, was estimated colorimetrically at 540 nm. Briefly, 20 µL of freshly drawn WBS was added to each tube and left stand at room temperature for 30 min. Tubes were centrifuged at 2000 rpm for 10 min and the optical density of the supernatants were determined colorimetrically at 540 nm against distilled water as a blank. The optical density of the tested sample in tube 16 was regarded as 100% haemolysis. The percentage of haemolysis was then calculated for each solution using the equation below and plotted against NaCl concentrations.

$$\text{Haemolysis (\%)} = \frac{100\times \text{ OD tested sample}}{\text{OD tube 16}}$$

The resulting osmotic fragility curve was then compared with that obtained with normal controls. The result of the test (osmotic fragility index, OFI) was expressed as the concentration of NaCl causing 50 % haemolysis.

Blood biochemical analysis

Blood biochemical parameters including GSH-Px (mU/mL) at 340 nm, plasma MDA (nmol/mL) at wavelength 534 nm, NO (µmol/L) at 540 nm, G6PD (U/g Hb) at 570 nm, blood serum Ca (mmol/L) and serum Pi (mmol/L) at 340 nm were measured spectrophotometrically using commercial test kits and UV spectrophotome-

ter (Humalyzer 3000, Human, Germany) according to manufacturer's instructions. A chemiluminescent immunoassay was adopted for *in vitro* quantitative determination of PTH in serum using Elecsys PTHSTAT immunoassay (Roche Diagnostics GmbH) according to manufacturer's instructions**.** The electrochemiluminescence immunoassay "ECLIA" was intended for use on Elecsys and Cobas immunoassay analyzers.

Statistical analysis

The current study was designed as 2×3 (with two types of animals: cattle & buffaloes and three treatments: control, mild to moderate & severe) factorial experiment conducted in Randomized Complete Block Design (CRD), and the statistical analysis model was as follows:

$$Y_{ij} = \mu + A_i + B_j + (AB)_{ij} + \varepsilon_i$$

Where Yij=the observation, μ=overall mean, Ai=effect of the i^{th} level of factor A (breed), Bj=effect of the j^{th} level of factor B (treatments), (AB)ij=effect of interaction between i^{th} level of factor A and j^{th} level of factor B, εij=the effect of the error related to individual observation. The statistical analysis was generated using SAS software (2013). Prior to analysis, data were tested for normality and transformations were performed when necessary. Comparisons between the different breeds and treatments were done using the Duncan Multiple Range Test, while the interactions were tested using LS means with PIDFF procedure. Test of significance was set up at $P<0.05$. Additionally, the correlation between all experimental variables was performed using proc corr; by SAS software (2013). Linear regression of OFI and biochemical parameters was also performed.

RESULTS

In the present study, PPH occurred in 20 buffaloes (aged 7.15±2.1 years) and 18 cattle (aged 6.28±1.6 years). The affected animals have recently calved. Intervals from calving to the onset of signs ranged from 20 to 60 days (35.7±12.9 days) in buffaloes and from 7–50 days (26.39± 11.4 days) in cattle (Table 2). Cases were reported in three seasons however the highest occurrence of PPH was observed in winter (65.8%), followed by spring (28.9%), and summer (5.3%). The examined cases showed various degree of severity based on PCV value (mild to moderate, >20%) and (severe, <20%). All examined cases showed haemoglobinuria. It was identified as passing out dark yellow urine in mild to moderate cases and red to coffee coloured urine in severe ones (Fig. 1A). The clinical examination of diseased animals revealed inappetence to anorexia, hypomotile to atonic rumen, normal to subnormal body temperature, normocardia to tachycardia, eupnea to polypnea. Severe cases were anorexic, hypothermic with tachycardia, exhibited marked decrease in milk yield, systolic murmurs, dyspnea, pale to icteric mucous membranes (Fig. 1B) and characteristic severe straining during defecation because of anal constriction (Fig. 1C). Detailed clinical signs were presented in Table 3.

Screening of haematological data revealed significant decrease ($P<0.05$) in the RBCs count, Hb concentration and PCV in both mild-moderate and severe cases of PPH-affected buffaloes and cattle. Detailed haematological data were presented in Table 4.

Cytomorphological study of peripheral blood RBCs (Fig. 2) revealed presence of an increased number of reticulocytes in mild-moderate and severe cases (5% and

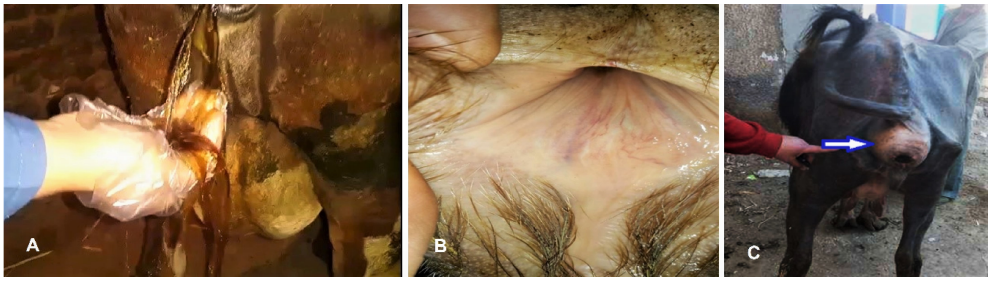


Fig. 1. Red urine (tea-like) in a PPH cow (**A**); marked paleness of vaginal mucous membrane and severe anemic in PPH in buffalo (**B**); anal constriction and difficult deification in a PPH buffalo (**C**).

Table 3. History and clinical signs in animals with post parturient haemoglobinurea

Examination	Healthy controls (n=20)	Mild to Moderate PPH (PCV > 20%) (n=16)	Severe PPH (PCV < 20%) (n=22)
Appetite	Good	Inappetence – depraved	Anorexia – depraved
Milk production	Good	Decreased	Markedly decreased
Temperature (^{o}C)	38.2 ± 0.08	38.06 ± 0.15	37.78 ± 0.12
Respiration (RC/min)	17.00 ± 1.86 Abdominal, regular	30.63 ± 2.20 Abdominal, regular	41.11 ± 2.86 Shallow rapid with harsh breath sound
Heart rate (beat/min)	66.00±2.08 Regular	86-25±1.25 Regular	94.44±3.77 Loud
Urine color	Pale yellow, clear	Clear, pale yellow	Pale red, red, dark red to coffee turbid
Character of feces	Semisolid, greenish to yellow brown	Hard and blackish, Constipation	Difficult, small offensive semisolid
Mucous membranes	Rosy, red, no lesion, no discharge	Pale red, with watery discharge	Pale yellowish, with watery discharge
Mortality rate	–	0	9.1%

9%, respectively) (Fig. 2A), nucleated RBCs and macrocytosis (Fig. 2B). Other form s of poikilocytosis and anisocytosis including blister RBCs (Fig. 2C), hypochromacia and target RBCs (Fig. 2D), tear drop RBCs (Fig. 2E), bite RBCs (Fig. 2F) were also reported in PPH. Both blister and bite RBC were mostly noted in PPH in buffaloes with G6PD deficiency. Total platelet count showed non-significant increase in severe cases as compared to healthy animals.

Biochemical analysis data showed significant decrease ($P<0.05$) of blood phosphorus level in mild-moderate and severe PPH-affected buffaloes and cattle. Blood calcium showed a significant decrease ($P\leq0.05$) in mild-moderate and severe cases in buffaloes. In diseased cattle, blood calcium level showed significant increase ($P\leq0.05$) in mild-moderate and no significant changes in the severe group. Ca:P ratio showed marked disturbance in the in mild-moderate and severe cases of

Table 4. Hematological indices (mean value ± SD) in healthy and diseased cattle and buffaloes

Parameter	Animals	Healthy controls	Mild to moderate PPH (PCV > 20%)	Severe PPH (PCV < 20%)
Hb (g/L)	Cattle	123.1 ± 10.9^{b}	78.2 ± 10.7^{c}	50.69± 10.58^{d}
	Buffaloes	141.8 ± 16.2^{a}	78.3 ± 10.1^{c}	53.78 ± 11.12^{d}
PCV (%)	Cattle	38.40 ± 0.96^{b}	24.00 ± 1.18^{c}	15.54 ± 0.92^{d}
	Buffaloes	44.40 ± 1.83^{a}	24.18 ± 1.04^{c}	15.33 ± 0.91^{d}
RBCs ($\times10^{12}$/L)	Cattle	5.73 ± 0.31^{b}	3.92 ± 0.24^{c}	2.30 ± 0.16^{d}
	Buffaloes	7.15 ± 0.28^{a}	3.53 ± 0.18^{c}	2.26 ± 0.19^{d}
MCV (fL)	Cattle	67.20 ± 2.67	61.00 ± 1.87	68.62 ± 3.20
	Buffaloes	62.80 ± 1.79	68.36 ± 1.56	69.22 ± 3.94
MCH (pg)	Cattle	21.90 ± 0.60^{b}	19.20 ± 0.58^{c}	21.92 ± 0.76^{b}
	Buffaloes	19.20 ± 0.63^{c}	22.73 ± 0.84^{ab}	24.44 ± 1.78^{a}
MCHC (g/L)	Cattle	323.0 ± 14.9^{ab}	314.0 ± 27^{b}	328.5 ± 24.44^{ab}
	Buffaloes	313.0 ± 11.5^{b}	323.6 ± 34.7^{ab}	345.6 ± 36.1^{a}
PLT ($\times10^{6}$/L)	Cattle	436.20±46.43^{ab}	466.80±88.67^{ab}	395.85±46.53^{ab}
	Buffaloes	318.90 ± 34.56^{b}	403.64±30.30^{ab}	523.56 ± 49.52^{a}
WBC ($\times10^{9}$/L)	Cattle	8.39 ± 0.88^{a}	11.50 ± 1.10^{a}	10.94± 1.05^{aa}
	Buffaloes	6.43 ± 0.47^{b}	9.56 ± 0.65^{ab}	10.74 ± 1.34
Neutrophils (%)	Cattle	53.60 ± 1.87^{b}	52.20 ± 4.98^{b}	50.08 ± 3.15^{bc}
	Buffaloes	52.20 ± 2.0^{b}	60.82 ± 2.34^{a}	58.33 ± 1.86^{b}
Lymphocytes (%)	Cattle	36.60 ± 1.87^{a}	38.80 ± 5.10	42.15 ± 2.86^{b}
	Buffaloes	40.00 ± 2.19^{b}	30.55 ± 2.22^{b}	34.56 ± 1.85^{a}
Monocytes (%)	Cattle	6.80 ± 0.42	6.00 ± 1.22^{b}	4.85 ± 0.46
	Buffaloes	4.80 ± 0.49	4.45 ± 0.34	4.11 ± 0.54
Eosinophils (%)	Cattle	3.00 ± 0.37	3.00 ± 0.84	2.92 ± 0.42
	Buffaloes	3.00 ± 0.56	3.18 ± 0.50	3.11 ± 0.39

buffaloes (5.06 and 4.46, respectively) compared to control one (1.64).

Serum PTH was significant decreased ($P<0.05$) in mild-moderate and severe groups of diseased buffaloes and cattle. Erythrocytic G6PD showed significant decrease ($P<0.05$) in mild-moderate and severe cases of buffaloes while in diseased cattle, it showed lower activity in mild-moderate and severe PPH. Screening data of GSH-Px (mU/mL) level in the RBC lysate in mild-moderate and severe cases revealed significant decrease ($P<0.05$) in PPH-affected buffaloes and cattle. Blood MDA concentrations were significantly higher ($P<0.05$) in mild-moderately and severely diseased buffaloes and cattle. Nitric oxide level (μmol/L) was slightly increased in diseased buffaloes. Detailed biochemical data are presented in Table 5.

Osmotic fragility is an important indicator of the stability and integrity of erythrocytes. The current study demonstrated a marked increased osmotic fragility of RBCs in diseased animals compared to healthy ones. The mean osmotic fragility index (% of saline solution) of erythrocytes in mild to moderate and severe cases of PPH-affected cattle was (0.66±0.02 and 0.71±0.02, respectively) compared to

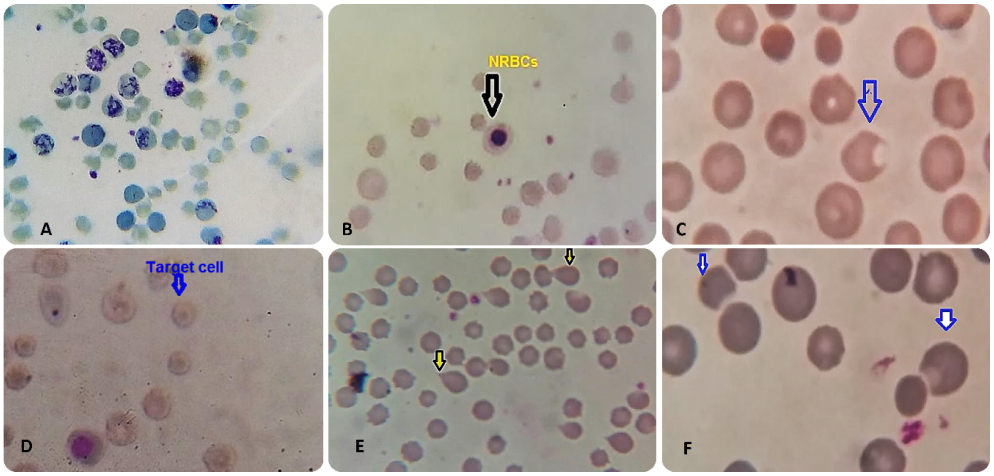
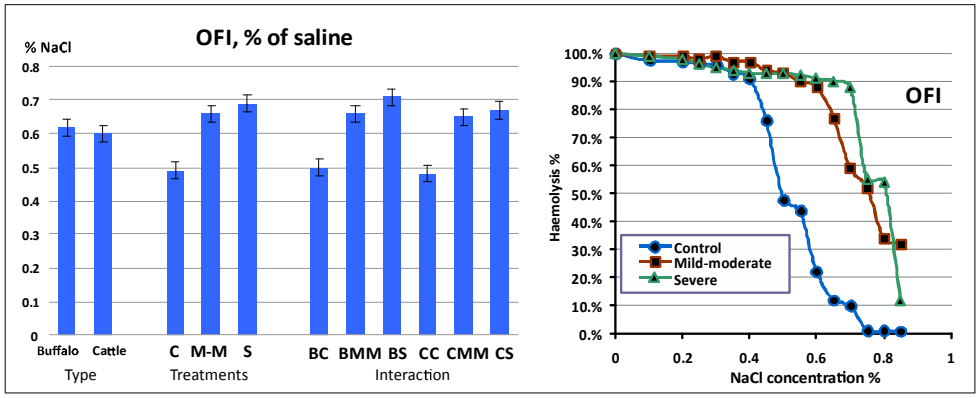


Fig. 2. Blood film showing reticulocytosis (**A**) and nucleated red blood cell and macrocytosis (**B**) in response to severe intravascular hemolysis in PPH. Characteristic "blister" RBCs (large arrow) (**C**), hypochromacia and target RBC (**D**), tear drop (**E**), bite cell (**F**) are noted in in a PPH buffalo with G6PD deficiency.

Table 5. Biochemical and hormonal indices (mean value ± SD) in healthy and diseased cattle and buffaloes.

Parameter	Animal	Healthy controls	Mild to moderate PPH (PCV > 20%)	Severe PPH (PCV < 20%)
Serum Pi (mmol/L)	Cattle	2.47 ± 0.29^{a}	0.37 ± 0.09^{b}	0.47 ± 0.11^{b}
	Buffaloes	2.05 ± 0.16^{a}	0.59 ± 0.14^{b}	0.62 ± 0.16^{b}
Serum Ca (mmol/L)	Cattle	2.20 ± 0.05^{b}	2.29 ± 0.12^{ab}	2.17 ± 0.14^{b}
	Buffaloes	2.61 ± 0.61^{a}	2.33 ± 0.09^{ab}	2.13 ± 0.12^{b}
Ca:P ratio	Cattle	1.13	8.05	6.00
	Buffaloes	1.64	5.06	4.46
PTH (pg/mL)	Cattle	121.90 ± 24.16^{a}	20.08 ± 6.05^{b}	37.85 ± 7.32^{b}
	Buffaloes	51.10 ± 7.41^{a}	36.36 ± 7.48^{b}	23.53 ± 6.22^{b}
G6PD (U/g Hb)	Cattle	9.81 ± 1.02^{b}	6.90 ± 1.79^{b}	6.15 ± 0.83^{b}
	Buffaloes	17.45 ± 1.67^{a}	7.07 ± 1.01^{b}	7.58 ± 1.04^{b}
GSHPX (mU/mL)	Cattle	442.37 ± 13.91^{a}	241.20 ± 31.55^{b}	181.45 ± 15.23^{c}
	Buffaloes	400.22 ± 21.49^{a}	285.50 ± 25.96^{b}	188.14 ± 28.08^{c}
MDA (nmol/mL)	Cattle	5.99 ± 1.26^{b}	10.54 ± 1.95^{a}	13.46 ± 2.29^{a}
	Buffaloes	7.26 ± 1.35^{b}	10.55 ± 1.60^{a}	13.12 ± 1.72^{a}
NO (µmol/L)	Cattle	24.69 ± 8.73^{a}	3.48 ± 1.03^{b}	$4.30 \pm 0.76b^{b}$
	Buffaloes	5.13 ± 1.51^{b}	6.21 ± 1.18^{b}	7.78 ± 1.39^{b}

healthy ones (0.50±0.02%). On the other hand, the mean OFI of erythrocytes in PPH-affected buffaloes was 0.65±0.02 and 0.67±0.02 in mild-moderate and severe cases, respectively compared to 0.48±0.02 in healthy ones (Fig. 3).



A **B**

Fig. 3. A. Osmotic fragility Index (mean± SD % of saline) in healthy and diseased animals; **B.** Plots demonstrating osmotic fragility index (OFI) in healthy and diseased animals. C (control), M-M (mild-moderate), S (severe), BC (buffalo control) BMM (buffalo mild-moderate), BS (buffalo severe), CC (cow control), CMM (cow mild-moderate), CS (cow severe).

Correlation study of osmotic fragility of RBCs revealed strong negative correlation between osmotic fragility and blood phosphorus level (r = –0.70), (Fig. 4), GSH-Px (r = –0.32) and G6PD (r = –0.50) (Fig. 5) while it showed strong positive correlation (r = 0.51) with MDA level (Fig. 6). There was no significant correlation between osmotic fragility and blood level of calcium and nitric oxide (Fig. 4 and 6, respectively).

DISCUSSION

Postparturient haemoglobinuria (PPH) represents a great threat to dairy cows and buffaloes during advanced pregnancy and early lactation in Egypt and worldwide and causes significant economic losses (Albayati *et al.,* 2020).

Hypophosphataemia and abnormalities in erythrocyte G6PD, and blood oxidant/ antioxidants levels have been implicated to PPH. However, their association and contribution to osmotic fragility of RBCs and development of PPH need to be explored.

Clinical observations of diseased animals revealed typical case of PPH. All examined cases showed haemoglobinuria (Fig. 2) and various degree of systemic disturbabces accroding to severity of PPH. Tachycardia, systolic murumer and inspiratory dyspnoea observed could be attributed to cardiovascular compensatory mechanisms secondary to anaemia. Excessive formation of hemosiderin and its deposition in the gastrointestinal mucosae in haemoglobinuria affected buffaloes could be responsible for gastrointestinal disturbances like ruminal stasis, constipation, straining and dark coloration of the faeces (Sharma *et al.,* 2014; Albayati *et al.,* 2020). Deaths in severe cases were attributed to anemic anoxia. Similar findings were reported by Tewari *et al.* (2014); Shalini *et al.* (2015); Jadhav *et al.* (2016); Shaikh *et al.* (2016), Deeba *et al.* (2019) & Albayati *et al.* (2020).

The present study revealed significant reduction (P<0.05) in RBCs count, Hb

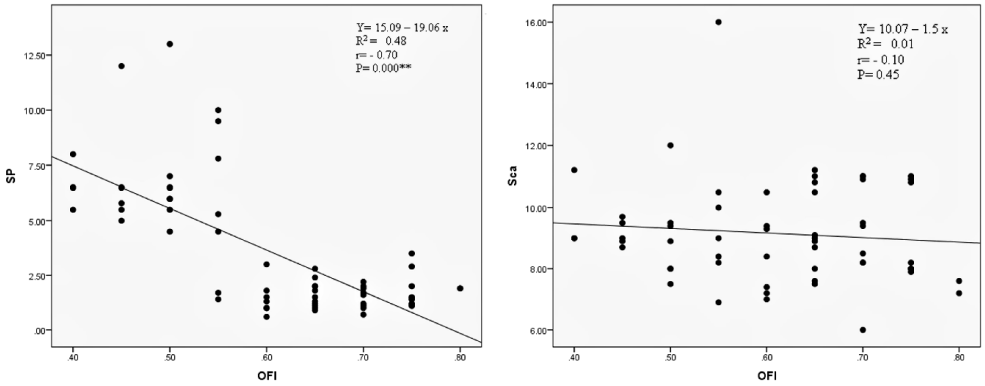


Fig. 4. Pearson's correlation (r) and linear regression analysis (R^2) analysis of OFI, blood P and Ca levels.

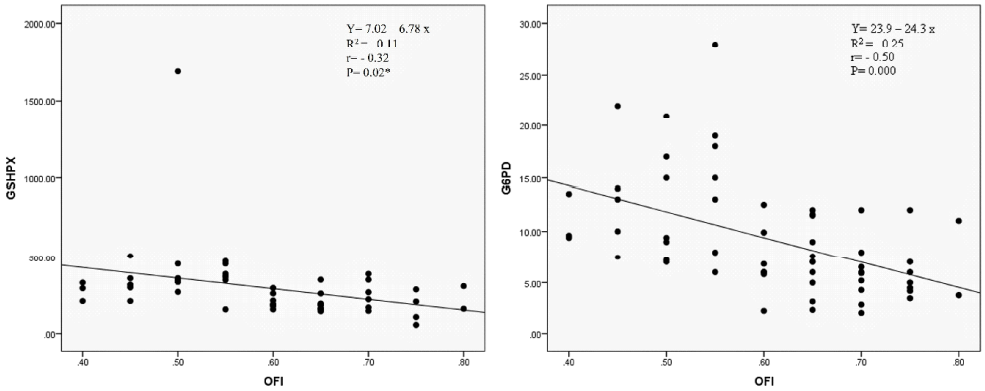


Fig. 5. Pearson's correlation (r) and linear regression analysis (R^2) analysis of OFI, blood GSH-Px and G6PD level.

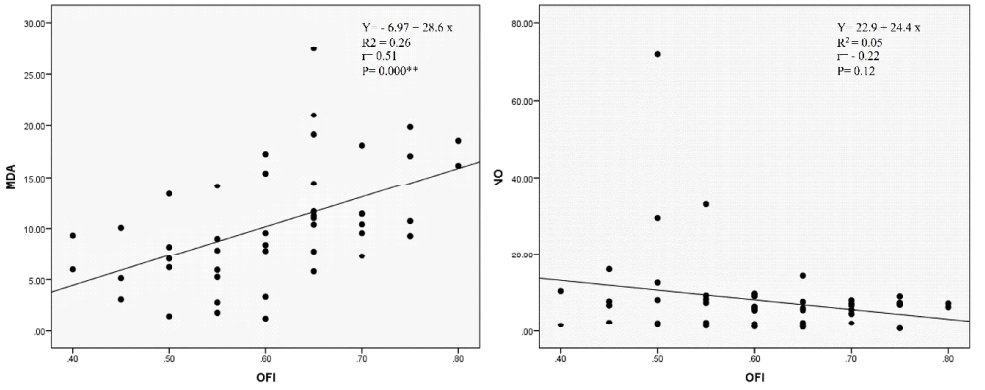


Fig. 6. Pearson's correlation (r) and linear regression analysis (R^2) analysis of OFI, blood MDA and NO level.

concentration, and PCV in PPH. Similar findings were obtained by Kumar *et al.,* (2019). Macrocytic hypochromic anaemia (regenerative type) was evident based on the obtained data of MCV and WBCs count. In addition to cytomorphological changes in RBCs that include reticulocytosis, presence of nucleated RBCs in peripheral blood and other abnormalities are characteristic of this type of anaemia (Mahmood *et al.,* 2013, Reddy *et al.,* 2014; Johri *et al.,* 2016, Zhang *et al.,* 2017; Albayati *et al.,* 2020). This could be attributed to intravascular haemolysis that is accompanied by increased bone marrow activity with the release of immature erythrocytes (erythroid regenerative response) and younger form of leukocytes (Coles, 1986). Although CBC revealed non-significant changes in MCH and MCHC in PPH, it was clearly shown hypochromacia in blood film (Fig. 2) of diseased animals. Similar findings were recorded in previous studies (Mahmood *et al.,* 2013; Albayati *et al.,* 2020). The authors inferred that in cases of haemolytic anaemia, MCH should not be considered an ideal measure.

The current study showed significant decrease ($P<0.05$) in the blood phosphorus level and alteration in blood Ca:P ratio in PPH-affected animals. Previous studies showed similar findings in both buffaloes (Samad *et al.,* 1997) and cattle (Stockdale *et al.,* 2005). This finding may be attributed to several factors including improper feeding management during pregnancy and lactation. Diet-rich in calcium but much lower in phosphorus content such as *Trifolium alexandrium* (Barseem), induced a serious imbalance of Ca:P ratio and decreases phosphorous level by interfering with its intestinal absorption (Akhtar *et al.,* 2007). Additionally, heavy drainage of phosphorus through milk particularly in high milk yielding animals, leads to hypophosphataemia (*Kumar et al.,* 2019). Phosphorus-deficient soils are common in dry tropical countries and contribute to hypophosphataemia.

The present study showed significant decrease ($P<0.05$) in the serum PTH in both diseased buffaloes and cattle. In advanced gestation, more phosphorus and calcium are required for the developing foetus. If supplementary phosphorus is not provided, thereby leading to hypophosphataemia. Moreover, high Ca:.P ratio diet during dry period may result in inactivity of parathyroid gland and decreases phosphorus absorption from the intestinal tract and ultimately leads to hypophosphataemia (Rahawy *et al.,* 2012).

Obtained result of erythrocytic G6PD showed significant decrease ($P<0.05$) in affected buffaloes. G6PD is an important metabolic enzyme. Among the two major pathways of glucose metabolism in RBCs, 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. Glutathione maintained in a reduced state protects RBCs from oxidative stress; thus, a deficiency of G6DP will result in oxidative stress and haemolytic anaemia (Luzzatto *et al.,* 2020). It worthwhile to mention that the study demonstrated a strong positive correlation between erythrocytic G6PD and blood phosphorus level. On the other hand, both blood phosphorus and G6PD showed a strong negative correlation with osmotic fragility suggesting the important role of G6PD in maintaining integrity of RBCs membranes.

Screening data of erythrocytic GSH-Px level revealed significant decrease

($P<0.05$) in affected animals. These results might be due to reduction in the process of glutathione generation secondary to suboptimal level of G6PD activity and increased production of reactive oxygen metabolites during early lactation (Sharma *et al.,* 2011; Deeba *et al.,* 2019). Correlation study revealed negative association between GSH-Px and OFI suggesting that low level of GSH-Px causes oxidative damage to erythrocytes with subsequent haemolytic syndrome (Deeba *et al.,* 2019).

Obtained data of blood level of MDA showed significant increase ($P<0.05$) in PPH indicating an enhanced lipid peroxidation in RBCs membrane. MDA is one of the well-known secondary products of lipid peroxidation after exposure to reactive oxygen species. It is important to pinpoint that there was strong positive correlation between blood MDA level and OFI suggesting the drastic effect of oxidative stress on the integrity of RBCs membranes. Increased level of MDA may be partially caused by suboptimal level of glutathione and stress of transition period. Nitric oxide is also a biomarker of lipid peroxidation. Although our data showed a slight increase in NO level in diseased buffaloes, diseased cattle showed unpredictable decrease in serum NO level.

Osmotic fragility index is an important indicator of the stability and integrity of erythrocytes. The recorded data in the current study demonstrated a marked increase in osmotic fragility of RBCs in PPH that leads to intravascular haemolysis with subsequent haemoglobinaemia, haemoglobinuria, and haemolytic anaemia. This was associated with low level of blood phosphorus, erythrocytic G6PD and GSH-Px. Hypophosphataemia results in decreased RBCs glycolysis, ATP synthesis and generation process of NADPH and glutathione. This defective metabolic pathway predisposes RBCs to alter their structure and function with subsequent loss of normal cell membranes integrity. This finding was in an agreement with Zhang *et al.,* (2017). Correlation data in the present study proved this concept. A strong association between osmotic fragility of RBCs and blood level of phosphorus, G6PD, MDA, and to some extent of GSH-Px was observed, suggesting their important role in the development of PPH. The severity of PPH has been strongly linked with blood level of phosphorus, G6PD and MDA and their effect on osmotic fragility. To our knowledge, the study demonstrated clearly metabolic pathway and the role of blood phosphorus and other related biomarkers and their association in the etiopathogenesis of PPH.

In conclusion, the study validated the hypothesis that phosphorus deficiency affects blood level of G6PD and alters generation process of GSH-Px predisposing RBCs to oxidative damage with subsequent intravascular haemolysis in PPH.

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Paper received 02.08.2023; accepted for publication 03.10.2023

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