STUDIES ON EXPERIMENTALLY INDUCED HYPOPHOSPHATAEMIA AND ITS INTERACTION WITH MOLYBDENOSIS IN BUFFALO CALVES

Thesis

Submitted to the Punjab Agricultural University
in partial fulfilment of the requirements
for the degree of

MASTER OF VETERINARY SCIENCE

in

VETERINARY MEDICINE

(Minor : Veterinary Physiology)

by

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1996

DEDICATED

TO

MY REVERED PARENTS

CERTIFICATE-I

This is to certify that the thesis entitled, **"Studies on experimentally induced hypophosphataemia and its interaction with molybdenosis in buffalo calves"** submitted for the degree of Master of Veterinary Science in the subject of Veterinary Medicine (Minor subject : Veterinary Physiology) of Punjab Agricultural University, is a bonafied research work carried out by **Ashwani Kumar** (L-93-V-278-M) under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE-II

This is to certify that the thesis entitled, **"Studies on experimentally induced hypophosphataemia and its interaction with molybdenosis in buffalo calves"** submitted by **Ashwani Kumar** (L-93-V-278-M) to the Punjab Agricultural University, Ludhiana in the partial fulfilment of the requirements for the degree of Master of Veterinary Science in the subject of Veterinary Medicine (Minor subject : Veterinary Physiology) has been approved by the student's Advisory Committee after an oral examination on the same in collaboration with an External Examiner.

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Ashwani Kumar
(ASHWANI KUMAR)

ABBREVIATIONS USED

@	at the rate of
Al	Aluminium
Ca	Calcium
Cu	Copper
CSF	Cerebro-spinal fluid
dl	decilitre
ESOD	Erythrocyte Superoxide Dismutase
Fe	Iron
FFD	Film focal distance
g	gram
h	hour
Hb	Haemoglobin
H&E	Haematoxylin and Eosin
Hg	Mercury
ISI	Indian Standard Institute
IU	International Units
Kg	Kilogram
KVp	Kilovoltage peak
mAs	Milliampere seconds
mg	Milligram
ml	Millilitre
mm^3	Cubic millimeter
Mn	Manganese
Mo	Molybdenum
MDA	Malondialdehyde
mm	millimeter
Na	Sodium

nmol	Nanomole
P	Phosphorus
ppm	Parts per million
r.p.m.	revolutions per minute
S	Sulphur
SEM	Scanning Electron Microscopy
S.E.	Standard Error
SOD	Superoxide dismutase
TEC	Total erythrocyte count
U	Units
Wt	weight
Zn	Zinc
%	Per cent
u	Micron
ug	Microgram
umol	Micromole

CONTENTS

CHAPTER	TITLE	PAGES
I	INTRODUCTION	1-4
II	REVIEW OF LITERATURE	5-28
III	MATERIALS AND METHODS	29-36
IV	RESULTS	37-55
V	DISCUSSION	56-76
VI	SUMMARY AND CONCLUSIONS	77-83
VII	BIBLIOGRAPHY	i-xiii

LIST OF TABLES

Table No.	Title	Page
1	Haematological indices of experimental buffalo calves	39
2	Alterations in erythrocytic enzymes of experimental buffalo calves	41
3	Minerals concentration in plasma of experimental buffalo calves	44
4	Minerals concentration in rumen liquor of experimental buffalo calves	46
5	Minerals concentration in CSF of experimental buffalo calves	48
6.	Haematological and biochemical alterations in experimental therapeutic trials in blood of buffalo calves	52
7.	Alterations in mineral constitutients of plasma in therapeutic trials of experimental buffalo calves	53
8.	Minerals alterations in rumen liquor and CSF in experimental therapeutic trials in buffalo calves	55

LIST OF FIGURES

Fig.1. Alopecia and discolouration of skin in experimental buffalo calf following induction of hypophosphataemia.

Fig.2. Alopecia along with reddening of hair (achromotrichia) following induction of hypophosphataemia and molybdenosis in experimental buffalo calf.

Fig.3. Lameness in hock joint and loss of musle mass of hind quarter following induction of hypophosphataemia in experimental buffalo calf.

Fig.4. Sunken eyes, cachexia, hide bound condition and weakness with inability to stand in hypophosphataemic buffalo calf.

Fig.5. Lateral recumbency following severe phosphorus depletion in experimental buffalo calf.

Fig.6. Comparative values of haemoglobin in three groups of experimental buffalo calves.

Fig.7. Comparative values of PCV in three groups of experimental buffalo calves.

Fig.8. Comparative values of TEC in three groups of experimental buffalo calves.

Fig.9. Alterations in ESOD at different time interval in three groups of experimental buffalo calves.

Fig.10. Alterations in °MDA values at different time interval in three groups of experimental buffalo calves.

Fig.11. SEM of erythrocuytes of healthy buffalo calves showing poikilocytosis x 22,200.

Fig.12. SEM of a typical biconcave erythrocyte from a healthy buffalo calf x 78,000.

Fig.13. Sphero-echinocytosis in hypophosphataemic buffalo calves x 12,000.

Fig.14. Discocyte with slight corrugation of the erythrocytic membrane in hypophosphataemic buffalo calves x 72,000.

Fig.15. SEM of a typical acanthocyte from hypophosphataemic animal x 72,000.

Fig.16. SEM of erythrocyte from hypophosphataemic buffalo calf depicting membrane alterations x 72,000.

Fig.17. SEM depicting echinocytes and discocytes with a few normal erythrocytes from hypophosphataemic and molybdenotic buffalo calves x 12,000.

Fig.18. SEM showing discocytes and acanthocytes in hypophosphataemic and molybdenotic buffalo calves x 11,400.

Fig.19. SEM of discocytes and echinocytes from hypophosphataemic animals x 21,000.

Fig.20. SEM indicating discocytes, spherocytes and acanthocytes in hypophosphataemic and molybdenotic animals x 27,000.

Fig.21. Alterations in the concentration of plasma inoprganic phosphorus in three groups of experimental buffalo calves.

Fig.22. Alterations in the concentration of plasma copper in three groups of experimental buffalo calves.

Fig.23. Relationship between concentration of plasma copper and molybdenum of hypophosphataemic and molybdenotic buffalo calves.

Fig.24. Relationship between concentration of plasma molybdenum and phosphorus of hypophosphataemic and molybdenotic buffalo calves.

Fig.25. Alterations in inorganic phosphorus contents of rumen liquor in three groups of experimental buffalo calves.

Fig.26. Alterations in inorganic phosphorus contents of CSF in T_2 and T_3 groups of experimental buffalo calves.

Fig.27. Dorsopalmar view of metacarpal from a healthy buffal calf.

Fig.28. Dorsopalmar view of the same calf 90th day post-induction depicting reduced joint space of fetlock.

Fig.29. Dorsopalmar view of metacarpal depicting reduced joint space of fetlock alongwith periosteal lipping 90th day post0induction.

Fig.30&31 Dorsopalmar view of metacarpal showing increased soft tissue density alongwith periosteal lipping.

Fig.32. Dorsopalmar view of metacarpal showing increased soft tissue density and periosteal reaction.

Fig.33. Section of liver indicating extensive degenerative changes viz. cloudy swelling, coagulative necrosis alongwith congestion and haemorrhages. H & E x 300.

Fig.34. Higher magnification of Fig.33 showing rarefaction of nuclei at certain places evidenced by vacuolation of nucleoplasm along with other changes. H & E x 600.

Fig.35. Section of kidney with degenerative changes comprising of cloudy swelling, coagulative necrosis, formation of casts inthe uriniferous tubules alongwith haemorrhages and increased Bowman's space. H & E x 300.

Fig.36. Section of kidney showing extensive coagulative necrosis in uriniferous tubules alongwith presence of the casts H & E x 300

Fig.37. Section of lung showing extensive emphysema and haemorrhage H & E x 150.

Fig.38. Higher magnification of Fig.37 showing extensive emphysema and haemorrhage H & E x 300.

Fig.39. Section fo bone indicating increased size of haversian canals H & E x 150.

Fig.40. Higher magnification of Fig.39 indicating increased size of haversian canals H & E x 300.

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ABSTRACT

The present investigations were conducted to study the effect of phosphorus deficiency and its interaction with molybdenum supplementation on various haematological, clinico-biochemical and histopathological alterations in buffalo calves. The study was conducted on 20 male buffalo calves out of which five were kept as healthy control (group T_1) whereas five were used for induction of hypophosphataemia (group T_3) and in ten of the experimental animals alongwith hypophosphataemia, molybdenum supplementation was given daily for 90 days (group T_2). Treatment was undertaken in five animals of group T_2 with oral administration of sterilised bone meal @ 35 g/100kg body weight/day for next 30 days.

The consistent clinical signs of hypophosphataemia and molybdenosis were loss of appetite, unthriftiness, hide bound condition, rough hair coat, alopecia, discolouration of skin and weakness with inability to stand followed by recumbency and death. Haematological findings revealed macrocytic normochromic anaemia with significant decline in Hb, PCV and TEC. The intra-erythrocytic enzyme viz. erythrocyte superoxide dismutase (ESOD) showed marked decline in its activity in animals of T_2 group as compared to those of T_3 group. The erythrocyte malondialdehyde (MDA) concentration representing lipid peroxidation showed variable fluctuations. Scanning electron microscopy (SEM) of erythrocytes of healthy animals revealed typical biconcave appearance with poikilocytosis whereas in phosphorus depleted and molybdenum supplemented animals, SEM of erythrocytes indicated presence of spherocytes and discocytes with slight corrugation of erythrocytic membrane. Plasma minerals analysis revealed significantly low levels of inorganic phosphorus in T_3 group whereas more intense decrease was recorded in later stages in animals of T_2 group. Mean plasma iron content showed inconsistent fluctuations. Mean copper concentration reflected significant

decline associated with a significant rise in T_2 group. Rumen liquor minerals analysis revealed parallel and significant decrease in mean inorganic phosphorus levels whereas iron showed non-significant increase with copper indicating significant decline in hypophosphataemic and molybdenotic animals. In CSF samples the decline in mean inorganic phosphorus was parallel to the phosphorus content of plasma and intensity was comparatively more marked in molybdenum supplemented animals. Similarly, mean copper CSF contents also decreased in molybdenotic animals. Radiographic examination of lower ends of metacarpal and metatarsal bones revealed mild periosteal lipping, reduced joint space of fetlock joint and increased soft tissue density indicating laminitis. Histopathologically, bones revealed increased haversian canals size indicating decreased mineralisation. Histopathology of lungs, liver and kidneys showed varying degrees of degenerative changes. Treatment with sterilised bone meal in animals of T_2 group resulted in significant improvement in ESOD and inorganic phosphorus and copper contents in plasma, rumen liquor and cerebro-spinal fluid which was associated with non-significant improvement in other haemato-biochemical constituents.

Ashwani Kumar
Signature of student

Signature of Major Advisor

CHAPTER-I

INTRODUCTION

World today is facing serious deficiency in food production caused mainly by the annual increase in population and decrease in available arable land and this scenario is particularly most relevant to India. The problem is being overcome by intensive cultivation system of land management and livestock production. The intensive cultivation has done serious damage to the health of soil resulting into inadequate availability of proper livestock feed in many parts of the world. This intensification of agricultural practices and overzealous use of some elements for increasing agricultural production has further accentuated the problem of the availability of mineral elements in soil-plant-animal chain which has caused a serious damage to the health of dairy animals.

Another aspect of this problem is the unequal distribution of some elements on earth depending upon geological material, soil type, climatic condition vegetation, quality and quantity of manures, fertilizers and water used practices etc. (McDowell 1992). This has resulted into severe mineral imbalances which are of main concern in global context and livestock are more susceptible thus directly affecting the human beings completely dependent upon animals for quality proteins.

Amongst these minerals, phosphorus is the second most abundant mineral element in the animals body. About 81 per

cent of this is present in bones and teeth and is an essential mineral of all the soft tissues. Phosphorus is probably the most versatile of all the mineral elements. It functions as a component of nucleic acids, maintains osmotic and acid-base balance, plays a vital role in energy utilization and transfer, in formation of phospholipid and proteins, in control of appetite and efficiency of feed utilization. (McDowell, 1992).

Phosphorus deficiency is very wide spread in dairy animals and it has been suggested that phosphorus followed by copper, in the tropical world, may be associated with most severe mineral deficiency disorder for grazing animals. With deficiency of phosphorus the protein content of the herbage falls so that the protein deficiency and frequently also a deficiency of available energy are precipitating factors in the malnutrition of livestock in phosphorus deficient areas(McDowell, 1992). Phosphorus deficiency in dairy animals has been associated with allotriophagy, post-parturient haemoglobinuria and infertility. Similarly, molybdenosis has also been implicated in the development of post-parturient haemoglobinuria, achromotrichia, vitiligo, stiff leg syndrome and infertility (Randhawa, 1993). Hypophosphataemia may also cause increased fragility of erythrocytes because it is a component of phospholipid and nearly half the mass of erythrocyte membrane is comprised of various phospholipid (Devlin, 1982).

Hypophosphataemia has also been associated with

rheumatoid syndrome in buffaloes (Malhotra, 1991). Enlargement around lower end of the metacarpus and metatarsus and a peculiar "stiff gait" are frequently observed. There is loss of body condition and decrease in milk yield.

Buffaloes form the major milch animals of the Punjab. In India buffaloes account for nearly 51 per cent to the world buffalo population. The buffaloes population in the country is less than one third of the cattle but they contribute 59 per cent of the total milk production (Dairy India, 1987). In Punjab most of the soils are deficient in phosphorus contents (Brar, 1979) and a part of such areas have high molybdenum content (Nayyar *et al* 1980) which could further potentiate phosphorus deficiency (Walker *et al* 1955). An obscure disease in Punjab manifested as progressive stiff gait, partial or complete anorexia and chronic debility leading to hide-bound condition had been reported in buffaloes with low plasma inorganic phosphorus (Arneja *et al* 1987)

Since there was paucity of scientific literature on induced as well as clinical form of hypophosphataemia in buffaloes and its interaction with molybdenosis, therefore, the present study was planned with following objectives:

I) To experimentally induce hypophosphataemia in buffalo calves by feeding phosphorus deficient diet and to study clinical symptomatology in the affected animals.

2) To investigate the effect of phosphorus deficiency alone or in combination with molybdenum

supplementation on haematological indices, erythrocytic morphology and mineral status especially molybdenum, copper and iron.

3) To study the combined effect of phosphorus deficient and molybdenum supplemented diets on erythrocyte morphology by scanning electron microscopy and intra erythrocytic enzyme viz. super oxide dismutase.

4) To undertake radiographic study of bones and investigate histopathological alterations in various tissues.

5) To undertake therapeutic measure in experimentally induced hypophosphataemia and molybdenosis.

CHAPTER II

REVIEW OF LITERATURE

Among all the diseases related to mineral deficiencies, hypophosphataemia is one of the most important as well as widely prevalent condition of grazing livestock (McDowell,1992). The condition occurs either due to low dietary uptake of phosphorus or due to combination of various other factors like improper absorption and interaction with other minerals like Ca, Mo and S etc. Phosphorus and molybdenum are known to interact in antagonistic manner in the fodder like oats and barseem (Pasricha, 1977). This interaction is especially important in a state like Punjab where most of the soils are deficient in phosphorus (Brar, 1979) and some of them are reported to be higher in molybdenum (Nayyar, *et al* 1980). While the sudden deficiency of inorganic phosphorus in high yielding cattle and buffaloes leads to haemoglobinuria, anaemia and death, a gradual deficiency of this mineral over a long period of time is reflected in poor growth rate, progressive emaciation, lameness, reproductive disorders, low production and anorexia.

The literature has been reviewed to understand the subject matter with regard to symptomatology, biochemical analysis of blood, minerals alterations in plasma, rumen liquor and cerebro-spinal fluid, radiographic and pathological changes in bones, changes in red blood cell morphology and other tissues of the body as well as

therapeutic measures instituted to correct hypophosphataemia in dairy animals.

2.1 INDUCTION

As early as 1929, Fish reported 25 per cent lowering of phosphorus level in the blood of recently calved cows.

Barnes and Jephcott (1959) recorded significantly lower serum inorganic phosphorus (2.25 mg per cent) in cattle after a drought season as compared to mean value of 5.06 mg per cent recorded in a good season.

Dhillon *et al* (1972) suggested that feeding of leguminous plants like berseem (*Trifolium alexandrinum)* in the Punjab from October to March caused phosphorus deficiency in animals. This was due to high up take of molybdenum by berseem which caused phosphorus deficiency in animals by decreasing its absorption from gastro-intestinal tract (GIT) and enhancing the elimination through urine.

Pasricha *et al* (1977) reported that molybdenum and phosphorus antagonise each other in soil and in fodder plants like berseem (*Trifolium alexandrinum)* and oats. He reported that application of single superphosphate to a molybdenum toxic soil markedly lowered the molybdenum content to safe level in the fodder.

McDowell *et al* (1985) stated that high amount of soil iron and aluminium accentuated phosphorus deficiency by forming insoluble non-absorbable phosphate complexes.

Crowe *et al* (1990) studied bio-availability of phosphorus in young dairy calves. It was observed that in calves fed on low phosphorus and high aluminium (0.20 %)

diet the serum inorganic phosphorus dropped to 1.62 mg/dl.

Blair-West *et al* (1992) induced phosphorus deficiency in heifers by loss of phosphate in saliva from a parotid gland fistula combined with a low phosphorus diet. Phosphorus level in plasma showed falling trend from start to end of experiment.

Gill (1992) and Haque and Verma (1992) experimentally induced phosphorus deficiency by feeding a phosphorus deficient ration to cross-bred cow calves. The phosphorus level of plasma reflected continuous decline from start of experiment till 75th day when it fell significantly as compared to control group.

Rodehuscord *et al* (1994) could detect hypophosphataemia in recently calved ruminants by feeding phosphorus deficient diet. Animals were showing symptoms after 10 days when the serum phosphorus level reached a minimum of 0.28 mM.

2.2 SYMPTOMATOLOGY

Typical symptoms of weakness, incoordination of gait, staggering, anorexia, inability to stand, rapid loss of flesh and anaemia were reported by various workers in post parturient haemoglobinuria, a disease associated with hypophosphataemia and molybdenosis (Farquharson and Smith, 1939, Madsen and Nielson, 1939, Parkinson and Sutherland, 1954 and Mullins and Ramsay, 1959).

Loss of appetite and craving for consumption of abnormal materials such as soil, wood, flesh and hair were the typical symptoms enlisted by Underwood (1981). In

hypophosphataemia. Brander *et al* (1982) stated that gradual deficiency of phosphorus over a long period of time was reflected as poor growth rate, progressive emaciation, lameness, reproductive failure, low production and anorexia.

Call *et al* (1986) ascribed the symptoms such as weight loss, rough hair coat, abnormal stance, lameness and spontaneous fractures that did not heal properly to phosphorus deficiency. They also observed impaired reproductive performance of the affected adult animals.

Verma and Gupta (1987) associated a rheumatism like syndrome in buffaloes in late gestation to hypophosphataemia. The clinical signs of aphosphorosis were retarded growth, emaciation, arched back with tucked up abdomen, reduced fertility, stiffness of joints and dirty brown discolouration of the skin with a rough and dry surface.

Blood *et al* (1989) described pica, poor growth, infertility and osteodystrophy as clinical symptoms of phosphorus deficiency.

Kaneko (1989) enlisted the clinical signs of molybdenosis as weight loss, anorexia, loss of colour (achromotrichia), anaemia, cartilaginous dysplasia, abnormal endochondreal ossification, sub-periosteal ossification and abnormal fibrogenesis.

Blair-West *et al* (1992), in the behavioural study on severe phosphorus depletion in cattle, demonstrated the symptoms of phosphorus deficiency as roughness of coat and

a change in colour from black to red-brown, alopecia, stiff and plodding gait with no tendency to run about, allotriophagy with avidly chewing tendency for old bones, failure to gain body weight and maintain condition and abnormal oestrous cycles.

Gill (1992) enlisted that symptoms of gradual deficiency of phosphorus in cross-bred cow calves over a long period were mutual plucking of hair, stiff gait, difficulty to stand up and arthritis like condition.

Haque and Verma (1992) induced phosphorus deficiency and enlisted the typical hypophosphataemic symptoms as complete loss of appetite, peculiar stiff gait, startling movement with peduncular gait and marked loss of body weight. The animals were unable to stand and there was tendency to fall down.

Randhawa (1993) reported the symptoms of molybdenosis as achromotrichia, vitiligo, anaemia, stiff leg syndrome and infertility.

Ghergaru *et al* (1994) recorded pica, stiffness, lameness and hypophosphataemia in cattle and buffaloes in Romania due to very low intake of phosphorus. However, the reason for presence of high phosphaturia could not be explained by them.

2.3 BLOOD ANALYSIS

2.3.1 HAEMOGRAM

2.3.1.1 HAEMOGLOBIN AND PACKED CELL VOLUME

Barnes and Jephcott (1955), in a clinical study, reported no correlation between "peg leg" and haemoglobin

level. In another study in 1959 the authors also recorded no correlation between serum inorganic phosphorus and haemoglobin levels.

Mullins and Ramsay (1959) inferred that in the endemic areas where aphosphorosis usually occurred, the haemoglobin and haematocrit were very low especially in anaemic cows. The values ranged from 4.1-10.9 g/dl and 23-30 per cent, respectively in the diseased animals.

Pandey (1980) observed nutritional anaemia in experimental calves by feeding wheat straw for 67 days. The distinct features were simultaneous decrease in PCV and Hb to as low as 18.0±0.54 per cent and 6.80±0.06 g/dl, respectively as compared to corresponding base value of 27.53±0.38 per cent and 11.06±0.35 g/dl.

Benjamin *et al* (1985) reported that haemoglobin content and packed cell volume in healthy bovines ranged between 9.25 -13.25 g/dl and 33-48 per cent, respectively.

Lesperance *et al* (1985) observed that experimental molybdenum toxicity depressed haematocrit from 36.0 to 24.0 per cent.

Kumar *et al* (1990) recorded that mean value of haemoglobin and packed cell volume for 12 months old healthy buffalo calves was 10.35±0.78 g/dl and 33.78±2.83 per cent, respectively.

2.3.1.2 Total erythrocytic count (TEC)

Mullins and Ramsay (1959) observed that erythrocytic counts of animals, on farms deficient in phosphorus, ranged between 4.10-5.04 x $10^6/mm^3$.

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Clegg and Evans (1962) reported that TEC on a deficient farm was ranging between 2.67-3.04x10^6/mm^3 with TEC in healthy cattle being 6.1-10.0 x 10^6/mm^3.

Patel *et al* (1969) stated the normal mean value of TEC in healthy Indian buffaloes was 6.1±0.09 x 10^6/mm^3.

Canfield *et al* (1984) studied haemogram of swamp buffalo *(Bubalus bubalis)* in Australia and reported that values of TEC ranged between 7.1±1.4 to 7.9±1.2 x 10^{12}/L in all the groups of animals.

Kumar *et al* (1990) analysed the mean TEC in healthy buffalo calves of 9-12 months of age to be 5.56±0.6 x 10^6/mm^3.

2.3.1.3 SCANNING ELECTRON MICROSCOPY (SEM) OF ERYTHROCYTES

Martinovich and Woodhouse (1971) showed that Heinz body anaemia was more prevalent in area where there was Cu-Mo imbalance.

Jain and Kono (1972) showed by SEM that bovine erythrocytes usually appeared to be uniformly outlined biconcave or uniconcave cells measuring 4 um in average diameter. The appearance of these cells varied with different profiles.

Singer and Nicolson (1972) visualized the cell membrane with electron microscopy and gave the concept of "the fluid mosaic model". As per hypothesis of that model, the proteins of cell membrane were particularly embedded in fluid bi-layer of phospholipid to form matrix of mosaic.

Schalm *et al* (1975) reported spindle, rod or spherical

shape of erythrocytes in clinically normal goats. They also observed acanthosis in apparently healthy cows. It was also detected that poikilocytosis was more in goat erythrocytes than any other species.

Shehata and Ibrahim (1984) reported that diameter of a bovine erythrocytes varied from 4.0-8.0 um with an average of about 5.8 um.

Guyton (1986) commented that like other membranes red cell membrane was also made up of proteins, lipids and carbohydrates. Of the lipids, phospholipid formed the major fraction of lipid bilayer and were integral part of the structure and function of biomembrane and also provide permeability barrier.

Bhardwaj (1988) did extensive work on scanning electron microscopy of erythrocytes of Indian buffaloes. He recorded that these erythrocytes generally appeared as biconcave disc but showed extensive poikilocytosis. Discocytes, stomatocytes, spherocytes and knizocytes were the shapes detected. He recorded that average diameter of buffalo erythrocytes was 4.8 um (range 4-5.6 um). It was also observed that buffalo erythrocytes were more sensitive to morphological changes than human and cattle erythrocytes. Buffalo erythrocytes also had greater depression than cattle erythrocytes.

Rana and Bhardwaj (1988) hypothesized that low intra erythrocytic ATP as well as membrane total phospholipid might be the cause of change in shape of erythrocytes. The studies on SEM of erythrocytes also ruled out possibility

shape of erythrocytes in clinically normal goats. They also observed acanthosis in apparently healthy cows. It was also detected that poikilocytosis was more in goat erythrocytes than any other species.

Shehata and Ibrahim (1984) reported that diameter of a bovine erythrocytes varied from 4.0-8.0 um with an average of about 5.8 um.

Guyton (1986) commented that like other membranes red cell membrane was also made up of proteins, lipids and carbohydrates. Of the lipids, phospholipid formed the major fraction of lipid bilayer and were integral part of the structure and function of biomembrane and also provide permeability barrier.

Bhardwaj (1988) did extensive work on scanning electron microscopy of erythrocytes of Indian buffaloes. He recorded that these erythrocytes generally appeared as biconcave disc but showed extensive poikilocytosis. Discocytes, stomatocytes, spherocytes and knizocytes were the shapes detected. He recorded that average diameter of buffalo erythrocytes was 4.8 um (range 4-5.6 um). It was also observed that buffalo erythrocytes were more sensitive to morphological changes than human and cattle erythrocytes. Buffalo erythrocytes also had greater depression than cattle erythrocytes.

Rana and Bhardwaj (1988) hypothesized that low intra erythrocytic ATP as well as membrane total phospholipid might be the cause of change in shape of erythrocytes. The studies on SEM of erythrocytes also ruled out possibility

of associating Heinz bodies with phosphorus deficiency in buffaloes.

Gill (1992) reported non-significant change in membrane phospholipid concentration of erythrocytes in induced phosphorus deficiency in calves.

2.4 BIOCHEMICAL ANALYSIS

2.4.1 SUPEROXIDE DISMUTASE

Ammerman (1970) stated that superoxide dismutase (SOD), a metalo-enzyme was a part of erythrocytes protective mechanism against oxidative stress. It was postulated that copper deficiency could depress SOD activity.

Bohenkamp and Weser (1976) inferred that only factor known to reduce Cu-SOD activity in swine, rats and sheep was copper deficiency itself.

Suttle (1986) showed the diagnostic potential of erythrocytic superoxide dismutase (ESOD) in cattle with primary copper deficiency.

Suttle and McMurray (1983) demonstrated that ESOD activity in hypocuprosis declined at only one-third to one-seventh of the rate shown by plasma copper.

Mason (1986) observed that thiomolybdate also appeared to be effective in tissues in that they reduce the effectiveness of the enzymes containing copper.

Suttle (1986) reported that diagnosis of copper deficiency was improved by addition of erythrocytes superoxide dismutase to the assay of copper status.

Xin (1991) studied SOD activity in red blood cell

lysate of healthy steers and reported a mean value of 0.6 U/mg Hb.

Ledwozyw and Stalarczyk (1992) reported mean value of ESOD to be 9.35±0.86 U/mg protein in healthy calves.

2.4.2 LIPID PEROXIDATION

Hammermuellar *et al* (1987) revealed that both dietary zinc and copper deficiencies cause lipid peroxidation in the microsome in rats. They stated that copper deficiency did not effect H_2O_2 production, however, it caused two to four fold increase in the concentration of iron in the lungs and liver microsome, respectively compared to copper adequate and *ad lib* fed controls and this increase in iron might be the possible cause of lipid peroxidation.

Jain and Williams (1988) investigated membrane lipid abnormalities and their possible role in copper deficiency anaemia by feeding rats a diet deficient in copper (1.1 mg/kg) and supplemented ones (6 mg/kg). They showed 43 and 38 per cent increase in cholesterol and phospholipid per cell, respectively in the copper deficient animals compared with copper supplemented rats. Thin layer chromatography of rbcs lipid showed an increased amount of phospholipid malondialdehyde adduct in Cu deficient rats compared with supplemented ones.

2.5 PLASMA MACRO- AND MICRO-ELEMENTS

2.5.1 PLASMA INORGANIC PHOSPHORUS

Parkinson and Sutherland (1954) observed phosphorus content of more than 4.0 mg/dl in normal cows.

Awad and Latif (1963) reported average serum inorganic

phosphorus level of 7.08±1.89 mg/dl in normal healthy buffaloes.

Dhillon *et al* (1972) advocated that low value of serum inorganic phosphorus, varying from 0.9-3.5 mg/dl with average content of 2.05±0.6 mg/dl, in 15 cases of haemoglobinuria in buffaloes was due to higher molybdenum content of blood.

Brooks *et al* (1984) observed that in a particular herd in New Zealand showing low milk production, ill thrift, infertility and osteophagia the levels of inorganic phosphorus were in the range of 1.86-5.77 mg/dl with mean value of 3.44 mg/dl which were quite low against the normal range of 4.03-7.13 mg/dl.

Canfield *et al* (1984) reported that average inorganic phosphorus level in healthy Swamp buffaloes was 7.44±1.55 mg/dl.

Lesperance *et al* (1985) indicated that in calves fed experimentally 100 ppm of molybdenum the plasma inorganic phosphorus was elevated (7.24 mg/dl), while those not receiving molybdenum had low serum inorganic phosphorus content (6.64 mg/dl).

Arneja *et al* (1987) reported mean plasma inorganic phosphorus level of 3.68±0.35 mg/dl in seven buffaloes exhibiting clinical signs of hypophosphataemia.

Haque and Verma (1990) induced hypophosphataemia by feeding a phosphorus deficient ration and showed that calves had lower serum phosphorus level (4.4±0.41 mg%) on

phosphorus level of 7.08±1.89 mg/dl in normal healthy buffaloes.

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Haque and Verma (1990) induced hypophosphataemia by feeding a phosphorus deficient ration and showed that calves had lower serum phosphorus level (4.4±0.41 mg%) on

65th day of experiment as compared to zero day (8.8±0.5 mg%).

Joshi *et al* (1991) observed that average plasma phosphorus content was 1.06±1.05 mg/dl in deficient mature buffaloes which developed signs of hypophosphataemia and haemoglobinuria.

Malhotra (1991) recorded a rheumatoid syndrome characterized by progressive stiffness of limbs with a consistent hypophosphataemia (2.24±0.56 mg%) compared to normal (4.71±0.6 mg %) buffaloes in Himachal Pradesh.

Gill (1992), during induction of phosphorus deficiency in cross-bred calves, observed significant decrease in phosphorus level in one group on 75th day (4.21±0.22 mg/dl) and on 90th day (3.54±0.36 mg/dl) in group supplemented molybdenum alongwith as compared to respective control group.

Blair-West *et al* (1992) demonstrated that plasma inorganic phosphorus fell down to 0.86±0.04 mM in the heifer after 6 months of phosphorus depleted diet and unilateral parotid saliva drainage.

Rodehuscord *et al* (1994) observed a plasma phosphorus level of 0.28 mM in recently calved ruminants after restricting phosphorus intake.

2.5.2 PLASMA MOLYBDENUM

The normal blood molybdenum concentration ranged from 6-10 ug/dl in healthy bovine (Cunningham, 1950, Fide Underwood, 1962).

Davis (1950) advocated that monogastric animals are

more tolerant to higher molybdenum levels as compared to ruminants; cattle being least and horse most tolerant among farm animals. Sheep, pigs and poultry came in between.

Clarke and Clarke (1967) showed that molybdenum reduced phosphorus content in the body by interfering with its absorption from the gastro-intestinal tract and also by enhancing elimination through urine.

Huber *et al* (1971) reported that blood molybdenum was greatly influenced by higher dietary molybdenum intake although requirement for it was very low in the animals.

Blood *et al* (1989) reported that concentration of molybdenum in blood of different species of farm animals ranged between 0.05-0.1 ppm.

2.5.3 PLASMA COPPER

Cunningham (1944) reported copper deficiency in cattle and sheep in New Zealand and found sub-normal copper content in liver and blood of animals grazing on copper deficient areas. In another study in 1954, he reported decrease in blood copper level in animals kept on pastures having excess molybdenum for a long period.

Wynne and McClymont (1956) reported significant hypocupraemia in animals fed on diets containing excess molybdenum and sulphates.

Mullins and Ramsay (1959) recorded decrease in plasma copper level (0.08±0.01 mg/dl) in 4 severely hypophosphataemic cows.

Corrigal *et al* (1976) indicated that during infectious diseases plasma copper level was increased and zinc level

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Corrigal *et al* (1976) indicated that during infectious diseases plasma copper level was increased and zinc level

decreased. However, in haemoglobinuria, a disease associated with hypophosphataemia, low level of plasma copper observed indicated its non-infectious origin.

Gardner *et al* (1976) observed plasma copper levels of 10-25 ug/dl in deficient as compared to 80-100 ug/dl in control herd and related the resultant copper deficiency to high molybdenum content of soil.

Parshad *et al* (1979) stated that normal mean plasma copper levels in lactating and dry buffaloes were 81.07±1.55 and 77.30±1.82 ug/dl, respectively.

Kincaid (1980) reported that mean plasma copper level increased from 69 to 110 ug/100 ml in experimental toxicity of ammonium molybdate induced @ 50 ppm added to drinking water of calves for 21 days.

Underwood (1981) remarked that whole blood or plasma copper concentration reflected the dietary status of the mineral and for large number of domestic animals the normal range was 60-150 ug/dl though high proportions of copper values were observed to be between 80-120 ug/dl.

Arneja *et al* (1987), in clinical cases of hypophosphataemia in buffaloes, reported mean blood copper level of 104.28±1.87 ug/dl.

Pandey and Misra (1987) detected mean plasma copper level of 227.88±8.07 ug/dl in healthy buffaloes.

Gill (1992) experimentally observed a rise in plasma copper from mean base value of 111.91±3.4 to 123.36±4.91 ug/dl in hypophosphataemic and molybdenotic cross-bred calves.

2.5.4 PLASMA IRON

Rys (1959) noticed decreased plasma iron content (average 126 mg/dl) in cows with hypocupraemia.

Patel *et al* (1969) reported that mean value of plasma iron in Surti buffalo was 53.9±6.57 mg per cent.

Underwood (1981) recorded mean plasma iron content of 146 ug/dl (range 89-253 ug/dl) in healthy cows.

Arneja *et al* (1987) revealed plasma iron level of 33.23±0.63 ug/dl in buffaloes showing symptoms of hypophosphataemia.

Reddy *et al* (1987) stated that in animals suffering from anorexia there is also reduced serum iron, total iron binding capacity and unsaturated iron-binding capacity. He indicated that iron concentration in plasma was around 142.38±11.07 ug/dl.

Blood and Radostits (1989) stated that plasma iron ranged between 57-162 ug/dl in healthy bovines.

Kaneko (1989) stated that plasma iron decline in severe iron deficiency, acute phase inflammatory reactions, hypoproteinaemia, hypothyroidism, renal disease and chronic inflammation however elevation in plasma iron occur in haemolytic anaemia, refractory anaemia, iron overload and liver diseases.

2.6 MACRO- AND MICRO-ELEMENTS IN RUMEN LIQUOR AND CEREBRO-SPINAL FLUID

The assay of various minerals in rumen liquor and cerebro-spinal fluid may also provide a valuable information about mineral status of the animal.

Dukes (1970) observed that there was an influence of ionic concentration of rumen fluid on acid-base and minerals balance between blood and rumen liquor.

Cakala and Albrycht (1975) stated that minerals in rumen liquor were indicative of their intake in the feed. The animals which were deprived of food and water showed lowered calcium, magnesium and phosphorus in the rumen fluid and raised sodium and pH after 12-24 hours.

Gerald (1978) observed that hay-making reduced molybdenum toxicity very dramatically and it was postulated that very high soluble protein of fresh pasture was readily hydrolysed in rumen and provided sulphur which combined with copper to produce insoluble copper sulphide. But when fodder was dried to make hay, protein content and solubility was lowered which resulted in lowered toxicity.

Matturi (1978) stated that low concentration of phosphorus observed in rumen liquor after unilateral parotid saliva drainage and phosphorus deficient diet feeding were close to the minimal requirement for optimal microbial fermentation and nutrition in ruminants.

Choudhuri *et al* (1980) observed mean phosphorus level of 12.10 mg per cent in the rumen liquor of two year old buffalo calves.

Compton *et al* (1980) and Wright *et al* (1984) inferred that phosphorus concentration of ruminant parotid saliva was high compared with other species and was ten times more than that of plasma.

Suttle *et al* (1983) described that molybdenum in rumen

react with sulphide to produce thiomolybdates. The subsequent formation of copper-thiomolybdate complexes isolated the copper from being biologically available.

Dua (1986) inferred that levels of phosphorus in rumen liquor and cerebro-spinal fluid were 14.23±0.76 and 1.67±0.38 mg/dl, respectively.

Reddy *et al* (1987) reported that in animals suffering from anorexia there was also reduced serum iron, total iron binding capacity leading to development of anaemia.

Kaneko (1989) gave the value of phosphorus in cerebro-spinal fluid in cows in the range of 0.9-2.5 mg/dl.

Blair-West *et al* (1992), through experimentation, revealed that with feeding of phosphorus deficient diet the phosphorus of parotid saliva also fell and this reduction in salivary phosphorus was associated with a fall in the phosphorus of ruminal fluid, consequently the disturbance in nutrition due to low phosphorus in saliva resulted into failure of weight gain in phosphorus depleted cows as compared to age match control cows on a daily phosphorus intake of 12g.

It was also observed that phosphorus in rumen liquor fell from 15 mmol/l to 5 mmol/l after 6 months of phosphorus depleted diet and unilateral parotid saliva drainage.

Rodehuscord *et al* (1994) observed that phosphorus concentration in rumen liquor started to fall immediately with changing phosphorus intake from adequate to low reaching a minimum of 0.28mM. Concentration rose immediately with return of phosphorus intake to adequate

and then returned to normal. Similar trend was observed in serum phosphorus concentration. He gave phosphorus values in rumen liquor of control, depleted and repleted animals as 30mM, 5.7mM and 30mM, respectively.

2.7 RADIOGRAPHIC AND MORPHOLOGICAL CHANGES IN BONES

Irwin *et al* (1974) detected lameness in cattle due to high molybdenum and sulphate resulting in secondary copper deficiency. The lesions in the distal metacarpal or metatarsal growth plates were characterized radiographically by widening of physis with considerable irregularity and fragmentation of metaphysis.

Butcher *et al* (1978) detected some decrease in bone density in growing beef cattle maintained on a diet containing 0.09 per cent phosphorus.

Pitt *et al* (1980) found exostosis and haemorrhages about long bones and loosened great trochanter of the femur of sheep which were allowed to graze on pastures sprayed three times with molybdenum @ 420 units/hectare.

Underwood (1981) described that in 'peg-leg', a term used to describe stiff gait movements of the phosphorus deficient grazing cattle, the basic defect was a failure or reduction in the mineralisation process so that the bones contained insufficient minerals to develop or maintain normal shape and strength and therefore to sustain their mechanical functions.

Hurtig *et al* (1991) observed symptoms of angular limb deformities, mild flexure deformities, epiphysitis and intermittent lameness in foals by restricting copper intake

for 2 months.

Blair-West *et al* (1992) reported that radiographs of bones of phosphorus deficient animals revealed increased bone radiolucency, coarser bone trabeculation and disruption of endochondrial ossification. The morphological changes were thinning of cortices of long bones and enlargement of marrow spaces. Bones were soft and reduction in their weight and specific gravity was observed.

Sharma *et al* (1995) observed thinned cortices and reduced density of medullary cavity radiographically in fore and hind limb bones of goats fed molybdenum experimentally.

2.8 PATHO-MORPHOLOGICAL CHANGES

Mullins and Ramsay (1959), on pathological examination of phosphorus deficient and haemoglobinuric animals, revealed that lesions of post-parturient haemoglobinuria and acute anaemia were seen and were mainly occurring in heart, fat, lungs and gall bladder. The heart was pale, grossly enlarged, flabby and musculature was pale with pronounced *khakhi* discolouration. Haemorrhages of varying degrees were present in different area of heart. Liver showed congestion and *khakhi* discolouration. Fat, lungs, small intestine and kidneys also showed *khakhi* discolouration of varying degrees.

Bones were fragile, medullary cavity appeared large and the marrow dark. The kidneys showed some congestion and haemorrhages. The spleen appeared normal or slightly swollen in size but the consistency of the pulp was soft.

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Bones were fragile, medullary cavity appeared large and the marrow dark. The kidneys showed some congestion and haemorrhages. The spleen appeared normal or slightly swollen in size but the consistency of the pulp was soft.

Joints showed no visible pathological lesions and tendon sheaths were not affected. Central lobular necrosis, suggestive of protein deficiency was observed in sections of liver.

Hill and Rajagopal (1962) observed that skeleton of a group of lame buffaloes from phosphorus deficiency area was poorer than from control area. However, evidence for a relationship between lameness and the quality of the skeleton was not strong.

Irwin *et al* (1974), in histopathological examination of bone sections of molybdenum induced hypocuprotic animals, observed focal widening of the growth plate consisting of tongues of uncalcified cartilage with delayed or impaired provisional calcification in the presence of active osteoblasts.

Suttle and Angus (1978) studied the effects of experimental copper deficiency on the skeleton of calves and inferred that copper deficiency contributed to the development of general matrix osteoporosis and overgrowth of the epiphyseal cartilage. Lesions were reported to be severe in costochondral junction and metatarsal region.

Randhawa (1993), on histopathological examination of molybdenotic buffalo calves, revealed focal widening, microfracture and marked decrease in osteoblastic activity of bones, degeneration of cardiac and Purkinje muscle fibres, fatty changes with hyperplasia in the liver and coagulative necrosis of renal tubules with glomerular atrophy and extensive haemosiderosis in various organs.

Sharma and Prihar (1994) experimentally induced molybdenosis in goats and noticed microscopic lesions indicative of haemosiderosis of lymph nodes, spleen, liver, lungs and intestine; loss of melanin from hair cortex and medulla alongwith degenerative changes in the liver, skeletal muscles and testes. Besides these, colloid goiter and arrested bone growth were also seen.

Blair-West *et al* (1992) reported emaciation in phosphorus depleted cows at the time of euthanesia. Post mortem examination confirmed cachexia with considerable individual variation. Most noticeable were reduced adipose tissues and skeleton mass. Light microscopy of rib sections showed thinning of cortices, thinner and less numerous medullary bone trabeculae and a reduction in the amount of haemopoietic bone marrow. The bone trabeculae showed little evidence of active osteoblastic and osteoclastic activity and bone formation at the costo-chondral junctions appeared to be appreciably reduced and distorted.

2.9 THERAPY

Madsen and Neilsen (1944) reported the blood level of phosphorus rose very rapidly in 5½ hours from 1.43 to 3.00 mg per cent after the cow was drenched with 200 g of bone meal.

Mullins and Ramsay (1959) advocated that effect of sodium acid phosphate in treatment of post-parturient haemoglobinuria was transient and bone meal should be given in addition.

Tillman and Brethour (1958) made comparison of

phosphoric acid and dicalcium phosphate as source of phosphorus in cattle and revealed no significant difference.

Barnes and Jephcott (1959) compared bone meal and sodium orthophosphate in the drinking water for treatment of phosphorus deficiency in cattle. It was observed that administration of sodium orthophosphate had alleviated the condition whereas bone meal had previously failed.

McTaggart (1959) reported a desirable elevation in plasma phosphorus levels of arthritic cattle following feeding half an ounce of sterilized bone meal for 30 days.

Dhillon *et al* (1972) advocated the administration of sulphate ion to haemoglobinuric buffaloes which was due to phosphorus deficiency, and administered 2 g of copper sulphate orally per day to each case for 3-4 consecutive days. It was reported that urine became clear within 3-5 days of therapy.

Peeler (1972) ranked various phosphorus supplements in decreasing order of available phosphorus as : sodium phosphate followed by phosphoric acid, monocalcium phosphate, dicalcium phosphate, deflourinated phosphate, bone meal and soft phosphate.

Blood and Radostits (1989) suggested that gross weekly requirements of phosphorus for adult cattle and calves (upto 150 kg body weight) were 225 g of bone meal. However, requirement of growing stock (over 150 kg body weight) and lactating cows was 350 g and one kg per week, respectively. These requirements were subject to change in amount from

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area to area.

Haque and Verma (1990) reported that serum phosphorus levels in phosphorus deficient calves following treatment with sodium acid phosphate achieved basal values as compared to tonophosphan and further suggested that sodium acid phosphate was better than tonophosphan as evident by rapid recovery, return to normal serum phosphorus and cent per cent cure rate.

Malhotra (1991) reported high efficacy of parenteral dose of phosphorus (Tonophosphan) as compared to oral supplementation with calfos AD_3 plus to alleviate phosphorus deficiency.

CHAPTER-III

MATERIALS AND METHODS

3.1 **Place and time of study** - The study was conducted in the Department of Veterinary Medicine, College of Veterinary Science, Punjab Agricultural University, Ludhiana from March, 1994 to January 1996.

3.2 **Experimental studies**

3.2.1 **Animals**

Twenty male buffalo calves of about one year of age and weighing between 68 to 106 kg with average body weight of 89 kg were used for the present study. The calves were kept under observation for about a month. The daily nutrient requirements were met by ***ad lib*** feeding of green fodder and wheat straw. The calves were dewormed with broad spectrum anthelmintic, freed from external parasites and were not under the effect of any medication prior to the start of study. The calves were maintained under identical management conditions during the entire period of experimental study. Before experimentation, the animals were screened for any parasitic infection by faecal examination. The rumination, defecation, urination, temperature, pulse and respiration rate of each animal was observed to be within normal range. The calves were randomly

divided into three groups viz. T_1, T_2, and T_3. Group T_1 had five animals which served as healthy control at PAU dairy farm. Group T_2 comprised of ten animals whereas group T_3 had five animals.

3.2.2 **Phosphorus deficient diet**- The basal phosphorus deficient diet was prepared by treating one quintal of wheat-straw with 3.5 kg urea dissolved in 40 liters of water. The urea treated wheat-straw was allowed to ferment naturally for 9 days. The basal ration contained 0.1 per cent phosphorus on dry matter basis (AOAC, 1980). The phosphorus free mineral mixture was prepared as per ISI specification by substituting copper with starch and phosphorus and calcium present as dicalcium phosphate with starch and calcium carbonate, respectively.

Composition of Phosphorus & Copper Free Mineral Mixture (All salts being 100% pure):Per Quintal

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

To this mixture vitamin A and vitamin D were

added @ 150 IU/kg of mineral mixture.

3.2.3 **Control values-** To establish control values (zero day) rumen liquor, blood and cerebro-spinal fluid (CSF) samples were collected simultaneously from each animal on two occassions at two days interval before experimental induction of hypophosphataemia. However, representative blood samples for Scanning Electron Microscopy (SEM) were collected from four healthy animals as well as two animals each of T_2 and T_3 group post-induction. For this one or two drops of free flowing blood from jugular venipuncture were taken directly in 2 per cent gluteraldehyde in 0.1 M phosphate buffer of pH 7.2, at room temperature in glass vials. The blood and gluteraldehyde were mixed slowly by rubbing the vials in hand and stopper applied.

3.2.4 **Radiographic examination of bones -** Radiographs of lower ends of metacarpal and metatarsal bones were taken in antero-posterior views. The factors given were mAs-20, KVp-65 and FFD-80 cms. Radiographs were analysed for bone density, texture and architecture.

3.2.5 **Induction of phosphorus deficiency-** Calves of group T_2 were kept on ***ad lib*** feeding of urea treated wheat-straw for 90 days. Basal ration was supplemented with phosphorus and copper free mineral mixture @ 15g/100 kg body weight. The

animals were fed molasses @ 50g/100kg body weight alongwith mineral mixture in daily diet. However, the calves of group T_2 were also given orally 10 per cent ammonium molybdate solution daily to provide molybdenum @ 3mg/kg body weight. The calves of control group T_1 were kept on ***ad lib*** feeding of green fodder and wheat-straw alongwith phosphorus and copper containing mineral mixture @ 15 g/100kg body weight.

3.3 **Clinical observations-** The animals were observed daily for body condition, hair coat, gait, feed intake and other clinical signs throughout the period of study.

3.4 **Collection of blood, rumen liquor and cerebro-spinal fluid-** Blood samples from all the animals were collected at 15 days interval for 120 days throughout the period of study. The treatment was instituted on 91st days. The blood samples were collected in 30 ml heparinised glass vials by jugular venipuncture. Five ml of blood was taken in glass vials and used for Hb, TEC and PCV. Two ml blood was used for the estimation of superoxide dismutase activity. Blood collected in 30 ml vials was centrifuged at 3000 rpm for 30 minutes to separate plasma. The plasma was stored in 5 ml plastic storage vials at -15°c and used for various mineral estimations. The erythrocytes were washed three times with

phosphate buffer saline (pH 7.4) and used for determination of lipid peroxidation in the erythrocytes (Stocks and Dormondy 1972). The rumen liquor samples were collected from each animal with the help of stomach tube in the morning before feeding at 30 days interval throughout the period of study and at 15 days interval after institution of treatment.

Rumen liquor was strained through double layer of muslin cloth and stored at -15°C for estimation of various minerals. Similarly, CSF from animals of group T_2 and T_3 was collected at 30 days interval throughout the period of induction of hypophosphataemia and at 15 days interval after instituting the treatment.

3.5 Analysis of rumen liquor, blood/plasma, tissues and cerebro-spinal fluid samples

3.5.1 Blood analysis

3.5.1.1 **Haemogram-** The haemoglobin was estimated by Drabkins cyanomethaemoglobin method (Raymond and Wilkinson, 1963). Total erythrocytic count (TEC) was determined by using haemocytometer (Benjamin, 1985) whereas the haematocrit was determined with haematocrit tubes (Wintrobe, 1962).

3.5.1.2 **Scanning Electron Microscopy (SEM) of erythrocytes**

Gluteraldehyde mixed blood was centrifuged at 500 rpm for two minutes and excess of gluteraldehyde was subsequently removed by washing once with phosphate buffer of 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 put on stubs with the help of micro-pipette, allowed to settle down and dry. Sputtering was done with gold using fine coat ion sputter[a]. The cells were then studied under desired magnification in Digital SEM- 6100[b].

3.5.2 **Blood/Plasma biochemical and minerals analysis**

3.5.2.1 **Superoxide dismutase in erythrocytes** - The activity of superoxide dismutase was determined by the method of Marklund and Marklund (1974). The assay of superoxide dismutase is based upon the ability of this enzyme to inhibit the auto-oxidation of pyrogallol in the presence of ethylene diamine tetra-acetic acid (EDTA).

3.5.2.2 **Lipid peroxidation in erythrocytes (rbcs)**- The lipid peroxidation in rbcs was determined by

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a) Fine Coat Ion Sputter, JFC-1100 (JEOL), Japan.

b) Digital SEM, JSM-6100 (JEOL), Japan.

measuring the malondialdehyde (MDA) produced using thiobarbituric acid (Stocks and Dormandy 1972). The values were expressed as nmole MDA produced/g Hb/hour.

3.5.2.3 **Plasma phosphorus and molybdenum-**Plasma inorganic phosphorus was estimated spectrophotometrically by using Span[a] Diagnostic Reagent kits by Gomorri's Method. Molybdenum was estimated spectrophotometrically, by acetone reduction of thiocyanate as per the method of Ellis and Olson (1950).

3.5.2.4 **Plasma copper and iron-** Plasma concentrations of copper and iron were determined by atomic absorption spectrophotometer (Model AA6, Varian Techtron, Melbourne, Australia) (Ludmilla, 1976).

For the minerals analysis, a known volume of plasma sample was digested with triple acid in the ratio of 10:3:1 (conc. nitric acid+ 70% perchloric acid+ conc. sulphuric acid). The final volume was made up to 8 ml with triple distilled water and the samples were run through atomic absorption spectrophotometer.

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a) Span Diagnostics Ltd. Surat, India.

3.6 **Rumen liquor and cerebro-spinal fluid analysis-** The minerals viz phosphorus, molybdenum and iron were determined in rumen liquor and cerebro-spinal fluid as described under 3.5.2.3 and 3.5.2.4.

3.7 **Radiographic and morphological changes in bones-** Radiographs of the fore limb and hind limbs below knee and hock joints were taken once 90 days post induction of hypophosphataemia.

3.8 **Pathomorphological changes:**

After death of each animal, post mortem was conducted, gross changes noted and various tissues viz lungs, spleen, liver, kidneys and bones were collected in 10% buffered formalin for histopathology. The tissues were processed as per standard procedure and stained with haematoxylin and eosin and various changes were noted microscopically. Similarly, pathological changes were observed in the bone sections after demineralisation, processing and staining with haematoxylin and eosin stain as per standard procedure.

3.9 **Therapeutic measure-** Treatments in 5 animals of group T_2 was undertaken at 91st day post-

induction of hypophosphataemia following oral administration of sterilised bone meal @ 35g/100kg body weight orally daily for 30 days.

3.10 **Statistical analysis-** The results were subjected to student's 't' test. Significance of difference of the mean values of various parameters between T_1 & T_2 and T_1 & T_3 groups of experimental buffalo calves was tested at 1 per cent and 5 per cent level of probability by applying simple t-test. (Snedecor and Cochran 1976).

CHAPTER IV

RESULTS

4.1 Studies on experimentally induced hypophosphataemia in buffalo calves

4.1.1 Clinical examination: All the animals were clinically healthy, alert, active and having fair body conditions. The mean body temperature, respiration and heart rate of the animals were within 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 (90th day) of clinical signs were 96.79 and 92.83 kg, respectively.

Animals of ground T_2 and T_3 showed general symptoms of rough hair coat, alopecia with discolouration of skin followed by slight depigmentation (Fig.1). Reddening of hair(achromotricia) was observed in all the animals of these groups (Fig.2). Loss of muscle mass, weakness, lameness (Fig.3) sunken eyes with lacrimation followed by recumbency and inability to stand (Fig.4). There was no mutual plucking of hair and pica was not observed. The clinical signs of weakness and other related symptoms were more marked in group T_2 as compared to group T_3. The increased intensity of clinical symptoms in groups T_2 and T_3 was evident on 60th and 70th day, respectively however sternal recumbency was evident on 70th day in group T_2 and 75th day in group T_3. The persistence of sternal recumbency lasted for several days with inability of the animal to remain in standing position followed by sudden falling when

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Fig.1. Alopecia and discolouration of skin in experimental buffalo calf following induction of hypophosphataemia.

Fig.2. Alopecia along with reddening of hair (achromotrichia) following induction of hypophosphataemia and molybdenosis in experimental buffalo calf.

Fig.3. Lameness in hock joint and loss of musle mass of hind quarter following induction of hypophosphataemia in experimental buffalo calf.

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Fig.4. Sunken eyes, cachexia, hide bound condition weakness with inability to stand hypophosphataemic buffalo calf.

Fig.5. Lateral recumbency following severe phosp depletion in experimental buffalo calf.

tried to walk or made to stand with physical support. At terminal stages animals were active, showing apathy and increased heart rate. The further deterioration of condition resulted in lateral recumbency (Fig.5) terminating in death of the animal.

4.2 Blood/ plasma analysis

4.2.1 Haemogram

The haematological indices of all the three groups of experimental buffalo calves have been presented in Table 1 (Fig. 6 to 8). The mean values of Hb, PCV and TEC in healthy control group (T_1) ranged from 9.49±0.27 to 11.47±0.39 g/dl, 31.32±0.84 to 37.6±1.21 per cent and 5.22±0.41 to 6.25±0.62x10^6/mm^3, respectively throughout the period of experimentation. The comparison of these values at different time intervals revealed non-significant fluctuations.

The mean values of Hb in group T_2 showed significant ($P<0.01$) decrease on 15th(9.19±0.35 g/dl), 30th (28.32±0.68 g/dl), 45th (8.53±1.26 g/dl), 60th (9.43±0.28 g/dl) and except 75th day (11.83±0.48 g/dl) where it showed significant ($P<0.01$) increase as compared to respective control values (9.49±0.27, 9.59±0.25, 9.79±0.29, 10.65±0.47 and 11.47±0.39 g/dl). The mean values of Hb in group T_3 also showed significant ($P<0.01$) decline on day 30th (8.98±0.15 g/dl), 60th (9.27±0.31 g/dl) and 75th (10.22±0.28 g/dl) and rise on day 45th (10.09±0.22 g/dl) as compared to respective control values.

The mean values of PCV in group T_2 showed significant

Table 1: Haematological indices of experimental buffalo calves

Parameters	Group	Days						
		0	15	30	45	60	75	90
Hb (g/dl)	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
PCV (%)	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 ($X10^6/mm^3$)	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

** Significant at one per cent level
* Significant at five per cent level

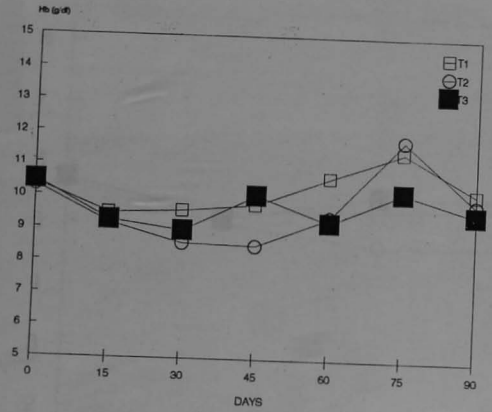


FIG 6:COMPARATIVE HAEMATOLOGICAL INDICES IN THREE GROUPS OF EXPERIMENTAL BUFFALO CALVES

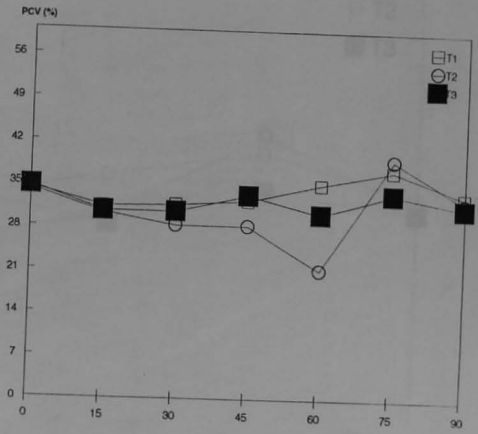


FIG. 7:COMPARATIVE VALUES OF PCV IN GROUPS OF EXPERIMENTAL BUFFALO CALVES

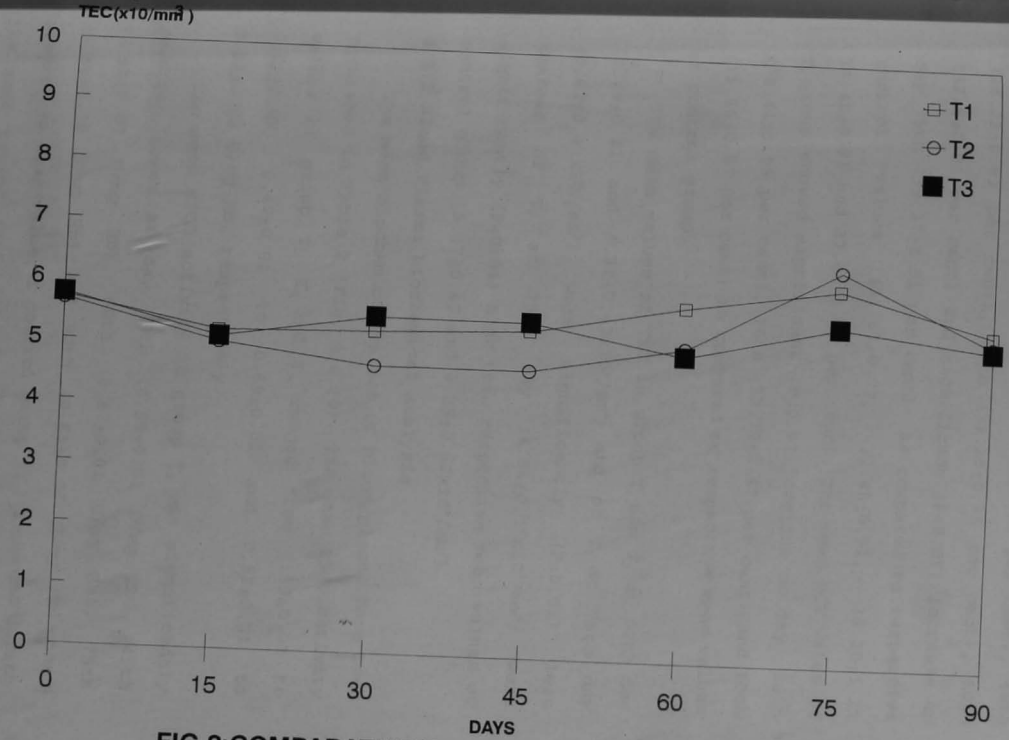


FIG.8:COMPARATIVE VALUES OF TEC IN THREE GROUPS OF EXPERIMENTAL BUFFALO CALVES

($P<0.01$) decline on day 30th (28.32±0.68 per cent), 45th (28.32±0.83 per cent), 60th (31.25±0.85 per cent), 90th (32.5±0.01 per cent) and significant ($P<0.05$) increase on day 75th (39.37±1.51 per cent) as compared to respective control values (31.67±0.77, 32.40±0.91, 35.20±1.21 33.42±0.91 and 37.60±1.21 per cent). The mean PCV value of T_3 group showed significant ($P<0.01$) decline on day 60th (30.41±1.09 per cent), 75th (33.75±0.87 per cent) and 90th (31.66±0.87 per cent) as compared to respective mean values of control group.

The mean values of TEC in group T_2 and T_3 at 90th day (5.39±0.81 and 5.31±0.43x10^6/mm^3) and of T_3 at 75th day (5.62±0.41x10^6/mm^3) were significantly ($P<0.01$) less whereas of T_2 at 75th day (6.56±0.75x10^6/mm^3) were significantly ($P<0.01$) high than respective mean values of control group (5.57±0.47 and 6.25±0.62x10^6/mm^3).

4.2.2 Blood/Plasma biochemical analysis

The mean biochemical values of blood/plasma have been furnished in Table 2 (Fig. 9 & 10). The mean ESOD activity values of group T_1, T_2 and T_3 ranged from 0.59±0.01 to 0.62±0.02, 0.47±0.01 to 0.59±0.01 and 0.55±0.01 to 0.62±0.02 U/mg Hb, respectively.

The mean ESOD activity in group T_2 was significantly ($P<0.01$) lower at day 15th (0.55±0.01 U/mg Hb), 45th (0.49±0.03 U/mg Hb), 60th (0.47±0.01 U/mg Hb), 75th (0.50±0.02 U/mg Hb) and 90th (0.51±0.01 U/mg Hb) than respective mean value of control group (T_1) recorded during the same period (0.61±0.01 U/mg Hb, 0.59±0.01 U/mg Hb,

Table 2: Alterations in erythrocytic enzymes of buffalo calves

Parameters	Group	Days						
		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	0.59 ±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
MDA (nmole/gHb/h)	T_1	90.165 ±11.34	92.94 ±11.71	117.04 ±14.82	278.08 ±36.37	222.27 ±90.14	316.05 ±39.52	110.8 ±42.30
	T_2	107.52 ±8.85	110.42 ±8.92	138.6 ±12.1	330.57 ±72.61	254.34 ±18.75	375.45 ±24.96	126.9 ±19.0
	T_3	95.46 ±21.60	99.18 ±22.60	122.12 ±27.4	253.35 ±24.29	254.31 ±9.20	264.87 ±77.63	151.72 ±27.41

** Significant at one per cent level
* Significant at five per cent level

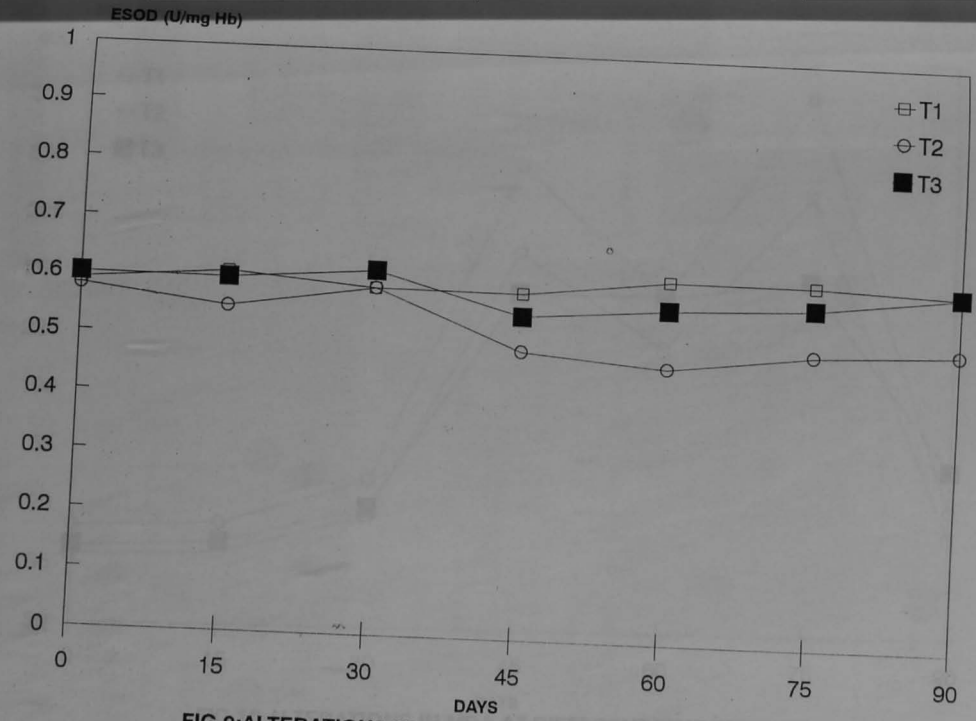


FIG.9:ALTERATIONS IN ESOD AT DIFFERENT TIME INTERVALS IN THREE GROUPS OF EXPERIMENTAL BUFFALO CALVES

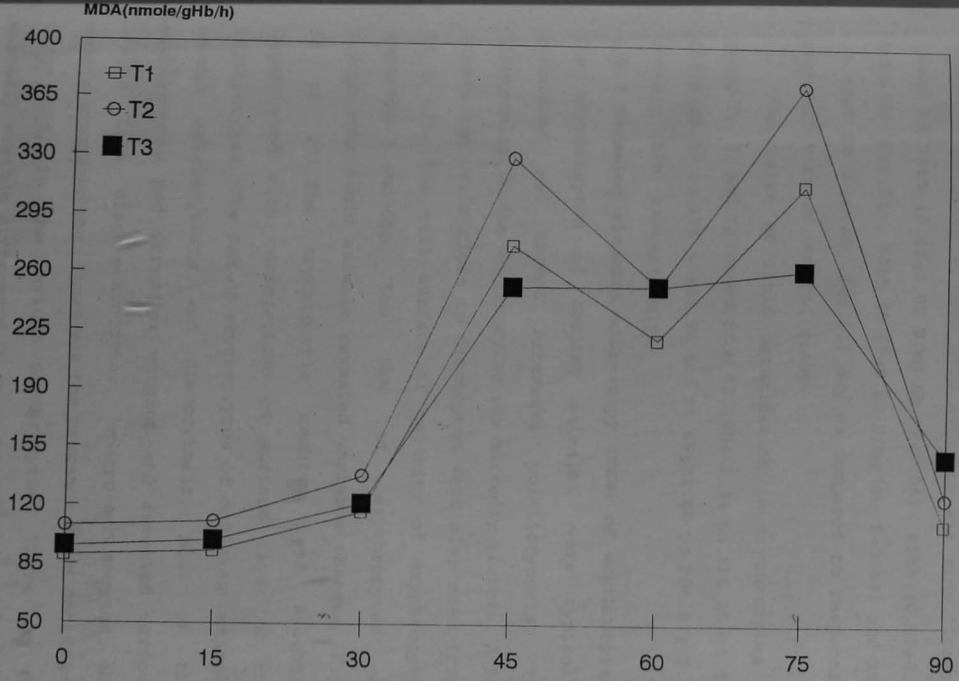


FIG.10:ALTERATIONS IN MDA AT DIFFERENT TIME INTERVALS IN THREE GROUPS OF EXPERIMENTAL BUFFALO CALVES

LS

0.62±0.01U/mg Hb, 0.62±0.02 U/mg Hb and 0.61±0.03 U/mg Hb). However, in group T_3 mean ESOD activity was significantly lower on 30th (0.62±0.02 U/mg Hb; $P<0.05$), 45th (0.55±0.01 U/mg Hb; $P<0.01$), 60th (0.57±0.01 U/mg Hb; $P<0.01$) and 75th (0.58±0.02 U/mg Hb; $P<0.01$) day as compared to respective mean activity of control group.

The value of lipid peroxidation in erythrocytes in group T_1, T_2 and T_3 ranged from 90.165±11.34 to 316.05±39.52; 107.52±8.85 to 375.45±24.96 and 95.46±21.60 to 264.87±77.63 nmole/g Hb/h, respectively.

4.2.3 Scanning electron microscopy (SEM) of erythrocytes- The erythrocytes of healthy animals were typically biconcave and showed extensive poikilocytosis. The biconcavity in the erythrocytes was markedly visible. A few uniconcave cells close to discocytes were also seen (Fig. 11 & 12). The cell surface of majority of erythrocytes appeared smooth. The SEM of erythrocytes of hypophosphataemic animals revealed variable changes (Fig. 13 to 20).The erythrocytic membrane was slightly degenerated with corrugations on surface layer of the erythrocytes. The SEM of erythrocytes of deficient animals showed spherocytosis and discocytosis. Most of the erythrocytes had irregular margins and revealed various shapes like disco-echinocytes, sphero-echinocytes and sphero-acanthocytes. There was the presence of unidentified large body in the various erythrocytes. There was no markedly visible difference in the erythrocytes of group T_2 and T_3.

Fig.11. SEM of erythrocytes of healthy buffalo calves showing poikilocytosis x 22,200.

Fig.12. SEM of a typical biconcave erythrocyte from a healthy buffalo calf x 78,000.

Fig.11. SEM of erythrocytes of healthy buffalo ca showing poikilocytosis x 22,200.

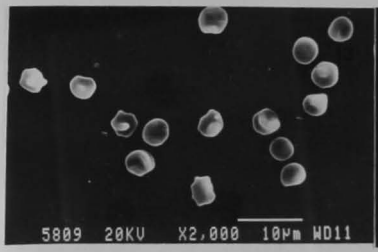
Fig.12. SEM of a typical biconcave erythrocyte healthy buffalo calf x 78,000.

Fig.13. Sphero-echinocytosis in hypophosphataemic buffalo calves x 12,000.

Fig.14. Discocyte with slight corrugation of the erythrocytic membrane in hypophosphataemic buffalo calves x 72,000.

Fig.15. SEM of a typical acanthocyte from hypophosphataemic animal x 72,000.

Fig.16. SEM of erythrocyte from hypophosphataemic buffalo calf depicting membrane alterations x 72,000.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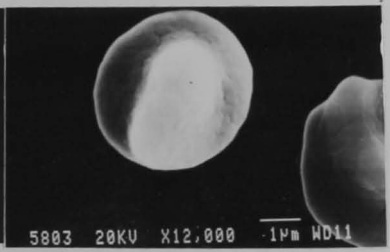
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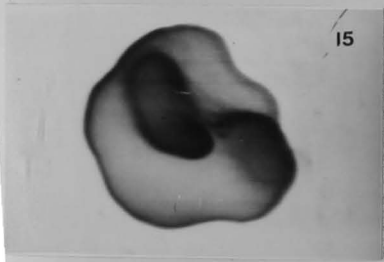
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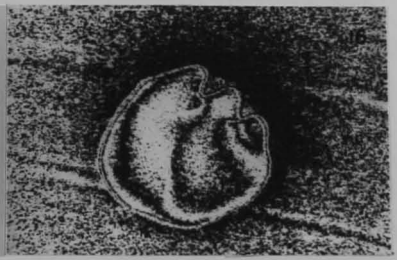
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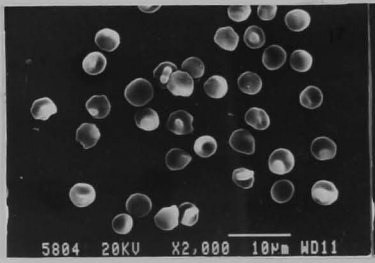
Fig.17. SEM depicting echinocytes and discocytes with a few normal erythrocytes from hypophosphataemic and molybdenotic buffalo calves x 12,000.

Fig.18. SEM showing discocytes and acanthocytes in hypophosphataemic and molybdenotic buffalo calves x 11,400.

Fig.19. SEM of discocytes and echinocytes from hypophosphataemic animals x 21,000.

Fig.20. SEM indicating discocytes, spherocytes and acanthocytes in hypophosphataemic and molybdenotic animals x 27,000.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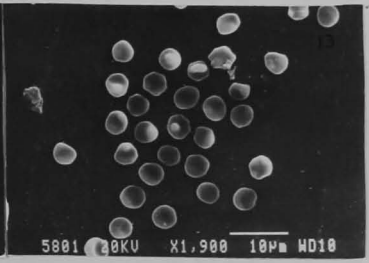
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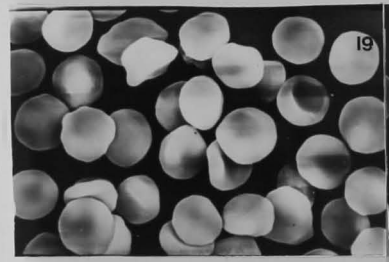
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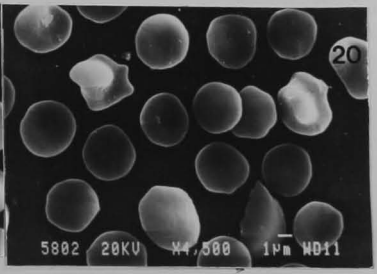
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4.3 Plasma Macro- and Micro-Elements

The alterations in the concentration of plasma macro- and micro- elements in all the three groups of experimental buffalo calves have been presented in Table 3 (Fig. 21 to 24).

The mean value of plasma inorganic phosphorus in control group T_1 ranged between 6.03±0.94 and 7.77±1.21 mg/dl throughout the period of study. While the values for group T_2 at 45th (4.73±1.27 mg/dl), 60th (4.03±0.49 mg/dl), 75th (3.03±0.63 mg/dl) and 90th (2.6±0.47 mg/dl) day were significantly ($P<0.01$) less than respective control mean values (6.03±0.94, 6.54±1.19, 6.97±0.81 and 6.72±0.47 mg/dl). Mean values of plasma inorganic phosphorus in group T_3 at 60th day (2.95±0.63 mg/dl) was significantly ($P<0.05$) lower which persisted even at 1 per cent level of significance at 75th (3.50±0.49 mg/dl) and 90th (2.72±0.49 mg/dl) days than respective control values (6.54±1.19 mg/dl, 6.97±0.81 mg/dl and 6.72±0.77 mg/dl).

The mean plasma copper values of group T_2 and T_3 ranged between 93.25±10.01 and 168.78±40.70 ug/dl and 106.6±0.00 and 119.9±13.32 ug/dl and were comparable to respective mean values of control group T_1 ranging between 105.2±15.01 and 127.9±12.84 ug/dl. The mean values of plasma copper of group T_2 were significantly ($P<0.01$) lower at day 45th (106.6±10.00 ug/dl), 60th (109.00±10.38 ug/dl), 75th (93.25±10.01 ug/dl) and 90th (96.72±8.2 ug/dl) than respective control values of 127.26±6.42, 127.9±12.84, 116.31±6.56 and 113.24±8.53 ug/dl.

Table 3: Minerals concentration in plasma of experimental buffalo calves

Parameters	Group	Days						
		0	15	30	45	60	75	90
Inorganic Phosphorus (mg/dl)	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
	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 (ug/dl)	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
	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 (ug/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
Molybdenum (ug/ml)	T_1	0.93 ±0.21	0.98 ±0.11	1.07 ±0.18	-	1.13 ±0.13	1.25 ±0.27	1.17 ±0.16
	T_2	1.06 ±0.16	2.11* ±0.81	3.71** ±1.78	-	6.22** ±0.83	8.15** ±0.91	9.11** ±1.02
	T_3	0.87 ±0.23	0.99 ±0.31	-	1.09 ±0.20	-	1.17 ±0.39	1.20 ±0.36

** Significant at one per cent level
* Significant at five per cent level

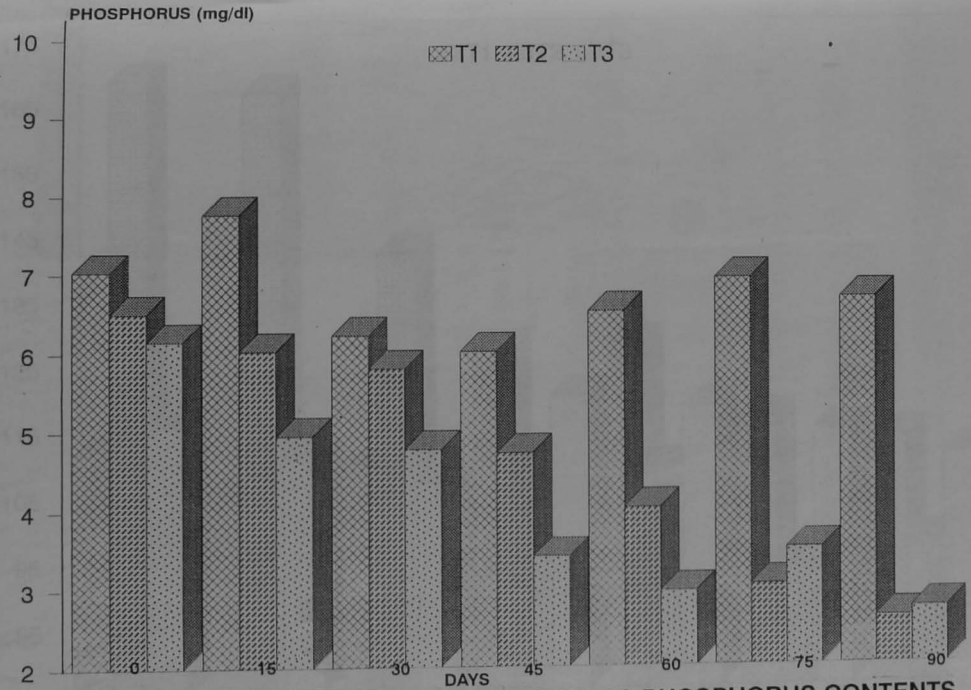

FIG.21:ALTERATIONS IN PLASMA INORGANIC PHOSPHORUS CONTENTS IN THREE GROUPS OF EXPERIMENTAL BUFFALO CALVES

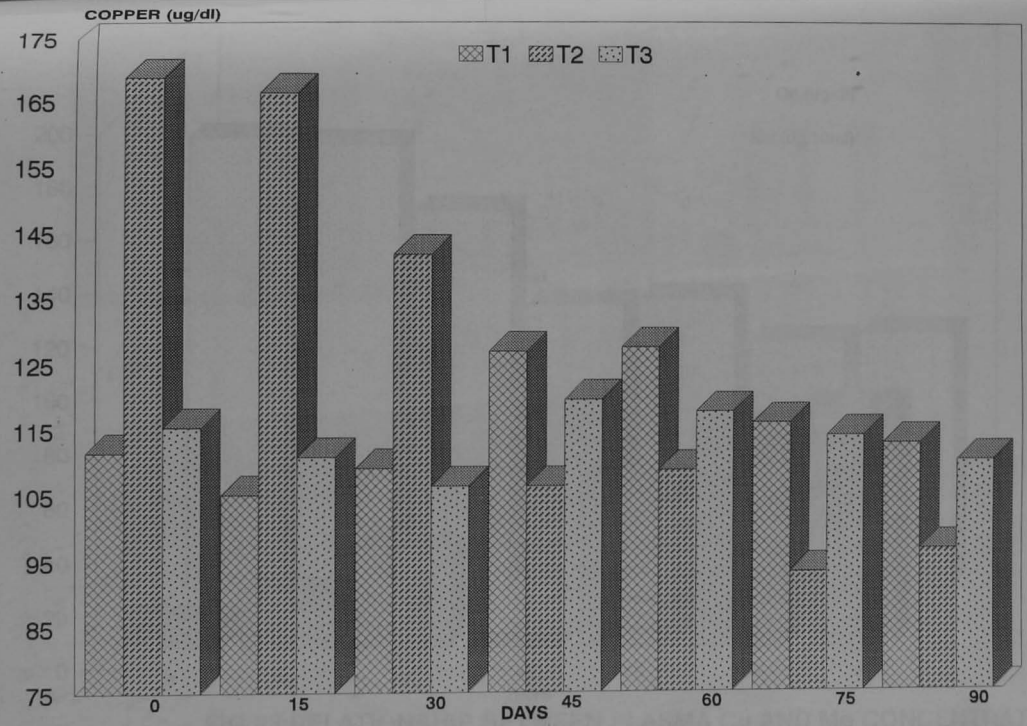

FIG.22:ALTERATIONS IN PLASMA COPPER CONTENTS IN THREE GROUPS OF EXPERIMENTAL BUFFALO CALVES

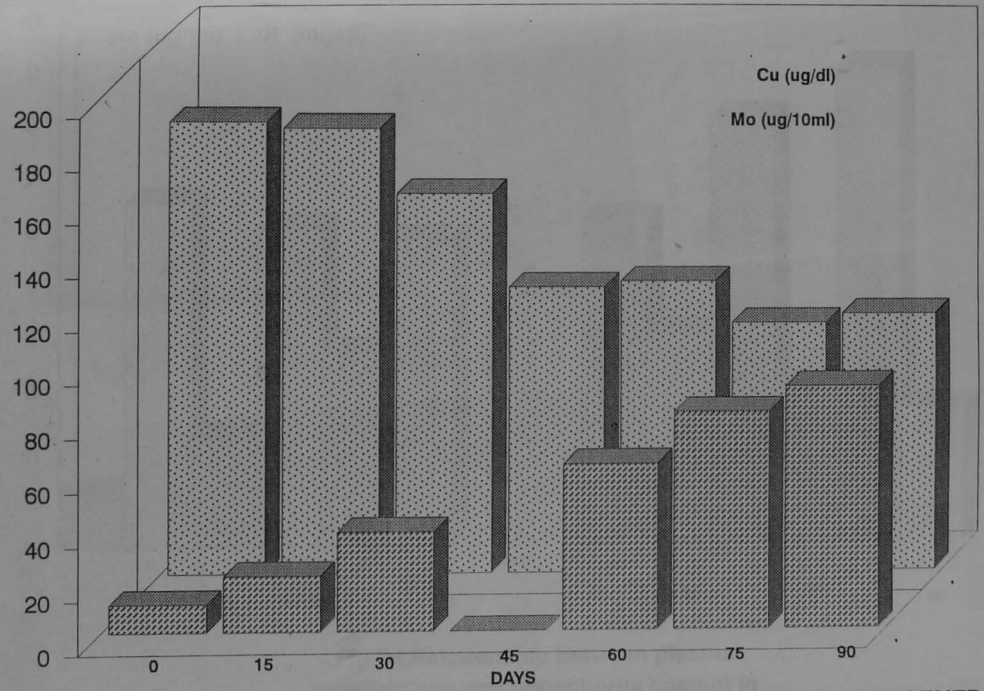

FIG.23:RELATIONSHIP BETWEEN PLASMA Cu AND Mo CONCENTRATION IN HYPOPHOSPHATAEMIC AND MOLYBDENOTIC BUFFALO CALVES

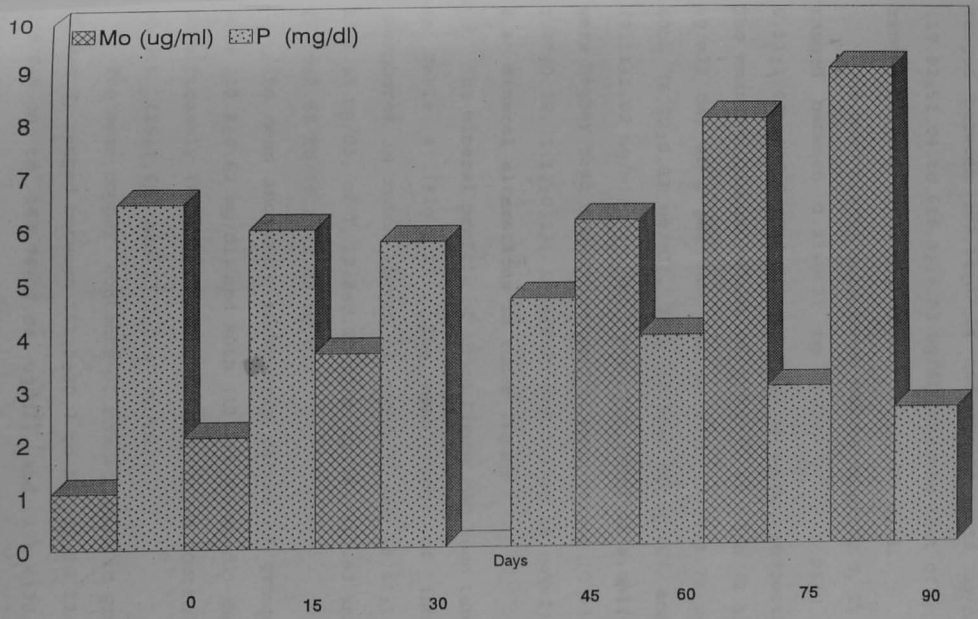

Fig.24:Relationship between plasma molybdenum and phosphorus content in hypophosphataemic & molybdenotic animals

The mean plasma iron concentrations of group T_2 and T_3 ranged between 302.14±67.04 and 624.30±16.58 ug/dl and 319.92±51.04 to 618.3±16.53 ug/dl, whereas that of group T_1 ranged between 338.85±18.36 and 616±17.73 ug/dl.

The mean plasma molybdenum values of group T_1, T_2 and T_3 ranged between 0.93±0.21 to 1.25±0.27 , 1.06±0.16 to 9.11±1.02 and 0.87±0.23 to 1.17±0.39 ug/ml, respectively. The mean molybdenum values of group T_2 at 15th (2.11±0.81 ug/ml) day at 5 per cent and on 30th (3.17±1.78 ug/ml), 60th (6.22±0.83 ug/ml), 75th (8.15±0.91 ug/ml) and 90th (9.11±1.02 ug/ml) day at 1 per cent level of significance were higher than respective control mean values (0.98±0.11, 1.07±0.18, 1.13±0.13, 1.25±0.27 and 1.17±0.16 ug/ml).

4.4 Mineral alterations in rumen liquor

The mineral profile of rumen liquor has been tabulated in Table 4 (Fig. 25). The mean range of inorganic phosphorus in rumen liquor of group T_1 was 15.41±1.21 to 18±1.65 mg/dl, of T_2 12.8±0.62 mg/dl to 16.93±0.83 and of T_3 12.66±2.68 to 16.13±3.28 mg/dl.

The mean inorganic phosphorus values of group T_2 at 60th (12.8±0.62 mg/dl) and 90th (13.2±0.47 mg/dl) day were significantly ($P<0.01$) lower than respective control group values (18±1.65 and 13.2±0.47 mg/dl).

The mean copper contents of rumen liquor in group T_1, T_2 and T_3 ranged between 151.0±30.7 to 186.55±30.77 ug/dl; 106.6±0.00 to 168.76±12.54 ug/dl; and 124.4±30.76 to 177.6±39.75 ug/dl, respectively. The mean values of copper in group T_2 and T_3 at 30th day (115.4±40.69 and 124.40±30.76

Table 4: Mineral concentration in Rumen Liquor of experimental buffalo calves

Parameters	Group	Days			
		0	30	60	90
Inorganic phosphorus (mg/dl)	T_1	15.92 ±1.40	17.82 ±2.92	18.00 ±1.65	15.41 ±1.27
	T_2	16.53 ±1.81	16.93 ±0.83	12.80** ±0.62	13.20** ±0.47
	T_3	16.13 ±3.28	15.73 ±3.19	12.66** ±2.68	13.40** ±0.31
Copper (ug/dl)	T_1	160.34 ±19.50	159.90 ±0.00	186.55 ±30.77	151.00 ±30.70
	T_2	168.76 ±12.54	115.40** ±40.69	159.90** ±67.00	106.06** ±0.00
	T_3	151.00 ±55.48	124.40** ±30.76	177.60 ±39.75	159.90 ±15.20
Iron (ug/dl)	T_1	2619.15 ±547.76	2412.80 ±502.80	2825.90 ±673.21	3199.00 ±1071.76
	T_2	2132.80 ±832.16	2532.80 ±673.20	4865.50** ±659.73	5199.00** ±689.23
	T_3	3065.90 ±757.05	2799.30 ±733.03	3599.20* ±188.37	2932.60 ±532.70

** Significant at one per cent level
* Significant at five per cent level

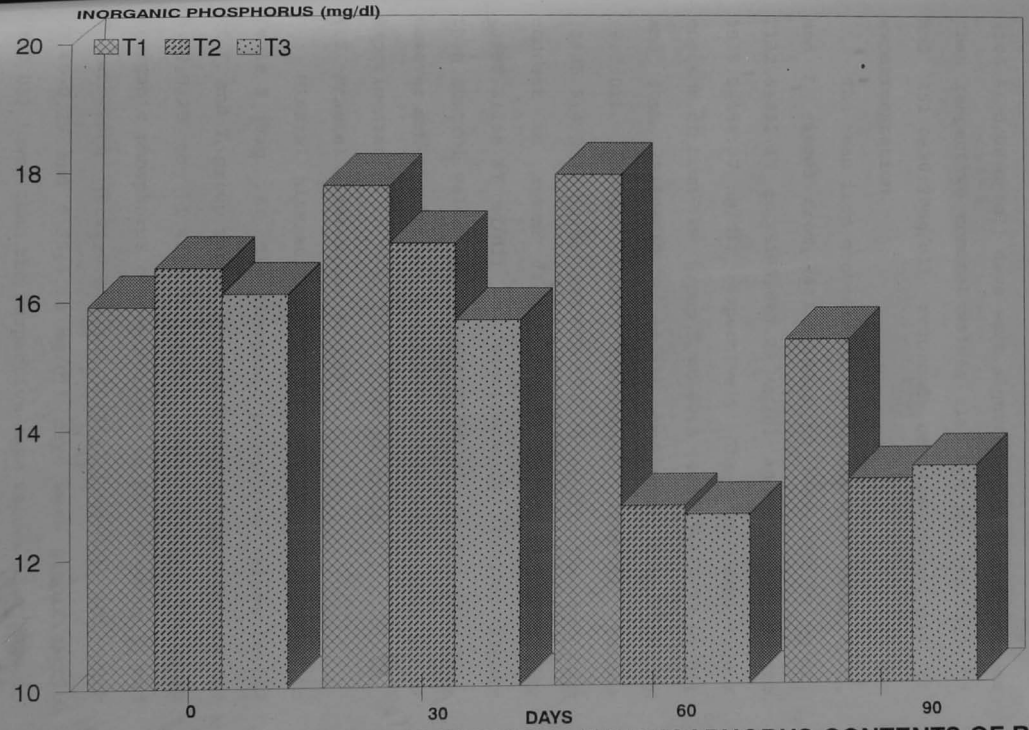

FIG.25:ALTERATIONS IN INORGANIC PHOSPHORUS CONTENTS OF RUMEN LIQUOR IN THREE GROUPS OF EXPERIMENTAL BUFFALO CALVES

ug/dl) and in group T_2 at 60th (159.9±67.00 ug/dl) and 90th (106.6± 0.00 ug/dl) days were significantly ($P \leq 0.01$) lower than respective control values (159.9±0.00, 186.55±30.77 and 151.0±30.70ug/dl) recorded on the same day of experimentation.

The mean iron contents of rumen liquor of group T_1, T_2 and T_3 ranged from 2412.8±502.8 to 3199±1071.76 ug/dl; 2132.8±832.16 to 5199±689.23 ug/dl and 2932.6±532.7 to 3599.2±188.37 ug/dl, respectively. The mean rumen liquor content of iron in group T_2 at 60th (4865.5±659.73 ug/dl) and 90th (5199±689.23 ug/dl) day were significantly ($P<0.01$) higher than respective control values (2825.9±673.21 ug/dl, 3199.0±1071.76 ug/dl). The mean iron content of rumen liquor of T_3 group at 60th day (3599.2±188.37 ug/dl) was significantly ($P<0.05$) higher than control value (2825.9±673.21 ug/dl) at the same day. However non-significant decline was recorded on 90th day of experimentation in mean iron value of group T_3.

4.5 Mineral alterations in CSF

Mineral alterations in CSF have been presented in Table 5 (Fig. 26). The inorganic phosphorus values in CSF of T_2 and T_3 group ranged between 0.21±0.17 to 1.0±0.14 and 0.40±0.29 to 1.46±0.40 mg/dl, respectively. The mean inorganic phosphorus concentrations in CSF of group T_2 and T_3 at 60th (0.21±0.17 and 0.56±0.11mg/dl) and 90th (0.40±0.29 and 0.59±0.18 mg/dl) days were significantly ($P<0.01$) lower than the respective base values of 0.98±0.13 and 1.46±0.40 mg/dl.

Table 5: Mineral concentrations in CSF of experimental buffalo calves

Parameters	Group	Days			
		0	30	60	90
Inorganic Phosphorus (mg/dl).	T_2	0.98 ±0.13	1.00 ±0.14	0.56** ±0.11	0.21** ±0.17
	T_3	1.46 ±0.40	1.41 ±0.96	0.59** ±0.18	0.40** ±0.29
Copper (ug/dl)	T_2	112.40 ±20.32	106.50 ±18.03	106.50 ±19.23	88.75* ±15.97
	T_3	106.50 ±19.17	100.50 ±17.06	71.00 ±12.78	75.45 ±13.58
Iron (ug/dl)	T_2	2400.20 ±432.00	2489.00 ±145.33	2222.50* ±400.05	2266.80 ±44.25
	T_3	2248.30 ±209.91	2311.00 ±416.03	2133.60 ±384.04	2103.80 ±357.90

** Significant at one per cent level
* Significant at five per cent level

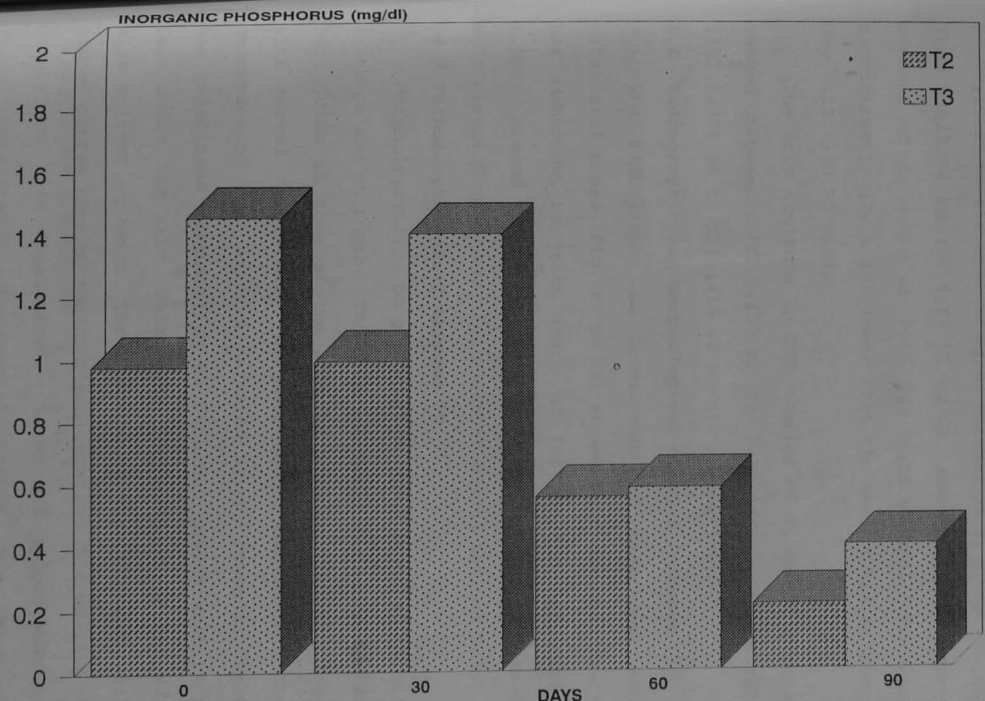

FIG.26:ALTERATIONS IN INORGANIC PHOSPHORUS CONTENTS OF CSF IN T2 AND T3 GROUPS OF EXPERIMENTAL BUFFALO CALVES

The mean copper contents of CSF in T_2 group ranged from 88.75±15.97 to 112.4±20.32 ug/dl whereas in T_3 group ranged from 71±12.78 to 106.5±19.17 ug/dl. However, CSF copper content of T_2 group at 90th (88.75±15.97ug/dl) day was significantly ($P< 0.05$) lower than the mean value at zero day (112.4±20.32 ug/dl).

The iron contents in CSF samples of T_2 and T_3 groups ranged between 2222.5±400.05 to 2489±145.33 ug/dl and 2103.8±357.90 to 2311±416.03 ug/dl, respectively.

4.6 Radiographic and morphological changes in bones

The radiographs of lower ends of metacarpal and metatarsal bones (Fig. 27 to 32) showed mild changes. There was reduction in joint space of fetlock and periosteal lipping around distal extremities of metacarpal. Increased soft tissue density was also observed around fetlock joint.

4.7 Pathomorphological changes

Morphologically, on post-mortem bones appeared normal in shape and strength. The synovial fluid was increased in hock joint in some of the animals however, it was clear with normal consistency. On post-mortem examination the carcasses of the animals revealed severe anaemia. There was gelatinous degeneration of fat. Fat depots were very less under the skin. Gelatinous degeneration of fat was also evident in heart. The musculature was yellowish with kidney cortex showing *khakhi* discolouration. The medulla in kidney was also degenerated. Liver and spleen were of normal consistency. Histopathologically, bone sections (Fig. 39 & 40) revealed enlargement of haversian canals.

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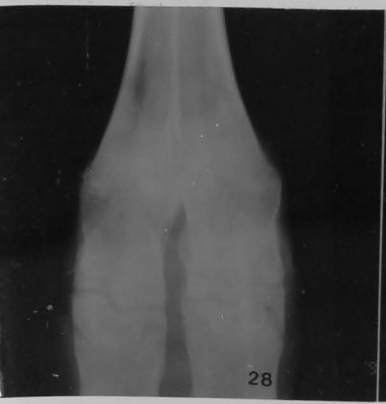
Fig.27. Dorsopalmar view of metacarpal from a healthy buffal calf.

Fig.28. Dorsopalmar view of the same calf 90th day post-induction depicting reduced joint space of fetlock.

Fig.29. Dorsopalmar view of metacarpal depicting reduced joint space of fetlock alongwith periosteal lipping 90th day post0induction.

Fig.30&31 Dorsopalmar view of metacarpal showing increased soft tissue density alongwith periosteal lipping.

Fig.32. Dorsopalmar view of metacarpal showing increased soft tissue density and periosteal reaction.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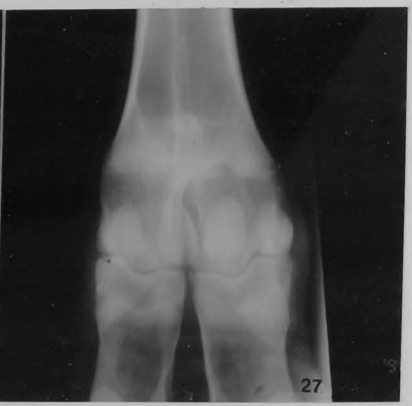
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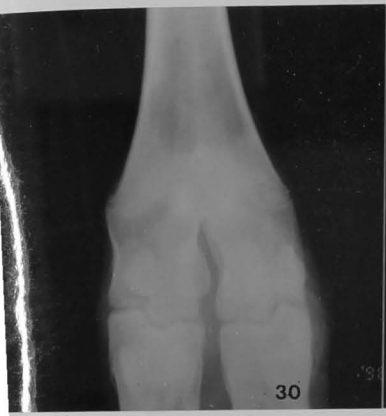
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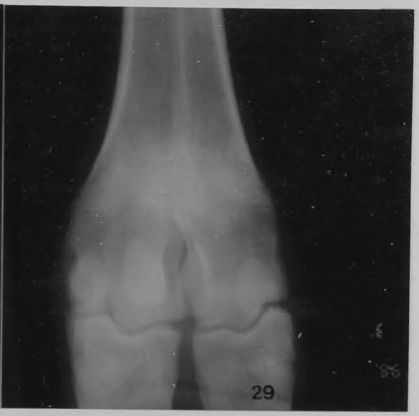
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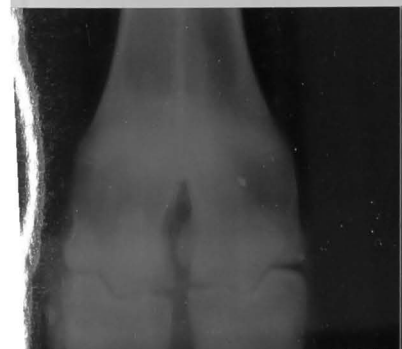
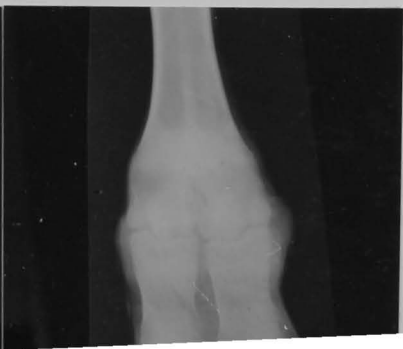




Rarefaction of bone was also evident. There was cloudy swelling and granular degeneration in liver at certain places (Fig. 33). Rarefaction of chromatin material in nuclei was evidenced by vacuolation in nucleoplasm (Fig. 34) at certain places.

In kidneys (Fig. 35 & 36) histopathological sections revealed coagulative necrosis. Extensive vacuolar degeneration with formation of casts in the uriniferous tubules alongwith haemorrhages was observed. Some of the tubules showed complete coagulative necrosis alongwith precipitation of degenerated protein material into the luminae. The glomeruli also showed vacuolation in between the capillaries.

There was extensive emphysema (Fig. 37 & 38) alongwith haemorrhages in lungs. However, there were no marked changes in spleen.

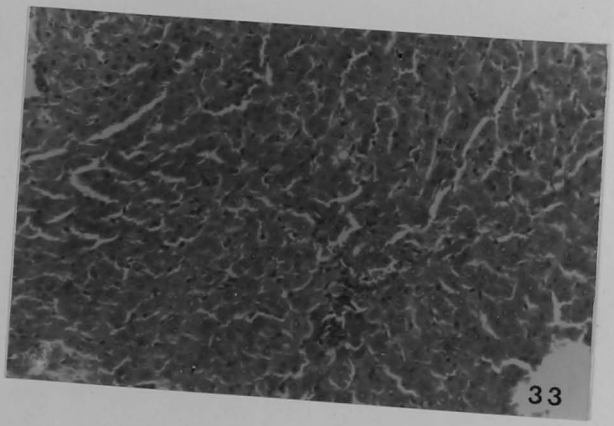
4.8 Therapeutic measure

4.8.1 Therapeutic trial in experimentally induced hypophosphataemia in buffalo calves

The treatment was undertaken in five animals of group T_2 with oral administration of sterilised bone meal daily at a rate of 35 g/100kg body weight. Before institution of treatment all the calves of group T_2 were showing clinical signs of muscular weakness, alopecia, sub normal body temperature by 60th day. However, after the commencement of treatment the chief clinical sign of weakness alleviated on 10th day of therapeutic trial followed by complete clinical recovery by 25th day post-treatment.

Fig.33. Section of liver indicating extensive degenerative changes viz. cloudy swelling, coagulative necrosis alongwith congestion and haemorrhages. H & E x 300.

Fig.34. Higher magnification of Fig.33 showing rarefaction of nuclei at certain places evidenced by vacuolation of nucleoplasm along with other changes. H & E x 600.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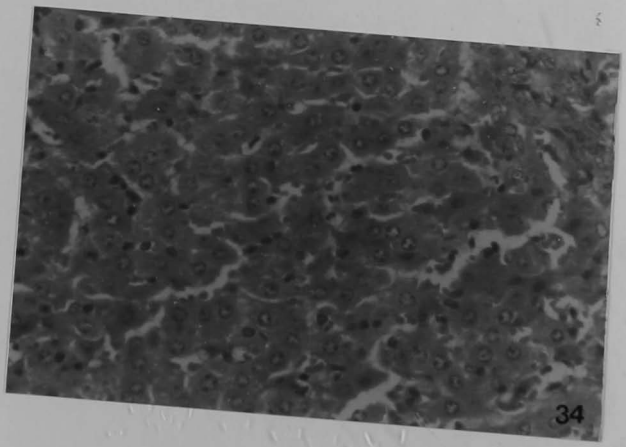
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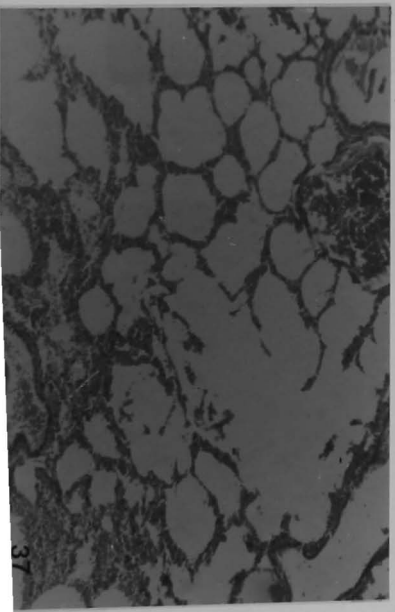
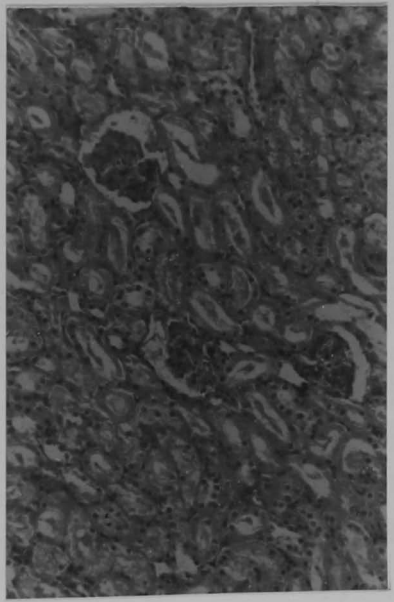
Fig.35. Section of kidney with degenerative changes comprising of cloudy swelling, coagulative necrosis, formation of casts inthe uriniferous tubules alongwith haemorrhages and increased Bowman's space. H & E x 300.

Fig.36. Section of kidney showing extensive coagulative necrosis in uriniferous tubules alongwith presence of the casts H & E x 300

Fig.37. Section of lung showing extensive emphysema and haemorrhage H & E x 150.





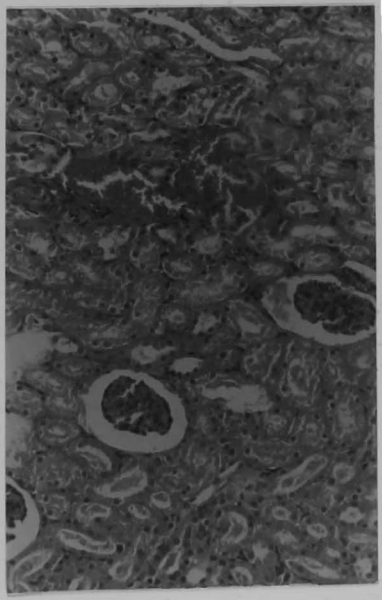
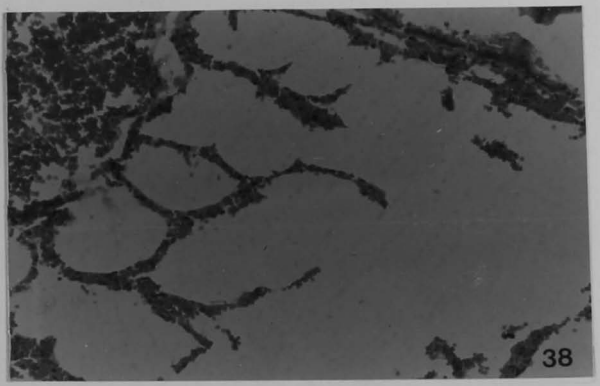


Fig.38. Higher magnification of Fig.37 showing extensive emphysema and haemorrhage H & E x 300.

Fig.39. Section fo bone indicating increased size of haversian canals H & E x 150.

Fig.40. Higher magnification of Fig.39 indicating increased size of haversian canals H & E x 300.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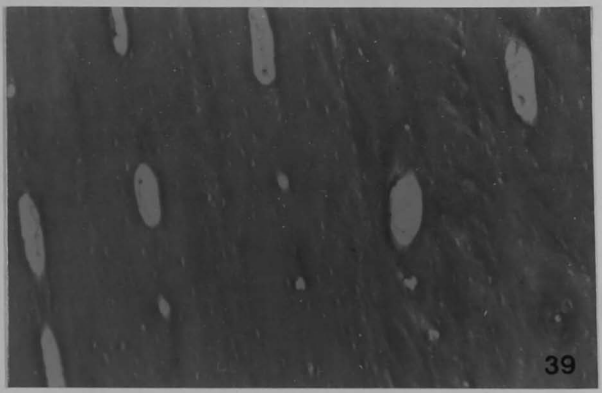
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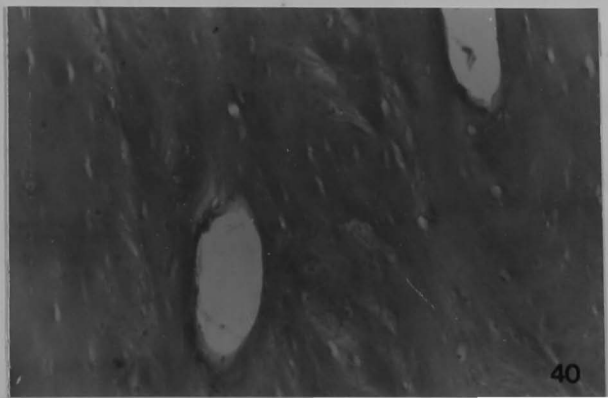
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4.8.2 Haemato-biochemical and mineral alterations in blood/plasma after therapeutic trial

The therapeutic regimen in 5 animals of group T_2 with sterilised bone meal resulted in non-significant improvement in Hb, PCV & TEC. The haemato-biochemical alterations in blood of experimental buffalo calves in therapeutic trials have been presented in Table 6.

The mean ESOD activity improved significantly at 15th day of treatment in T_2 group (0.51±0.01 U/mgHb) as compared to control value on same day (0.61±0.03 U/mgHb). There was no significant improvement in mean MDA value even after adoption of therapeutic measure. The mineral alterations in plasma following treatment have been shown in Table 7.

The mean value of inorganic phosphorus improved significantly in treated animals at 15th day of treatment (5.92±0.76 mg/dl) and was comparable to control mean value on same day (6.77±0.81 mg/dl) whereas at 30th day of treatment it (6.40±0.97 mg/dl) reached to almost same at that of control (6.52±0.63 mg/dl), respectively.

The mean value of plasma copper improved significantly ($P< 0.05$) at 30th day of treatment (159.9±8.07 ug/dl) as compared to control mean value of same day (127.54±10 ug/dl). However there was no significant change in mean value of plasma iron even after 30 days of treatment with sterilised bone meal.

4.8.3 Mineral alterations in rumen liquor and cerebro-spinal fluid after therapeutic measure

The mineral alterations in rumen liquor and CSF

Table 6: Haematological and Biochemical Alterations in experimental Therapeutic Trials in Blood of Buffalo Calves

Parameters	Group	Days		
		0	90	105
Hb(g/dl)	T_1	10.38 ±0.23	10.21 ±0.33	11.93 ±0.29
	T_2	10.30 ±0.21	9.87** ±0.11	9.79** ±0.31
PCV (%)	T_1	34.26 ±0.71	33.42 ±0.91	34.50 ±0.91
	T_2	34.20 ±0.27	32.50** ±0.01	32.50** ±0.85
TEC ($X10^6/mm^3$)	T_1	5.71 ±0.36	5.57 ±0.47	6.58 ±0.44
	T_2	5.66 ±0.27	5.39** ±0.81	5.39** ±0.43
ESOD (u/mgHb)	T_1	0.59 ±0.01	0.61 ±0.03	0.59 ±0.02
	T_2	0.58 ±0.02	0.51** ±0.01	0.58 ±0.01
MDA (nm/gHb/h)	T_1	90.165 ±11.34	110.80 ±42.30	131.64 ±17.82
	T_2	107.52 ±8.85	126.90 ±19.00	145.36 ±14.20

** Significant at one per cent level
* Significant at five per cent level

Table 6: Haematological and Biochemical Alterations in experimental Therapeutic Trials in Blood of Buffalo Calves

Parameters	Group	Days		
		0	90	105
Hb(g/dl)	T_1	10.38 ±0.23	10.21 ±0.33	11.93 ±0.29
	T_2	10.30 ±0.21	9.87** ±0.11	9.79** ±0.31
PCV (%)	T_1	34.26 ±0.71	33.42 ±0.91	34.50 ±0.91
	T_2	34.20 ±0.27	32.50** ±0.01	32.50** ±0.85
TEC ($X10^6/mm^3$)	T_1	5.71 ±0.36	5.57 ±0.47	6.58 ±0.44
	T_2	5.66 ±0.27	5.39** ±0.81	5.39** ±0.43
ESOD (u/mgHb)	T_1	0.59 ±0.01	0.61 ±0.03	0.59 ±0.02
	T_2	0.58 ±0.02	0.51** ±0.01	0.58 ±0.01
MDA (nm/gHb/h)	T_1	90.165 ±11.34	110.80 ±42.30	131.64 ±17.82
	T_2	107.52 ±8.85	126.90 ±19.00	145.36 ±14.20

** Significant at one per cent level
* Significant at five per cent level

Table 7: Alterations in mineral constituents of plasma in therapeutic trial of experimental buffalo calves

Parameters	Group	Days			
		0	90	105	120
Inorganic phosphorus (mg/dl)	T_1	7.03 ±0.77	6.72 ±0.77	6.77 ±0.81	6.52 ±0.63
	T_2	6.50 ±1.06	2.60** ±0.47	5.92 ±0.76	6.40 ±0.97
Copper (ug/dl)	T_1	111.57 ±15.38	127.90 ±12.84	127.26 ±8.60	127.54 ±10.00
	T_2	168.78 ±40.70	106.50 ±8.20	133.25 ±4.90	159.90** ±8.07
Iron (ug/dl)	T_1	417.16 ±20.30	616.00 ±17.73	727.80 ±20.40	663.80 ±44.68
	T_2	377.68 ±104.04	619.17 ±14.23	658.92 ±122.46	666.40 ±18.70

** Significant at one per cent level
* Significant at five per cent level

following treatment have been presented in Table 8. The inorganic phosphorus values of CSF after treatment at 30th day (1.0±0.00 mg/dl) was significantly ($P<0.01$) more than at 90th post-induction (0.21±0.17 mg/dl) whereas there was no significant change in mean value of copper and iron in CSF.

The pre-treatment post-induction mean inorganic phosphorus value of rumen liquor of group T_2 was 13.20±0.47 mg/dl which, following adoption of therapy at 30th day of treatment, increased to 15.20±0.78 mg/dl which was comparable to mean control group value of the same day (17.07±1.42 mg/dl).

The value of copper in rumen liquor at 30th day of treatment (159.9±2.00 ug/dl) was comparable to respective mean value of control group (165.8±23.7 ug/dl).

The iron content of rumen liquor at 15th (5199±689.23ug/dl) and 30th day (4532.2±515.21 ug/dl) of treatment was significantly ($P<0.05$) more than respective mean control values (3199±1071.76 and 2909.8±1085.15 ug/dl).

Table 8: Mineral Alterations in Rumen Liquor and CSF in experimental Therapeutic Trials of Buffalo Calves

Parameters	Group	Days		
		0	90	120
Rumen Liquor				
Inorganic Phosphorus (mg/dl)	T_1	15.92 ±1.40	15.41 ±1.27	17.07 ±1.42
	T_2	16.53 ±1.81	13.20 ±0.47	15.20 ±0.78
Copper (ug/dl)	T_1	160.34 ±15.15	151.00 ±30.70	165.80 ±23.70
	T_2	168.76 ±12.54	106.6** ±0.00	159.9 ±2.00
Iron (ug/dl)	T_1	2619.05 ±547.76	3199 ±1071.76	2909.8 ±1085.15
	T_2	2132.8 ±832.16	5199** ±689.23	4532.2** ±515.21
CSF				
Inorganic Phosphorus (mg/dl)	T_2	0.98 ±0.13	0.56 ±0.00	1.00 ±0.00
Copper (ug/dl)	T_2	112.40 ±20.32	106.50 ±19.23	112.40 ±21.21
Iron (ug/dl)	T_2	2400.0 ±432.00	2222.5 ±400.05	2489.2 ±448.05

** Significant at one per cent level
* Significant at five per cent level

CHAPTER-V

DISCUSSION

In experimentally induced hypophosphataemic buffalo calves, the general clinical finding of poor growth, progressive emaciation, loss of muscle mass, weakness, lameness with staggering gait and anorexia were similar to those described by Underwood(1981), Verma and Gupta (1987), Blood *et al* (1989) and Haque and Verma (1992) in different animal species. The presence of alopecia could be due to deficient protein status of the body. Discolouration of skin and depigmentation of hair (achromotrichia) might be due to depletion of copper due to more intake of molybdenum as copper is a prosthetic group of enzyme tyrosinase responsible for hydroxylation of tyrosine to form DOPA (dihydroxy phenyl alanine) in the melanocytes. This was supported by findings of Damir *et al* (1988)and Randhawa (1993) in hypocuprotic animals and by Gill (1992) in phosphorus deficient animals.

The absence of allotriophagia and mutual plucking of hair was in disagreement with the findings of Gill (1992), Underwood (1981) in cross-bred cow calves. Fraser *et al* (1991) also stated that allotriophagia was not a cardinal symptom of phosphorus deficiency however it could be there in number of deficiencies or could be a vice. The increase in heart rate and force of contraction evident by increased pitch of heart sounds could be due to anaemia as supported by Brooks *et al* (1984), Gill (1992) and Haque and Verma

(1992). The more intensity of clinical symptoms observed in group T_3 could be due to the potentiating effect of molybdenum in causing phosphorus deficiency (Walkar, 1955) and was also observed by Gill (1992) in cross-bred calves.

There was a marked decline in the values of Hb, PCV and TEC reflecting development of anaemia. The macrocytic normochromic anaemia was observed by Mullins and Ramsay (1959) and Gill (1992) in phosphorus deficiency in cattle whereas Jacob and Amsden (1971) reported acute haemolytic anaemia due to severe phosphorus deficiency in human beings.

The anaemia could be ascribed to the development of copper deficiency due to low dietary intake of copper and feeding of molybdenum. Copper is responsible for absorption of iron from gastro-intestinal tract, its release from body stores and utilisation of iron in haemoglobin synthesis, so a deficiency of this decreases the life span of erythrocytes. Copper is involved at functional levels in the formation of porphyrin molecules of haemoglobin (Guyton, 1991). Lesperance *et al* (1985) and Soodan (1996) also reported significant decrease in TEC in molybdenum supplemented animals. Copper deficiency might cause decrease of cytochrome oxidase (Gallaghar, 1957) within the mitochondria thus slowing down reduction of ferric to ferrous ions required for haemoglobin formation (Kaneko, 1989). Another factor could be less feed intake leading to starvation like condition thus causing protein deficiency (Underwood, 1981) which caused reduced erythropoietin

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production (Benjamin, 1985) thereby causing a maturation block at the rubricyte level. Slight fluctuation or increase at one time or other in Hb, PCV and TEC values might be due to development of dehydration associated with reduced intake of feed and water.

Deficiency of phosphorus and molybdenum toxicity leading to concurrent deficiency of copper had caused alterations in the activity of intra-erythrocytic superoxide dismutase (ESOD) enzyme in the present study. In the red blood cells constant production of free radicals during physiological conversion of oxyhaemoglobin to methaemoglobin had been recorded (Chiu *et al*, 1982). Detoxification of these free radicals (oxidants) is brought about by several enzymatic and non-enzymatic antioxidant mechanisms.

Copper present in erythrocytes is mostly associated with ESOD which protect the red blood cells from superoxide anions generated by autoxidation of Hb. If there is oxidation stress the generation of superoxide anions increases further. These superoxide anions could cause inactivation of vital enzymes, denaturation of haemolglobin, production of Heinz bodies and peroxidation of red cell membrane lipids with subsequent hemolysis (Chiu *et al*, 1982).

There was significant ($P<0.01$) decline of mean ESOD values from 15th day and 30th day onwards in group T_2 and T_3, respectively. The decline could be due to unavailability of copper due to deficient intake or formation of copper

thiomolybdate complexes and was concordant with the findings of BohenKamp and Weser (1976), Suttle and McMurray (1983), Gartner and Weser(1983), Master *et al* (1990) and Xin *et al* (1991). Masters *et al* (1990) observed a close relationship (r=0.75) between E-Cu and ESOD on pasture rich in molybdenum.

The mean values of lipid peroxidation in group T_2 and T_3 at different days were comparable to control group throughout the period of experimentation. The significant variation in mean values in healthy controls during different days may be ascribed to seasonal effects. However, these findings were not in agreement with Parthasarthy and Steinberg (1992) and Rayssiguier *et al* (1993). The gradual increase in activity of MDA might be due to high iron concentration in blood plasma (Wills 1972, Koster and Koster, 1986 and Thomas and Aust, 1985).

Although minerals do not yield energy in the body yet they act as intermediator in the number of reactions in the body. Various minerals in the body are involved directly or indirectly in reactions controlling intermediate metabolism, as electrolytes concerned with maintenance of osmotic pressure and acid-base balance and as structural components of certain organs and tissues. Although mineral elements are only a small part of total cost of feed, a considerable reduction in net income can occur when performance is affected adversely by deficiencies, excesses or imbalances (Miller, 1974). Providing suitable amounts of essential mineral elements usually is easy, the key part is

knowing what is needed. For some of the minerals the requirements may be extremely variable and greatly influenced by many factors; the best known of these being interaction with other minerals. Phosphorus is probably the most versatile of all the mineral elements, the 20 per cent of phosphorus not present in the skeletal tissues is widely distributed in various fluids and soft tissues of the body where it serves a variety of essential functions. The mean values of plasma inorganic phosphorus in group T_2 and T_3 from 45th day onwards were significantly($P<0.01$) lower than respective mean control values.

This finding was in agreement with those of Haque and Verma (1992), Blair-West (1992) and Gill (1992) who reported decline in levels of plasma phosphorus with decrease in phosphorus intake. Underwood (1981) stated that first response to a dietary deficiency of phosphorus was a fall in the inorganic phosphate fraction of blood plasma and withdrawl of its reserves in the bones.

The hypophosphataemia observed in the present study could be ascribed to feeding of phosphorus deficient diet (Hill and Rajagopal, 1962); cattle and buffaloes being more susceptible than sheep and horses (Jubb *et al*, 1985) and due to gradual depletion and withdrawl of its reserves in the bones (Underwood, 1981). Moreover normal range of phosphorus in young calves (6-8 mg/dl) was more than that of adults (4-6 mg/dl) (Underwood, 1981) and stores being less thus their requirement might be more due to rapidly developing skeleton and particularly due to decreased

intake of phosphorus deficient diet as in the present study these experimental animals appeared to be more prone to deficiency.

A number of other factors could have also contributed to the development of hypophosphataemia. It might be due to wider or disturbed Ca: P ratio due to deficiency of phosphorus in feed (Haque and Verma 1989, Brooks *et al* 1984) or high dietary molybdenum intake which has been reported to be associated with disturbed phosphorus metabolism (Davis 1950, Arrington and Davis 1953 and Dhillon *et al* 1972). The value of plasma inorganic phosphorus in group T_2 was consistently more upto 75th day and then fell more than group T_3 on 75th and 90th day. This might be due to gradual mobilisation of phosphorus from skeletal reservoirs (Shirley *et al* 1950, Clarke and Clarke 1967). Lesperance *et al* (1985) also recorded higher plasma phosphorus level in calves supplemented with 100 ppm of molybdenum in their daily diet whereas Dhillon *et al* (1972) ascribed the presence of low serum inorganic phosphorus to high dietary molybdenum intake. The more intense decrease in plasma phosphorus in group T_2 as compared to T_3 after 75th day might be due to decrease of skeletal phosphorus reserves and more excretion of phosphorus through urine and faeces (Clarke and Clarke, 1967), or due to damage to liver and kidneys as revealed on histopathology, thus unable to maintain its normal levels. Gill (1992) reported rapid decrease of plasma phosphorus level in molybdenotic calves at terminal stages after 90 days.

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The mean phosphorus concentration in rumen liquor of group T_2 and T_3 were also significantly ($P<0.01$) less than control animals on 60th and 90th days. This could be ascribed to low dietary intake due to feeding of deficient diet. These findings were in agreement with those of Blair-West *et al* (1992) who reported fall in mean phosphorus from 15 to 4 mmol/L in six months after feeding phosphorus deficient diet and unilateral parotid saliva drainage.

Phosphorus concentration in rumen liquor is directly linked to the feed intake and in animal feeds cereal seeds and their bye-products are the main source of phosphorus. Phosphorus content of the roughage vary with the soil on which they are grown as well as fertilizer provided (Dutt, 1977). Phosphorus content in plant decreases with age being minimum at ripening stage. Thus depleted concentration of rumen liquor inorganic phosphorus could be ascribed to feeding of urea treated wheat straw and phosphorus deficient mineral mixtures. The concentration of inorganic phosphorus in rumen liquor in the present study decreased considerably on 60th day whereas significant decrease in plasma phosphorus concentration occurred on 45th day. This variance in rumen liquor and plasma inorganic phosphorus might be because phosphorus concentration of ruminant parotid saliva was higher compared to other species and there was 10 fold more concentration of phosphorus in saliva as compared to arterial plasma due to active transport which extracts 50 per cent of the phosphorus from arterial blood passing through the gland (Compton *et al*,

1980)

High phosphorus concentration in rumen liquor was also necessary for optimal microbial fermentation and nutrition in ruminants. Thus decrease in phosphorus content in body fluids analysed in the present study might explain the failure of phosphorus deficient animals to maintain weight and body condition as compared to control calves.

The ability of animal to absorb and use phosphorus depends on the supply of vitamin D (Underwood, 1981) and presence of other antagonistic elements like Mo, Ca, Al and Fe. Hypophosphataemia condition could develop if antagonistic factors were predominant in the GIT even though adequate supply of phosphates was there in the diet.

The phosphorus content of CSF decreased gradually and significantly with decrease of phosphorus in blood. The present finding was contrary to that of Blair-West *et al* (1992) who recorded a consistently constant level of phosphorus in CSF, throughout the study in phosphorus depleted animals. It might be ascribed to increased mobilisation of phosphorus from various organs of the body due to molybdenum intake. The blood-brain barrier is only slightly permeable to the electrolyte (Guyton, 1986) so decreased concentration of phosphorus could be due to less synthesis of ATP thereby inability on the part of membrane to transport phosphorus from blood to CSF.

The mean values of plasma copper in group T_2 from 45th day onwards were significantly ($P<0.01$) less than respective controls. However, plasma copper in group T_2

first increased non-significantly upto 45th day and then decreased.

The initial increase in plasma copper might be due to the increased mobilisation of copper from hepatic stores due to supplementation of molybdenum. This was also supported by the observations of Kincaid (1980) who showed elevated copper plasma levels in calves orally administered 50 ppm molybdenum dissolved in water. This elevation in plasma copper had been ascribed to mobilisation of hepatic copper store with corresponding decline of liver copper concentration (Mills and Fell 1960 and Hogan *et al* 1968). Miller (1974) stated that tissue deposition and withdrawl were key routes in copper homeostasis. Therefore, plasma copper is not a reliable indicator of the nutritional copper status during periods of elevated intake of molybdenum. These findings explained the elevation of plasma copper in group T_2. The mean plasma copper level in group T_3 in hypophosphataemic animals was similar to the findings of Gill (1992) and Mullins and Ramsay (1959). Gill (1992) also reported lower value of copper in hypophosphataemic animals kept on farms with narrow Cu: Mo ratio (1.26) as compared to control farms (ratio 3.59).

The mean concentration of copper in group T_2 in rumen liquor was significant ($P<0.01$) low from 30th day onwards. This might be due to the fact that molybdenum in combination with sulphur produced from fermentation of feed stuff in rumen, reacted with copper to form copper-thiomolybdate complexes which were biologically unavailable

to the system (Suttle and Field 1983, Mason 1986 and Blood and Radostits 1989).

The decrease of copper in rumen liquor may also be due to the fact that thiomolybdate formed were more readily absorbed than oxygen analog molybdate (Hidiroglou *et al* (1990).

The CSF copper concentration showed a non-significant declining trend in group T_2 and T_3 throughout the period of experimentation. This might be due to the dietary deficiency of copper. The decline was more in group T_3 as compared to T_2 and was coinciding with the blood copper content which was more in group T_2 due to general tissue mobilisation because of molybdenum ingestion which was similar to the findings of Howell (1964) and Ivan *et al* (1990) in brain and Ivan *et al* (1990) in spinal cord.

Mean Iron concentration in plasma of group T_2 and T_3 showed a continuous but non-significant rising trend. These alterations were ascribed to developing anaemia associated with non-utilization of iron due to formation of copper-thiomolybdate complexes thus making copper unavailable for heme synthesis.

The mean iron content in rumen liquor in group T_2 showed significant ($P<0.01$) increase from 60th day onwards. This may be due to adequate availability of iron in the feed and due to less production of apoferritin (Guyton, 1986), a transport protein synthesised in liver, due to impairment of liver function because of molybdenum toxicity and phosphorus deficiency as evident on histopathological

examinations. Miller *et al* (1974) also inferred that absorption and tissue deposition were the very important means of homeostasis in case of iron.

Due to deficiency of phosphorus and toxicity of molybdenum resulting in changes in bones (Davis, 1950), the synthesis of blood cells in the bone marrow could be impaired thus iron probably remained circulating in the plasma bound with protein thus more negative feedback was provided to the absorption thereby making its concentration more in the rumen liquor. More concentration in rumen liquor might also be due to poor absorption because of defective digestion due to low phosphorus and copper in the feed. A non-significant increase in iron levels in the hair and tissues was suggestive of defective reutilization of endogenous iron because of decreased ceruloplasmin concentration (Jubb *et al*, 1985). The CSF iron concentration in group T_2 and T_3 also maintained a non-significant continuous decreasing trend due to low absorption because of impaired ATP production and active transport.

The mean plasma molybdenum level of group T_2 showed a rising trend and was significantly ($P<0.01$) higher after 30th day as compared to control group whereas in group T_3 was comparable to control values. The higher molybdenum level could be ascribed to increased intake of molybdenum at a rate of 3 mg/kg body weight in group T_2 and is in agreement with Bingley and Anderson (1972) and Lesperance (1985). It may also be due to the fact that thiomolybdates

are more readily absorbed (Hidiroglou *et al*, 1990) thus, making their concentration more in plasma. However, comparable concentration of T_3 and control group was according to the findings of Ward (1978) and Radostits (1994). Miller (1974) also indicated that tissue deposition and excretion in milk are the two very important means of homeostasis in case of molybdenum.

Scanning electron microscopy of erythrocytes of healthy buffaloes in the present study revealed extensive poikilocytosis. There was presence of different sizes of erythrocytes in healthy buffaloes as reported by Bhardwaj and Rana (1988). However, majority of biconcave erythrocytes were detected in healthy buffalo calves which was comparable to finding of Bhardwaj and Rana (1988). The biconcave erythrocytes in various species of domestic animals (dog, cat, cow, horse, sheep and goat) have been shown by Jain and Kono (1972). They showed varying degrees of concavity in healthy erythrocytes of these animals. Poikilocytosis as observed in healthy buffalo calves had also been reported in goat erythrocytes by Schalm *et al* (1975). Schlam *et al* also observed spindle, rod or spherical shape of erythrocytes from healthy goats and acanthocytosis in healthy cows. Wart like projection observed on ovine erythrocytes by Jain and Kono (1972) were absent on buffalo erythrocytes in the present study.

Scanning electron microscopy of erythrocytes of phosphorus deficient animals in the present study indicated extensive spherocytosis and discocytosis varying from

echinocytosis to acanthocytosis which was comparable to the findings of Rana and Bhardwaj (1988) in phosphorus deficient animals.

Wall of erythrocytes is two molecule thick with a lipid bilayer inside and hydrophilic end outside. The phospholipid form the major portion of the lipids bilayer (Deuticke, 1968). Singer and Nicolsen (1972) stated that phospholipids are integral part of the structure and function of biomembranes and nearly half of mass of cell membrane is provided by various phospholipids (Devlin, 1982). The synthesis of phospholipids takes place in the liver and they are liberated in plasma which can be exchanged with red cell membrane phospholipids(Agar and Board, 1983). Decrease of total phospholipids might also be there because of degenerative changes in the liver leading to impairment of functions of liver resulting in decreased synthesis of phospholipids which in turn led to decrease in the plasma phospholipids and consequently phospholipids of erythrocytic membranes. Normally red blood cells has a great 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 (Guyton, 1991). With the 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 and during circulation while passing through capillaries or

reticuloendothelial network of spleen these cells might be lysed.

Various workers reported decrease in ATP production in phosphorous deficiency (Mata and Bhardwaj 1985, Singh 1990, Gill 1992). The reduced ATP production unable to maintain the shape and rigidity of cell and might result in discocytosis or sphero-echinocytosis (Lux and John, 1974). Weed *et al* (1969) reported spherocytosis in ATP-depleted red blood cells, ineffective Na-K ATPase pump and decreased phosphorylation to dephosphorylation ratio. Depletion of ATP in rat, chicken and sheep cells had been reported to effect the susceptibility of the exterior membrane phospholipids to chemical and enzymatic modifications and under such conditions more phospholipids were available for hydrolysis (Gazitt *et al* 1976).

The long bones of animals, examined radiographically, indicated periosteal lipping and reduction in joint spaces. The bone alterations could be due to deficiency of various enzymes, due to developing phosphorus and copper deficiency, like lysyl oxidase necessary for cross linking of collagen and elastins (Jubb *et al* 1985) and amine oxidase (Rucker *et al* 1975) as also supported by findings of Irwin *et al* (1974) and Smith *et al* (1975). Underwood (1981) described lameness and stiffness of gait, enlarged and painful joints, bending, deformation or fractures of the pelvis and long bones, arching of the back with posterior paralysis in pigs, facial enlargements involving particularly the submaxillary bones in horses and

malformation of the teeth and jaws, especially in young sheep as characteristic of phosphorus deficiency in domestic animals. The basic defect was a failure or reduction in the mineralization process so that the bones contained insufficient minerals to develop or maintain normal shape and strength and therefore to sustain mechanical functions.

Blair-West *et al* (1992) also indicated loss of weight and specific gravity of bones in phosphorus deficiency. Morphologically cited changes were thinning of cortices of long bones and enlargement of the marrow spaces.

Blair-West *et al* (1992) observed radiographically, increased bone radiolucency, coarser bone trabeculation and disruption of endochondreal ossification in phosphorus deficiency. Hill and Rajagopal (1962) also reported coarser bones, reduced ash contents and reasonable close relationship between quality of skeleton and blood phosphorus concentration in buffaloes in N.E. Malaya. However, in none of the bones retained (mandibles, cervical vertebrae and radii) were any exostoses or other abnormality in gross anatomy observed. Close agreement between the results of ash analysis and radiological values of vertebrae were also observed.

Histopathologically, in the present study there was marked increased in size of haversian canals in demineralised bones. This might be ascribed to increased bone resorption and decreased mineralisation due to phosphorus deficiency.

Guyton (1991) argued that due to decreased Ca and phosphorus the reserves of bones start coming in the blood. Even upto 10 per cent reserves of bones if depleted have no effect on bone structure and function. When due to prolonged deficiency the depletion is more the changes in the bone are evident. There is increased activity of osteoclasts thereby causing resorption of bone and decreased activity of osteoblasts causing decreased mineralisation of bone. As a result, bones become soft and spongy. The bones affected are ribs, vertebrae, sternum and cancellous ends of long bones which are lowest in ash followed by compact shaft of long bones like femur, tibia and humerus (Underwood, 1981).

The most striking effect of phosphorous depletion was increased plasma level of 1,25 dihydroxy vitamin D in rats, chicks, pigs and humans (Bar and Wesserman 1973, Dominguez *et al* 1976, Fox *et al* 1978, Harmelin *et al* 1990, Tanaka and DeLuca, 1973). Blair-West *et al* (1992) argued that both experimental and clinical phosphorus depletion states have been found to provide an overriding stimulus to 1,25 dihydroxy vitamin D production, causing increased intestinal Ca absorption and net bone resorption. These changes in turn caused increase in plasma Ca concentration which caused decreased plasma PTH concentration. Ultimately the decreased PTH concentration caused decreased plasma osteocalcin concentration which probably reflected a decreased bone formation rate due to suppression of PTH secretion.

On post-mortem examination, the carcass was severely anaemic. There was gelatinous degeneration of sub-cutaneous fat. The signs of anaemia were evident in all the viscera. In kidneys the medulla appeared like a jelly. Noticeable changes like reduced adipose tissue and skeletal mass were observed. Blair- West (1992) also reported similar findings in phosphorus depleted cows. The cachexia, anaemia and degeneration of adipose tissue might be due to inability of animal to maintain normal energy supply due to impaired metabolism of carbodydrates, because of severe phosphorus deficiency leading to decreased intake of feed as evident by failure of animal to gain body weight. The degeneration of fat might also be linked to decreased phospholipid (Gill 1992, Singh, 1990) synthesis and utilisation of adipose tissue due to starvation like condition observed in terminal stages. Decreased muscle mass might be due to decreased synthesis of proteins. Blood (1989) reported that inorganic phosphates, which might be ingested as such or liberated from esters during digestion or in intermediate metabolism, were utilized in the formation of proteins and tissue enzymes and were withdrawn from the plasma inorganic phosphate for this purpose.

The cloudy swelling, granular degeneration in liver at certain places and rarefaction of chromatin material in nuclei evidenced by vacuolation in nucleoplasm at certain places were attributed to combined effect of phosphorus deficiency, toxic effect of molybdenum and decreased level of copper leading to inactivation of various vital enzymes

viz. cytochrome oxidase which might have affected the generation of ATP molecules through interference in oxidative phosphorylation in accordance with findings of Fell *et al* (1975), Mills *et al* (1976) and Underwood (1981) in copper deficient cattle. Vacuolation of nuclei of hepatocytes observed in present study could be due to defective synthesis of nucleotides due to decreased ATP synthesis (Bhardwaj and Rana, 1988) and due to toxic effect of molybdenum.

Copper is distributed throughout the body forming stable complexes and is found to be associated with amino acids, purines, pyrimidines, nucleotides, DNA, RNA, enzymes and proteins. So the deficiency of this markedly affected the function of these cellular constituents resulting in degeneration in various organs (Evans 1973).

The degenerative changes in the liver could be attributed to inactivity of the enzyme cytochrome oxidase, as also confirmed on histoenzymological studies (Soodan, 1996) which might have affected the generation of ATP molecules in oxidative phosphorylation through interference in the ionic movements (ATP dependent Na+ pump) across the membrane (Fell *et al* 1975 and Mills *et al* 1976).

The kidneys section showed coagulative necrosis, vacuolar degeneration of uriniferous tubular epithelium with precipitation of degenerated protein material into the luminae. These findings were comparable to nephropathy due to feeding semipurified diet with low copper content in ovine (Richardson *et al* 1979). These alterations could also

be due to direct toxic effect of thiomolybdate complexes excretion through urine as per findings of Mason (1986). Tokarnia *et al* (1960) also reported degenerative changes in kidneys of animals having lower liver copper contents.

Presence of emphysema in lungs indicated expiratory distress. Dairy animals are more prone to emphysema due to well developed interstitial tissues. Bovine lungs are highly susceptible to emphysema from many different causes, not all of them respiratory in origin (Blood, 1989). Pulmonary emphysema might be there because of inadequate strength of the supporting tissues which were thus unable to support the alveolar wall during exertion or coughing. Pulmonary congestion could be due to decreased SOD activity in relation to developing molybdenosis (Soodan, 1996). This could have resulted in decline in the protection of phagocyctic cells from oxidation mediated by free radicals, thereby leading to autotoxic damage to mononuclear system associated with lungs, thereby causing proliferation of organisms in lungs and thus pneumonia.

The oral administration of sterlized bone meal daily @ 35g/100kg body weight for one month resulted in significant improvement in the clinical condition of animals. The animals became active, showed normal gait and their feed intake was also increased. However, the mean values of Hb, PCV and TEC did not show much change fifteen days after institution of the therapy. This might be due to severe depletion effect and phosphorous supplementation was unable to restore blood parameters to the normal.

Associated damage to liver and kidneys done by toxic effect of molybdenum and due to prolonged deficiency of phosphorus and slow formation of rbcs due to decreased bone marrow functioning might have interfered with restoration of haemotological indices 15 days post-treatment. The present findings were not in agreement with those of Gill(1992) who reported restoration of blood parameters to normal after 15 days of therapy with dicalcium phosphate and sodium acid phosphate in phosphorus depleted cross-bred cow calves. The slow restoration in haematological values might be ascribed to the fact that animals started taking feed and water normally so no dehydration was evident clinically, consequently the increased volume of blood plasma might have masked the improvement of Hb, TEC and PCV.

The ESOD in T_2 group showed significant improvement and the mean values were comparable to the control group 15 days after institution of treatment after stopping of molydbenum supplementation and feeding of balanced mineral mixture. This might be due to improved energy status of the animal, inhibition of formation of thiomolybdate due to stoppage of intake of molybdenum, and increased functional status of the cellular constituents like amino acids, purines, pyrimidines, nucleotides, DNA, RNA, enzyme and proteins with which copper was associated (Evans, 1973). The lipid peroxidation was non-significantly improved in all the groups and was comparable with control values.

The mean inorganic phosphorus values showed significant improvement after 15 days and at 30th day was

comparable to mean zero day value so proving that therapy with bone meal should be regular and prolonged. These findings were in accordance with that of Medsen and Neilsen 1944, Mullins and Ramsay 1959 and McTaggart, 1959. Blood .(1989) rated the biological values of bone meal for pigs in terms of phosphorus at 56 per cent. The plasma copper increased to base level only at 30th day however maintained a continuous rising trend. This might be due to the fact that depleted copper stores might have got repleted by that time or more copper than normal present in mineral mixture was needed for deficient animal. Mean iron value, 30th day after therapy, was much more than base value indicating more absorption from GIT due to improved copper status but less utilisation in bone marrow or liver due to their extensive damage.

The mean inorganic phosphorus and copper contents of rumen liquor were comparable to respective base values 30th day post-treatment and showed a continuous rising trend. The rumen liquor mean iron content of the group T_2 was decreased at 30th day post-treatment which could be due to better absorption because of more copper intake and improved energy status of the animal. Mean CSF copper and iron contents also followed the same trend.

CHAPTER-VI

SUMMARY AND CONCLUSIONS

Health of dairy animals is the most fundamental factor that governs the production. The high producing dairy animals always stand on the verge of abnormality due to their higher nutritional requirements. Among essential nutrients required for good health, at least fifteen mineral elements are of concern for the bovines. Although minerals do not yield energy in the body, yet one or more of these elements are involved directly or indirectly in various metabolic processes in the living system. Among these minerals, phosphorus deficiency is one of the most widespread occurring in various parts of the world. The deficiency of this mineral is precipitated by excess of molybdenum in soil and fodder. There was paucity of scientific literature on the haematological, biochemical and radiological alterations associated with hypophosphataemia in buffaloes and scanning electron microscopic changes in erythrocytes especially in phosphorus deficiency occurring due to high molybdenum intake. The present study covered some of the important facets of this syndrome in buffaloes which might help in understanding its etio-pathogenesis with particular reference to its effect on erythrocytes and bones and for evolving suitable diagnostic and therapeutic measures.

Experimental studies were conducted in three group of buffalo calves comprising of five (T_1 group), ten (T_2 group)

and five animals (T_3 group) of about 8-9 months of age with average body weight of 89 kg. Before the start of experiment, all the animals were dewormed freed from external parasites and acclimatised for about a month. Hypophosphataemia was induced in ten animals of group T_2 and five animals of group T_3 by *ad-lib* feeding of urea treated wheat-straw (containing about 0.1% phosphorus on dry matter basis) and phosphorus and copper free mineral mixture @ 15g per quintal body weight for about 90 days. Alongwith this, animals of group T_2 were given molybdenum @ 3 mg/kg of body weight orally. Therapeutic trial was conducted on five animals of group T_2 on 90th day following induction of hypophosphataemia. Sterilised bone meal @ 35 g/quintal body weight was given daily to these animals for 30 days alongwith phosphorus containing mineral mixture. Feeding of molybdenum was discontinued in all the animals undergoing therapeutic trial. Five animals of group T_1 served as healthy control.

For establishing the normal values, venous blood, rumen liquor and cerebro-spinal fluid were collected on two occasions from all the animals of group T_1, T_2 and T_3. Radiographs of lower ends of metacarpal and metatarsal of healthy animals were taken before start of experiment. The venous blood was analysed at 15 days interval for various haematological, biochemical and mineral alterations i.e. Hb, PCV, TEC, ESOD activity, lipid peroxidation of erythrocytes and plasma inorganic phosphorus, iron, copper and molybdenum concentrations. The rumen liquor and CSF

were also analysed for various minerals. The SEM of erythrocytes was undertaken to observe various surface changes in erythrocytes in induced hypophosphataemia. Radiographic examination of bones was conducted at the peak of phosphorus deficiency. The histopathological examination of bones was also done after induction of phosphorus deficiency in animals of group T_2. After death of animals post-mortem was performed for observing gross abnormalities and various tissues and organs pieces were collected for histopathological examination.

In therapeutic trials, the treatment was undertaken with sterilised bone meal @ 35g/quintal body weight orally for 30 days. The blood samples were collected twice following institution of treatment at 15 days interval. The effect of feeding of sterilised bone meal on haematological and biochemical constituents of blood and mineral contents of various body fluids viz. plasma, rumen liquor and CSF was also analysed.

The general clinical signs of phosphorus deficiency observed in all the animals of group T_2 and T_3 were rough hair coat, alopecia with discolouration of skin followed by mild depigmentation, weakness with sunken eyes followed by recumbency and inability to stand, staggering and plodding gait and tucked up abdomen in last stages of deficiency. The clinical signs of weakness and other related symptoms were more marked in group T_2 as compared to group T_3 . The main clinical signs of deficiency were evident on 60th and 70th day in group T_2 and T_3, respectively, however sternal

recumbency was followed on day 70th and 75th, respectively. At terminal stages, animals were active, however showing apathy and there was increased heart rate. Lateral recumbency was followed by further intensification of clinical signs and death.

The haematological indices revealed significant decline in Hb, PCV and TEC reflecting macrocytic normochromic anaemia. The SEM of erythrocytes of T_2 and T_3 groups revealed presence of spherocytosis and discocytosis varying from mild echinocytosis to mild acanthocytosis. The normal erythrocytes were however biconcave with varying degree of concavity and showed poikilocytosis. Biochemical examination of blood showed significant increase in ESOD in hypophosphataemic and molybdenotic animals as compared to control group and group T_3. However, lipid peroxidation of erythrocytes of hypophosphataemic and molybdenotic animals was comparable to that of healthy control. Mineral analysis of plasma revealed significant decline in plasma inorganic phosphorus concentration in group T_2 and T_3. The decline in phosphorus concentration in group T_2 was slow but was more severe in later stages, as compared to group T_3 which showed consistent decline throughout the induction of deficiency. The plasma copper revealed a significant and consistent decline in molybdenum supplemented animals after 45th day. However, the plasma iron showed non-significant fluctuation in both the groups throughout the study.

Rumen liquor mineral analysis revealed decreased inorganic phosphorus concentration in animals of both the

groups, whereas mean rumen liquor copper concentration was reduced in animals getting molybdenum supplement alongwith. Mean rumen liquor iron showed significant increase after 60th day in phosphorus depleted and molybdenum supplemented animals. In CSF mean inorganic phosphorus level was decreased with decreasing plasma phosphorus content but other minerals indicated non-significant fluctuations. Radiological examination of lower end of metacarpal and metatarsal revealed widening of joint space, lipping of periosteum and there was increased soft tissue density though the degrees of changes were very mild. Histopathological study of bones revealed widening of haversian canals due to demineralisation and increased activity of osteoclasts. Gross post-mortem lesions included gelatinous degeneration of fat, loss of muscle mass and generalised anaemia. Histopathological changes in various organs viz. liver, kidneys and lungs were also recorded. In liver sections, there was cloudy swelling, granular degeneration at various places and rarefaction of chromatin material evidenced by vacuolation in nucleoplasm at certain places. Kidneys showed coagulative necrosis with precipitation of degenerated protein material into the luminae and extensive vacuolar degeneration of uriniferous tubular epithelium. In lungs, there was emphysema alongwith haemorrhages.

The treatment in five animals of group T_2 with oral feeding of sterilised bone meal for 30 days revealed 80 per cent recovery rate. The clinical signs subsided gradually and marked improvement in health of animals was observed.

The feed intake was improved and there was significant improvement in various biochemical and mineral constituents. The mean inorganic phosphorus concentration of various body fluids like plasma, rumen liquor and CSF was restored to normal levels 30 days post-treatment.

CONCLUSIONS

1) The consistent signs of induced hypophosphataemia in buffalo calves were reduced appetite, stiff gait, hide bound condition, general weakness, arched back with pale mucous membranes followed by recumbency and death.

2) The haematological alterations included macrocytic normochromic anaemia.

3) Significantly low levels of plasma inorganic phosphorus was a consistent finding in hypophosphataemic animals.

4) The molybdenum supplementation caused gradual but more intense decrease in plasma phosphorus level.

5) Significantly low activity of ESOD was a consistent finding.

6) Hypophosphataemia was associated with parallel and significant decline in phosphorus contents of rumen liquor and cerebro-spinal fluid.

7) Histopathological findings of bones depicted decreased mineralisation and increased haversian canals size.

8) Hypophosphataemia and molybdenosis led to degenerative changes of varying degrees in liver, kidneys and lungs.

9) Scanning electron microscopy of erythrocytes revealed spherocytosis and discocytosis with mild corrugation of erythrocytic membrane.

10) Oral administration of bone meal for 30 days resulted in marked clinical recovery and restoration of haemato-biochemical alterations.

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