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Changes in haematology, blood mineral profile, ultra structure and superoxide dismutase activities in erythrocytes in hypophosphetemic buffaloes given excess molybdenum

A K SHARMA[1], S N S RANDHAWA[2], S K UPPAL[3] and R RANJAN[4]

Guru Angad Veterinary and Animal Sciences University, Ludhiana, Punjab 141 004 India

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Phosphorus (P) deficiency, a common problem in dairy animals, is characterized by loss of muscle mass, allotriophagia, weakness, anemia, haemoglobinuria and bending of legs (Radostits *et al.* 2000). Low level of P in diet or secondary to high molybdenum concentration in diet result into poor bioavailability of dietary P (Hansen *et al.* 2008). High molybdenum concentration in soil and fodder occurs naturally in many parts of the Punjab state (Hundal *et al.* 2006).

Hypophosphataemia ultimately leads to altered functions and structure of erythrocytes in cattle (Ogawa *et al.* 1989). Lactating buffaloes are highly susceptible to deficiency of trace minerals and phosphorus (Akhtar *et al.* 2012). However, changes in ultra structure of erythrocytes, blood mineral profile and oxidative stress indices during phosphorus deficiency and molybdenum toxicity in buffaloes have not been investigated so far. The present study was designed to study the effects of phosphorus deficiency and molybdenum toxicity on erythrocytes in buffaloes.

Male buffalo yearlings (20), with average body weight of 89 kg, were dewormed, acclimatized for 20 days and randomly divided into T_1, T_2 and T_3 groups. Group T_1 (5 animals) served as healthy control; group T_2 (10 animals) used for induction of hypophosphatemia and molybdenosis and group T_3 (5 animals) for induction of hypophosphatemia alone. Animals of group T_1 were given green fodder and wheat straw *ad lib.,* along with balanced mineral mixture @ 15g/100kg body weight. Phosphorus (P) deficiency was induced in T_2 and T_3 by feeding phosphorus deficient diet for 90 days. The diet consisted of daily *ad lib.* feeding of urea treated wheat straw supplemented with P and Cu deficient mineral mixture @ 15 g/100 kg body weight and molasses @ 50 g/100 kg body weight. The P and Cu free mineral mixture was prepared by substituting Cu and P with starch (composition: lime stone powder, 59.50 kg, sodium chloride, 23 kg, magnesium oxide, 8.3 kg, zinc oxide, 400 g, manganese sulphate, 310 g, cobalt chloride, 40 g, potassium iodide, 30 g, ferrous sulphate, 250 g, starch, 8.17 kg, vitamin A and D 150 IU/kg). The calves of group T_2 were also given 10 % ammonium molybdate solution orally once daily so as to provide molybdenum @ 3 mg/kg body weight.

To establish control values (zero day), blood sample was collected from each animal on 2 occasions at 2 days interval before starting the experiment. During experimentation blood was collected by jugular venipuncture at 15 days interval for 120 days. Disodium EDTA was used as anticoagulant for blood collected for measurement of hematological parameters. Blood samples (5 ml) were also collected in acid free heparinized vials, plasma was harvested and used for mineral estimation, while packed RBCs were used for measurement of superoxide dismutase activity.

The haemoglobin (Hb) was estimated as per Raymond and Wilkinson (1963), total erythrocyte count (TEC) by haemocytometer and the packed cell volume (PCV) by microhaematocrit method (Benjamin 1985). The activity of erythrocytic superoxide dismutase was estimated as per Marklund and Marklund (1974). Inorganic phosphorus in plasma was estimated using kits (Gomorri 1942). Copper and iron were estimated by atomic absorption spectrophotometer.

Scanning electron microscopy (SEM) of erythrocytes of 4 healthy animals and 2 animals each from T_2 and T_3 was done at zero day and on 80–90th day of experiment by taking 1 or 2 drops of free flowing blood from jugular venipuncture in glass vials containing 2 % gluteraldehyde in 0.lM phosphate buffer (pH 7.2) at room temperature. The blood drops were slowly mixed with gluteraldehyde for fixing, centrifuged at 500 rpm for 2 min and excess of gluteraldehyde was removed by washing once with phosphate buffer (pH 7.2), and twice with double distilled water. The stubs were prepared and silver film was fixed on them. Suspended erythrocytes in distilled water were

Present address: [1]Department of Veterinary Medicine (ashgadvasu@yahoo.com), [2]Director of Research (sarnarinder @gmail.com), [3]Professor (sk.uppal@yahoo.co.in), Department of Veterinary Medicine, [4]Assistant Professor (rakesh_ranjan3 @rediffmail.com), Department of TVCC.

put on stubs with the help of micropipette and allowed to settle down and dry. These were sputtered with gold using fine coat ion sputter. The cells were then studied under desired magnification in digital scanning electron microscope.

Values of a given parameter obtained on different day of observation within a group were compared with day 0 value using t- test (Snedecor and Cocharan 1994).

All the animals before starting experiment were clinically healthy, alert, active and having fair body condition. The mean rectal temperature, respiration and heart rate of the animals of different groups were within the normal range. Average body weights of group T_2 and T_3 at the start of the experiment were 95 and 89 kg and at the peak of clinical signs (after about 3 months) were 96.79 and 92.83 kg, respectively. Animals of group T_2 and T_3 showed general symptoms of rough hair coat, alopecia with discoloration of skin, followed by slight depigmentation. Reddening of hair (achromatrichia) was observed in all the animals of these groups. The other clinical signs observed included loss of muscle mass, weakness, lameness, sunken eyes, recumbancy and inability to stand. No mutual plucking of hairs or pica was observed at any stage. The clinical signs of weakness, lameness and achromotrichia were more marked in group T_2 as compared to group T_3. The high intensity of clinical symptoms observed in group T_2 could be due to the simultaneous potentiating effect of excess Mo in addition to P deficiency (Underwood 1981). The increased intensity of clinical symptoms in groups T_2 and T_3 were evident after 60th day. Similarly, the sternal recumbency in some animals exhibited by prolonged sitting and difficulty in getting up was noticed after 70th day in T_2 and T_3 animals. It lasted for about a week with inability to stand, followed by sudden falling when tried to walk or made to stand with physical support during this period. The animals continued to have moderate appetite during this period. The deterioration of condition in 2 animals resulted in lateral recumbancy terminating in death. At terminal stages animals were dull, showing apathy and increased heart rate.

The general clinical symptoms of poor growth, progressive emaciation, loss of muscle mass, weakness, lameness with staggering gait and anorexia observed in phosphorus deprived buffalo calves were similar to those described by Underwood (1981) and Radostits *et al.* (2000) in different animal species. Ozkan *et al.* (2012) also observed weight loss, weakness, unwillingness to walk, lameness and extremity fracture in young turkeys suffering from phosphorus deficiency. The presence of alopecia could be due to deficient protein status of the body associated with anorexia. Discoloration of skin and depigmentation of hair (achromatrichia) could be due to excess Mo induced depletion of Cu because Cu is a prosthetic group of enzyme tyrosinase responsible for hydroxylation of tyrosine to form DOPA (dihydroxy phenyl alanine) in the melanocytes (Randhawa *et al.* 2009). Copper concentration in forages usually remains suboptimal, hence it must be supplemented in high yielding lactating animals (Khan *et al.* 2011).

Hb, PCV and TEC decreased consistently in P deficient and molybdenotic calves after 60 days (Table 1). Cu

Table 1. Haematological indices of experimental buffalo calves

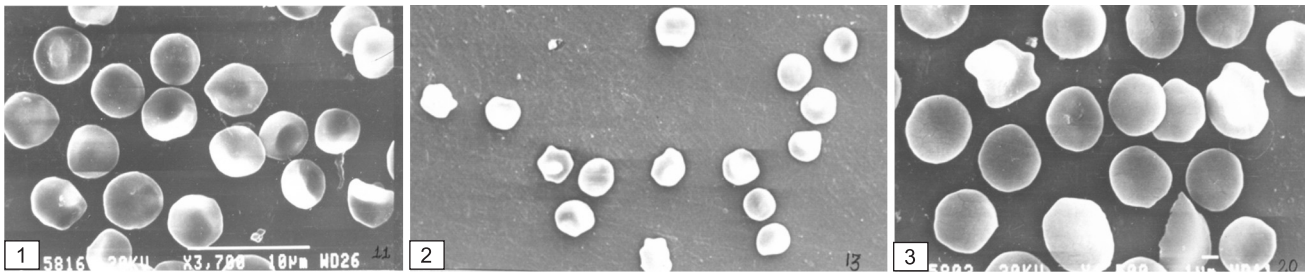
Parameters	Group	Day of observation						
		0	15	30	45	60	75	90
Hb (g/d1)	T_1	10.38±0.23	9.49±0.27	9.59±0.25	9.79±0.29	10.65±0.47	11.47±0.39	10.21±0.33
	T_2	10.30±0.21	9.19**±0.35	8.58**±0.21	8.53**±0.26	9.43**±0.28	11.83**±0.49	9.87**±0.11
	T_3	10.46±0.29	9.37*±0.24	8.98**±0.15	10.09**±0.22	9.27**±0.31	10.22**±0.28	9.57**±0.31
Haematocrit (%)	T_1	34.26±0.71	31.32±0.84	31.67±0.77	32.40±0.91	35.20±1.21	37.60±1.21	33.42±0.91
	T_2	34.20±0.27	30.33±1.03	28.32**±0.68	28.32**±0.83	21.25**±0.85	39.37**±1.51	32.50**±0.01
	T_3	34.33±0.81	30.60±0.69	31.57±0.39	33.32±0.70	30.41**1.09	33.75**0.87	31.66**±0.87
TEC ($\times 10^6$/mm^3)	T_1	5.71±0.36	5.22±0.41	5.29±0.77	5.39±0.91	5.87±1.21	6.25±0.62	5.57±0.47
	T_2	5.66±0.27	5.03±0.52	4.72±0.41	4.73±0.41	5.19±0.83	6.56**±0.75	5.39**±0.81
	T_3	5.75±0.61	5.12±0.35	5.53±0.37	5.53±0.33	5.06±0.54	5.62**±0.41	5.31**±0.43

** Values differ from day zero value at $P < 0.01$; * values differ from day zero value at < 0.05; T_1, control; T_2, hypophosphataemic animals given excess molybdenum; T_3, hypophosphataemic animals.

Table 2. Alterations in erythrocytic superoxide dismutase (ESOD) activities

Parameters	Group	Day of observation						
		0	15	30	45	60	75	90
ESOD (U/mg Hb)	T_1	0.59±0.01	0.61±0.01	0.59±0.02	0.62±0.01	0.62±0.01	0.62±0.02	0.61±0.03
	T_2	0.58±0.02	0.55**±0.01	0.59±0.01	0.49**±0.03	0.47**±0.01	0.50**±0.02	0.51**±0.01
	T_3	0.60±0.02	0.60±0.02	0.62**±0.02	0.55**±0.01	0.57**±0.01	0.58**±0.02	0.61±0.02

** Value differ from day zero value at $P < 0.01$; * value differ from day zero value at $P < 0.05$; T_1, control; T_2, hypophosphataemic animals given excess molybdenum; T_3, hypophosphataemic animals.



Figs 1–3. **1.** SEM of erythrocytes of healthy buffalo calves showing poikilocytosis. × 22,200. **2.** Sphero-echioncytosis in hypophosphataemic buffalo calves. × 12,000. **3.** SEM indicating discocytes, spherocytes and acanthocytes in hypophosphataemic and molybdenotic anaimals. × 27,000.

deficiency causes anemia due to impaired iron absorption, release from stores and utilization in hemoglobin synthesis. Cu is involved at functional levels in the formation of porphyrin molecules (Guyton 1991) and regulates cytochrome oxidase, which is required for reduction of iron to form hemoglobin (Radostitis *et al.* 2000). SEM of erythrocytes in healthy animals revealed typical biconcave appearance and extensive poikilocytosis. A few uniconcave cells close to discocytes were also seen (Fig. 1). The cell surface of majority of erythrocytes appeared smooth. SEM of erythrocytes of hypophosphataemic animals revealed variable changes (Figs 2 and 3). The erythrocyte membrane slightly degenerated with corrugations on surface layer of the erythrocyte. Erythrocytes exhibited spherocytosis and discocytosis. Most of these erythrocytes had irregular margins and revealed various shapes like disco-echinocyte, spheroechinocytes and sphero-acanthocytes. Large unidentified bodies were present in many erythrocytes. Erythrocytes of group T_2 and T_3 revealed no significant differences. The different size of erythrocytes in buffalo calves with majority showing biconcave appearance is in accordance with Bhardwaj and Rana (1988). The reduced ATP production, inability to maintain the shape and rigidity of the cell might result in discocytosis or spherocytosis (Guyton 1991). The poikilocytosis observed in buffalo calves was similar to that reported in goat erythrocytes by Schalm *et al.* (1975). They observed spindle, rod or spherical shapes of erythrocytes in healthy goats and acanthocytosis in healthy cows. Normally RBCs have an excess of cell membrane for the quantity of material inside, deformation does not stretch the membrane greatly and consequently does not rupture the cell, as would be the case with many other cells. With decrease of total phospholipids the excess membrane might have decreased and RBCs changed to spherocytes or discocytes from the normal biconcave appearance and their stretchability or deformability was reduced. During circulation while passing through capillaries or reticuloendothelial network of spleen these cells might get lysed. It was reported that hypophosphatemia results in decrease erythrocyte glycolysis and ATP synthesis and the subnormal concentration of ATP predisposes erythrocytes to altered functions and structure. An increase in fragility may lead to hemolysis, hemoglobinemia and hemoglobinuria. Copper deficiency is also one of the etiological factors of hemoglobinuria as its deficiency reduces the activity of Cu-containing enzyme superoxide dismutase, which is a part of erythrocyte protective mechanism against oxidative stress.

There is a growing evidence of excess production of free radicals beyond the endogenous counteracting mechanism in various infectious, inflammatory and deficiency diseases in buffaloes (Dimri *et al.* 2008). The erythrocytes membrane is rich in polyunsaturated fatty acid and, thus, prone to oxidative insult by pro-oxidants (Clemens and Waller 1987). Decrease in SOD activity in phosphorus deficient animals

Table 3. Mineral concentrations in plasma of experimental buffalo calves

Parameters	Group	Day of observation						
		0	15	30	45	60	75	90
Phosphorus	T_1	7.03±1.06	7.77±1.21	6.23±0.81	6.03±0.94	6.54±1.19	6.97±0.81	6.72±0.77
(mg/dl)	T_2	6.50±1.06	6.03±0.83	5.81±1.18	4.73±1.27**	4.03±0.49**	3.03±0.63**	2.60±0.47**
	T_3	6.15±1.13	5.95±1.17	4.78±1.27	3.41±0.78	2.95±0.63**	3.50±0.49**	2.72±0.49**
Copper	T_1	111.57±15.38	105.2±15.01	109.31±7.34	127.26±6.42	127.90±12.84	116.31±6.56	113.14±8.53
(µg/dl)	T_2	168.78±40.70	166.60±26.25	142.15±107.70	106.60±10.00**	109.00±10.38**	93.25±10.01**	96.72±8.20**
	T_3	115.48±15.38	111.03±7.67	106.60±0.00	119.90±13.32	118.00±60.10	114.34±20.52	110.47±20.22
Iron (µg/dl)	T_1	417.16±20.30	338.85±18.36	352.54±8.51	413.24±21.62	542.10±40.68	606.50±18.80	616.00±17.73
	T_2	377.68±104.04	302.14±67.04	533.22±21.52	423.22±17.48	564.30±18.70	624.30±16.58	619.17±14.23
	T_3	357.72±62.34	319.92±51.04	513.30±18.94	406.54±34.62	595.42±10.77	612.55±13.92	618.30±16.53

**Value differ from day zero value at $P < 0.01$; * value differ from day zero value at $P < 0.05$; T_1, Control; T_2, hypophosphataemic animals given excess molybdenum; T_3, hypophosphataemic animals.

might be responsible for enhanced oxidative insult to RBCs. Excess molybdenum further appears to increase the oxidative damage to RBCs. Superoxide dismutase (SOD) is a primary antioxidant enzymes present in mammalian cells. It catalyses the formation of O_2 from reactive oxygen species. Mean erythrocytic SOD values decreased significantly after 45 days in group T_2 and T_3 as compared to healthy control. Decrease in SOD (a copper dependent enzyme) in RBCs could be due to decreased plasma Cu level (Xin *et al.* 1991). However, the variation in mean values of lipid peroxidation in healthy control as well as T_2 and T_3 groups at different time interval was observed, which could not be explained.

From our results it can be concluded that phosphorus deficiency induces marked abnormalities in erythrocytes in buffaloes and excess molybdenum supplementation further aggravates the condition. Increased oxidative damage due to reduced antioxidant enzyme level may contribute changes in erythrocytes in phosphorus deficiency and molybdenum toxicity.

SUMMARY

Phosphorus deficiency in buffaloes resulted into development of macrocytic, normochromic anaemia with significant decline in haemoglobin, packed cell volume and total erythrocyte count. Erythrocytic superoxide dismutase activity was markedly decreased in hypophosphataemic animals given excess molybdenum (T_2) in comparison to hypophosphataemic animals not given molybdenum (T_3). Scanning electron microscopy of erythrocytes in both T_2 and T_3 animals showed presence of spherocytes, discocytes and slight membrane corrugations; while erythrocytes in healthy control (T_1) appeared biconcave with mild poikilocytosis. From the present study it can be concluded that phosphorus deficiency induces ultrastructural abnormalities in erythrocyte, which is further aggravated by excess molybdenum supplementation. Increased oxidative damage may contribute structural changes in phosphorus deficiency and molybdenum toxicity.

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