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CLINICOPATHOLOGICAL STUDIES ON THE EFFECT OF HYPOPHOSPHATEMIA IN BUFFALOES

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CONTENTS

List of Abbreviations

	Abbr.Description
A/G	: Albumin/globulin ratio
ALP	: alkaline phosphatase
ALT	: Alanine aminotransferase
AST	: Aspartate aminotransferase
ATP	: Adinosine tripophste
Ca	: Calcium
Calc	: Calcitonin
CPK	: Creatine Phosphokinase
DNA	: Deoxyribonucleic acid
DBp	: Vitamin D binding protein
EDTA	: Dipotassium salt of Ethylene Diamine Tetra Acetic acid
ELISA	: Enzyme Linked Immunosorbent Assay
ESR	: Erythrocytic sedimentation rate
G-6-P	: Glucose-6-phosphate dehydrogenase
GPx	: Glutathione peroxidase
H_2PO_4	: Dihydrogen phosphate
Hb	: Hemoglobin
HPO_4	: Monohydrogen phosphate
LDH	: Lactate dehydrogenase
MCH	: Mean corpuscular hemoglobin
MCHC	: Mean corpuscular hemoglobin concentration
MCV	: Mean corpuscular volume

MDA	: Malondialdehyde
P	: Phosphorus
PCV	: Packed cell volume
PI	: Inorganic phosphate
PO	: Organic phosphate
PO4	: Phosphate
PTH	: Parathyroid hormone
RBCs	: Red blood corpuscles
RNA	: Ribonucleic acid
SOD	: Superoxide dismutase
WBCs	: White blood cells
T.P	: Total proteins
fl	: Femtoliter
ng	: Nanogram
pg	: Picogram
dl	: Deciliter
mg	: Miligram
g	: Gram
2,3 DPG	: 2,3 diphosphoglycerate
23 FGF23	: 23 fibroblast growth factor
25(OH)D3	: 25-hydroxy vitamin D3
1,25(OH)2	: 1,25 dihydroxycholecalciferol

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INTRODUCTION

INTRODUCTION

Phosphorus has an important biological functions that make it essential for human being and or animal health. Oxidative phosphorylation, oxygen delivery, glycolysis and maintenance of cellular structural integrity are among these processes **(Grunberg, 2008)**.

Phosphorus is the major component of the bone. It is necessary for generation of adenosine triphosphate (ATP) which is important for many physiological processes. In addition to phosphorus compounds containing phosphate ion are the components of DNA, RNA also phospholipids which form all cell membranes. Severe decrease in ATP concentration in erythrocytes can lead to hemolytic anemia and hemoglobinuria **(Ziwei, 2017)**. Phosphorus (P) is an important macro-mineral. It united with oxygen atom forming phosphate (PO_4). This is establish in the body in two forms either in organic (PO) and inorganic phosphate (PI) **(Grunberg, 2014)**.

The organic phosphate (PO) (non-bone P): present in intracellular fluid. It bound to a molecule containing carbon and it is found in phospholipids, phosphate esters, phosphoproteins, nucleic acid, ATP. This form is not measure in biochemical assays **(Forrester and Moreland, 1989)**. Where the inorganic phosphate (PI) that called orthophosphate. This form of phosphate is fundamentally free (not bound to any carbon-containing molecules). It is found in the extracellular fluid and is measured in serum or plasma **(Grunberg,**

2008). The inorganic phosphate level in the body depends on the correct absorption of P ions, mainly in small intestine, ions, correct excretion in urine and mobilization from in bone **(Kurek et al., 2010)**.

The endocrine regulation of extracellular phosphate hinge on the active form of vitamin D (1-25 dihydroxycholecalciferol) and parathyroid hormone (PTH) also parenchymal organs such as kidney and liver are involved **(Bjorkman et al., 1994)**.

The decrease in serum inorganic phosphate level in dairy cattle is one of the most common metabolic disorders. This drop is considerably following a sudden loss of P as it occurs at the onset of lactation through mammary gland also hypophosphatemia can be a result of shifting P from extracellular fluid into intracellular fluid **(Montiel et al., 2007)**.

Hypophosphatemia has been associated with a number of clinical signs such as anorexia, pica, muscle weakness or recumbence, intravascular hemolysis and more recently disturbed liver function **(Grunberg, 2008)**.

Aim of the work

1- Studying the hematological and biochemical alterations associated with hypophosphatemia.

2- Appraise interrelation between phosphorous deficiency and vitamin D and parathyroid hormone

3- Studying the effect of hypophosphatemia on the oxidative status of the animal.

4- Assessment the effect of phosphorous deficiency on musclular markers.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Body distribution of phosphorus

Knochel (1985) mentioned that phosphorus is distributed through the body as 80-85% in the skeleton and the rest in the soft tissue.

Fraser et al. (1987) recorded that phosphorus exits intracellular in the body as organic phosphate in form phosphorus esters. Most intracellular phosphorus is organic form but most inorganic phosphate (Pi) present extracellular.

Organic phosphate

Horst (1986) reported that organic phosphate is present in many compounds including phospho-proteins, nucleic acids, enzymes and phospholipids.

Forrester and Moreland (1989) recorded that phospholipids are a component of external plasma membranes and intracellular membranes that form organelles and play an important role in maintaining cellular integrity. Phospho-proteins are the components of mitochondria used in electron transport system.

Inorganic phosphate (Pi)

Tietz et al. (1987) recorded that most phosphorus in extracellular fluid is inorganic phosphate, only 12-15% of inorganic phosphate is protein bounded and the rest is freely exists as monohydrogen phosphate (HPO_4) or dihydrogen phosphate (H_2PO_4).

Forrester and Moreland (1989) stated that inorganic phosphate is a substrate for many vital processes in the body including oxidative phosphorylation, the production of 2, 3 diphosphoglycerate (2,3 DPG) in erythrocytes and glycolysis in kidneys and liver. Only small portion of intracellular phosphorus exists as inorganic phosphate which is vital to cell function be the source of ATP which is the energy source for several physiological processes as sodium potassium pump and synthesis of chemical compounds.

Grunberg et al. (2006) recorded that the ratio between monohydrogen phosphate and dihydrogen phosphate depend on blood pH. When alkalemia occurs (blood pH > 7.4) the ratio increases and inorganic phosphate is present in the divalent form. When academia is present (blood < 7.4) the ratio decreases and more phosphate exists in monovalent form.

Role of phosphorus in the body

Knochel (2006) stated that phosphorus is an essential mineral that is required by every cell in the body for normal function, bBound to oxygen in all biological systems. It is found as phosphate (PO_4) in the body. Approximately 85% of the body phosphorus is found in bones and teeth. Also, it is the major structural component of bone in the form of calcium phosphate salt called hydroxyapatite. Phospholipids are the major structural components of cell membranes. All energy production and storage are dependent on phosphorylated compounds such as (ATP) and creatine phosphate. Nucleic acids (DNA and RNA) are long chains of phosphate-containing molecules. A number of enzymes, hormones and cell signaling molecules depend

on phosphorylation for their activation. Phosphorus also helps in maintaining normal acid base balance (pH) by acting as one of the body's most important buffers.

Navjot et al. (2017) mentioned that erythrocytes depend upon extracellular glucose for their energy requirement. The inorganic phosphorus promote uptake of glucose by erythrocytes thus hypo-phosphatemia results in decreased red cells glycolysis and ATP synthesis. Subnormal concentration of ATP predisposes red blood cells to altered function and structure, loss of normal deformability and increase in fragility and hemolysis.

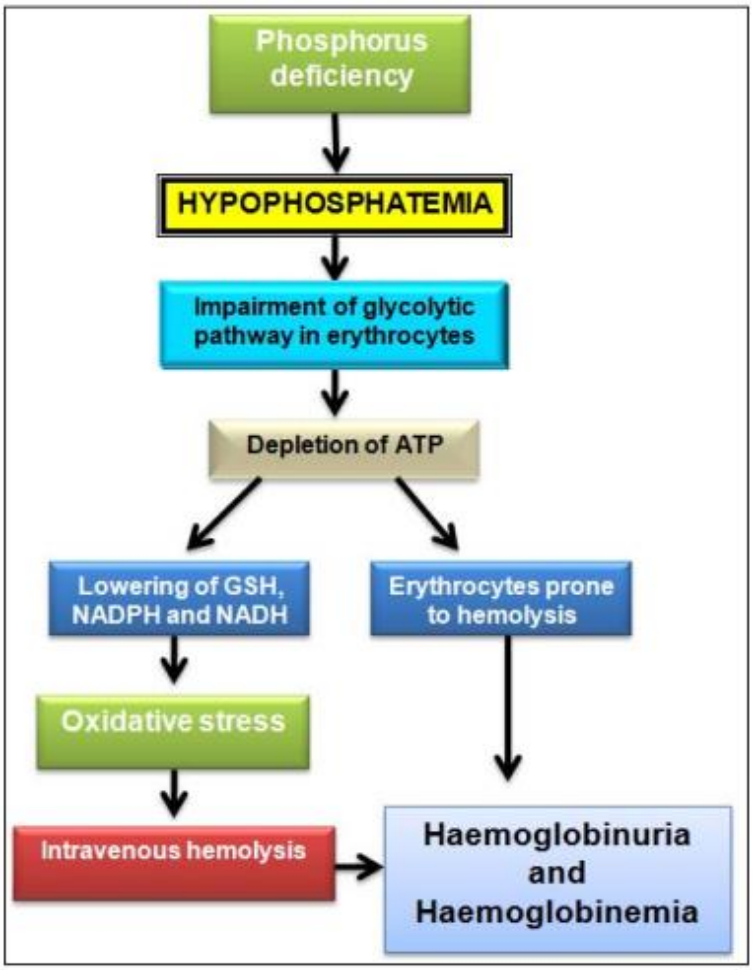

Fig. (1): Possible mechanism of hemoglobinuria and hemoglobinemia (Navjot et al., 2017)

Phosphorus hemostasis

Regulation of extracellular phosphate

Horst (1986) stated that phosphorus concentration in serum or plasma is regulated through intestinal absorption rate, salivary, renal excretion rate and through mobilization of phosphorus from bone.

Forrester and Moreland (1989) reported that intestinal absorption of phosphate is an active energy dependent process. Increased absorption occurs with decreased dietary calcium and increased acidity of intestinal content. Growth hormone enhances intestinal absorption of phosphate. The active form of vitamin D (1, 25 dihydroxycholecalciferol) stimulate intestinal phosphate absorption.

Lunn and Mcgurirk (1990) recorded that renal phosphorus excretion under physiological condition is only of minor importance with renal threshold approximately 7 mg/dl. Renal re-excretion increases when the plasma (Pi) increase than the renal threshold with decreasing urine pH due to decreased tubular P reabsorption. Parathyroid hormone is the most important regulator of renal phosphorous transport.

Morse et al. (1992) concluded that milk production has a strong impact on P homeostasis in dairy cows as milk contain considerable amount of P. Effective P losses through mammary gland can be achieved by decreasing milk production, increasing milk production results in increasing phosphorous demand.

Grumberg (2008) stated that ruminants' salivary gland excrete large amount of phosphorous in saliva which help rumen microflora in the metabolism also it act as a buffer. A large proportion of salivary phosphorous is reabsorbed by intestine and the reminder contributing to fecal losses.

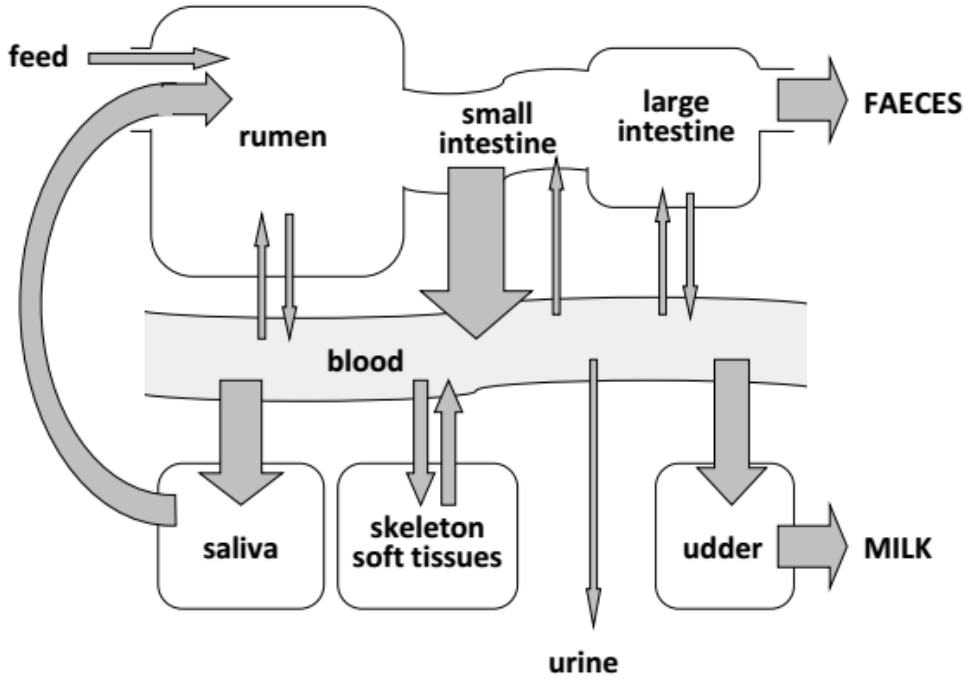

Figure (2): Simplified scheme of phosphorus pools (boxes drawn) and transport routes (arrows) in the ruminant (Goselink et al., 2015).

Endocrine regulation of extracellular phosphate

Kurek et al. (2010) reported that the phosphorous homostasis depends on problematical hormonal regulation which embraces parathyroid hormone (PTH), calcitonin (Calc), fibroblast growth factor, 23(PGF23), phosphatine, active metabolic form of vitamin D and parenchymatous organs as liver and kidney are involved.

(1) Vitamin D3 (1,25 dihydroxycholecalciferol):

Allen and Weingand (1985) reported that active form of vitamin D stimulate intestinal phosphate transport. Vitamin D deficiency and mal absorption syndrome resulted in decreasing phosphate absorption.

Portale et al. (1989) stated that the phosphorous directly regulate the production of $1,25(OH)_2$ vitamin D by the kidney cells in culture and in vivo.

Breves and Schroder (1991) mentioned that phosphorous depletion was found to increase vitamin D receptors binding affinity in lactating goat.

Condamine et al. (1994) concluded that the low level of serum $1,2(OH)_2$ vitamin D may reflect a response to direct effect of phosphorous on the renal synthesis of $1,25(OH)_2$ vitamin D.

Puggard (2012) reported that there is a stimulatory effect of low phosphorous on vitamin D3.

Jakobsen et al. (2015) stated that vitamin D3 production is induced by hypophosphatemia and hypocalcemia.

Jacquillet and Unwin (2019) recorded that vitamin D3 (chole calciferol) is produced in the skin depending on the intensity of ultraviolet irradiation and exposure then it imported to liver by vitamin D binding protein (DBP) then hydroxylated by 25 hydroxulase enzyme and 25-hydroxy vitamin D3 ($25(OH)D_3$ yielded. $25\ (OH)D_3$ is biological inactive formula and is required to be hydroxylated to active one $1,25(OH)_2$. So it bounded to DPB and

imported to the kidney where it is filterated and taken by proximal tubules under the effect of 1αhydroxylase.

(2) Parathyroid hormone (PTH)

Knox and Haramati (1985) reported that parathyroid hormone is the most important regulator hormone for renal phosphate transport. Parathyroid hormone diminishes reabsorption of bicarbonate, sodium, calcium and phosphate in the proximal tubules, while it augments calcium, reabsorption in the distal tubules. So, the net effect is occasioned in increased serum calcium concentration and phosphate urea.

Horst (1986) recorded that the increase of parathyroid hormone secretion results in the elevation of salivary and renal phosphorous excretion.

Berndt et al. (2005) stated that parathyroid hormone affect renal excretion of phosphorous by lessening tubular reabsorption and thus increasing urinary phosphorous concentration.

Silver and Naveh (2009) stated that the increase in extracellular phosphate leads to increase PTH secretion which is phosphaturic hormone and the decrease in serum phosphorous level found to decrease serum PTH level.

Bergwtiz and Juppner (2011) concluded that the parathyroid glands directly respond to the change in serum calcium through Ca-sensing receptors, resulting in increasing PTH manufacture. It does not directly respond to intravenous phosphorous infusion but

indirectly respond to extracellular phosphorous concentration by their effect on mRNA stability coding for PTH synthesis which destabilizes in cases of hypophosphatemia.

Jacquillet and Unwin (2019) reported that PTH is the abundant modulator of bone and mineral metabolism through its effect on Ca and Pi hemostasis. Synthesis of PTH occurs in parathyroid gland following two successive cleavage of 115 amino acids pre-pro PTH into 90 amino acids pro-PTH then cleaved into 84 amino acids (active mature PTH). The secretion of PTH is under control of ca+2 and serum pi levels as pi play an important role in the regulation of PTH mRNA.

(3) Calcitonin

Puggard (2012) reported that calcitonin is produced in the thyroid gland and it's involved in calcium balance with moderate role in phosphorous homeostasis. It increases and decreases with high and low extracellular phosphorous concentrations respectively leading to lower and higher resorption of bone.

Felsenfeld and Lexine (2015) stated that calcitonin is amino acid hormone secreted by C-cells in thyroid gland and it is stimulated by increasing serum calcium concentration protect against the development of hypocalcemia. Calcitonin is known to stimulate renal $1,25(OH)_2$ vitamin D production at proximal tubules.

Xie et al. (2020) mentioned that calcitonin is a peptide hormone that reduces calcium level in the systemic circulation. The

hypocalcemic effect produced due to inhibition of bone resorption or suppression of calcium release from bone.

(4) Phosphatonins

Berdnt et al. (2005) reported that phosphatonins are factors that reducing phosphate balance by increasing renal excretion and reducing vitamin D3 production in the kidneys and parathyroid hormone production in parathyroid glands.

Sapir-Koren and Livshits (2014) concluded that fibroblast growth factor 23 (FGF-23) is the most ample in this group. It is produced mainly in osteoblasts and osteoclasts.

Tan et al. (2014) stated that both FGF_{23} and PTH are phosphaturic hormones that act independally to inhibit activity of renal sodium phosphate contransporter so the result is phosphaturia.

Kassianides and Bhandari (2021) mentioned that FGF23 is a bone derived hormone. It is secreted a response to increased calciterol, PTH and hyperphosphatemia. It is secreted only by osteocytes and act on the kidney through reduction of Napi contraceptors activity in proximal tubules so the net effect is phosphaturia. Moreover increasing level of FGF23 magnify PTH synthesis.

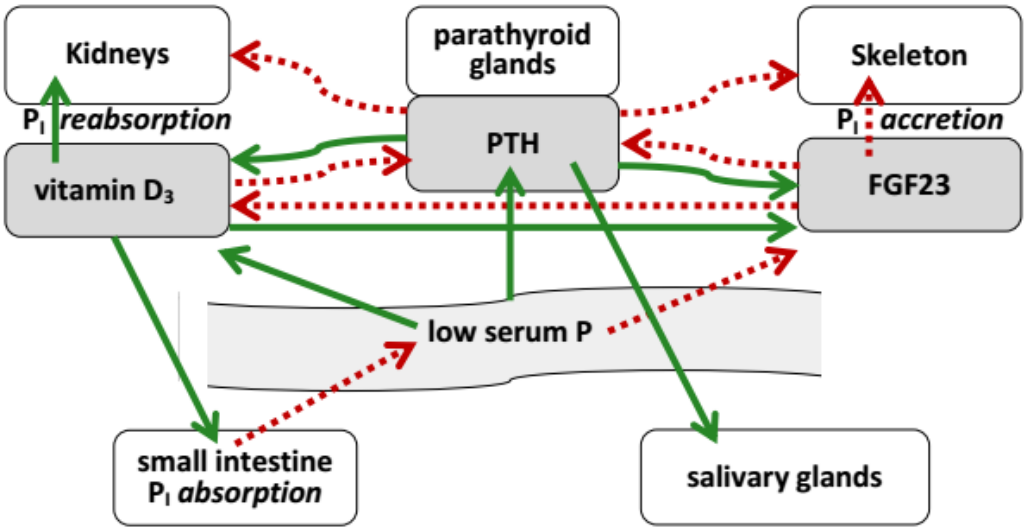

Figure (3): Simplified scheme of regulation of phosphorus metabolism in case of decreased extracellular phosphorus. Positive actions (activation) are shown as green solid arrows; negative actions (inhibition) in red, dotted arrows **(Goselink et al., 2015)**.

PI - inorganic phosphorus; PTH - parathyroid hormone; FGF23 - fibroblast growth factor 23

Etiology of hypophosphatemia

Forrester and Moreland (1989) reported that the general causes of hypophosphatemia are decreased intestinal absorption, transcellular shifts of phosphorous from blood into cells and increased renal excretion of phosphate.

Grunberg (2008) mentioned that hypophosphatemia occurs as a resulting of increasing renal excretion of phosphate. Hyperparathyroidism causes phosphaturia and hypophosphatemia.

Grunberg et al. (2017) recorded that acute phosphorus losses associated with hypophosphatemia is well recognized problem in high

yielding dairy cows that occurs at the onset of lactation due to phosphorous losses through mammary gland and decrease feed intake around parturition also impaired intestinal absorption and increase losses of phosphorus through urinary tract are involved. Chronic phosphorus deficiency is common caused by inadequate feed intake for long period. This can be seen in grazing animals in region with low phosphorus content in the soil. It can also occur as a result of primary hyperparathyroidism and renal tubular disease.

Purohit et al. (2018) mentioned that the common cause of hypophosphatemia is dietary deficiency of phosphorus as feeding on cabbage lucerene or barseem, wheat, rice and straw. Also, phosphorus deficiency can also occur due to the presence of inhibitory factors such as metabolic ions which interfere with absorption of phosphate. Areas with soil deficient in phosphorus will be a result of hypophosphatemia. Copper deficiency is also etiological factor of hypophosphatemia as its deficiency reduces activity of copper containing enzymes as superoxide dismutase (SOD) which is a part of erythrocytic protective mechanisms against oxidative stress.

Rahmati et al. (2021) mentioned that incorrect ratio between Ca/P, increasing activities of parathyroid hormone and increase renal and salivary functions are from the causes of hypophosphatemia. Also, copper deficiency has been an important etiological factor for post parturient hemoglobinuria.

Pathogenesis of hypophosphatemia

Sarma et al. (2014) stated that a possible mechanism for post parturient hemoglobinurea could be the reduction in glutathione

content in red blood cells and increasing malondialdehyde levels in erythrocytes of affected buffaloes suggested oxidative stress. Also, low activity of glucose-6-phosphate dehydrogenase was included.

Navjot et al. (2017) recorded that hypophosphatemia actually related to oxidative stress that is leads to low ATP production and subsequent weaking of antioxidant system of the body such as catalase, superoxide dismutase, glutathione peroxidase. Subnormal concentration of ATP predispose red blood cells to alter function, structure, loss of normal deformability and increase fragility and hemolysis.

Purohit et al. (2018) concluded that the pathogenesis of erythrocyte destruction leading to anemia is poorly known. There is an association with hypophosphatemia and low dietary intake of phosphorous. The onset of lactation cause further depletion of phosphorous. The deficiency of phosphorous has been documented to be the result of ATP depletion and also 2-3 diphosphoglycerate synthesis in RBCs. The depletion of ATP predispose red blood cells to alter function and structure causing loss of normal formability and increase fragility leading to hemolysis.

Rashid et al (2021) mentioned that phosphorous deficiency alters the process of glycolysis and ATP synthesis in RBCs. Several metallo enzymes which include super oxide dismutase, glutathione peroxidase and catalase were altered by phosphorus deficiency leading to oxidative damage of RBCs and intravascular hemolysis.

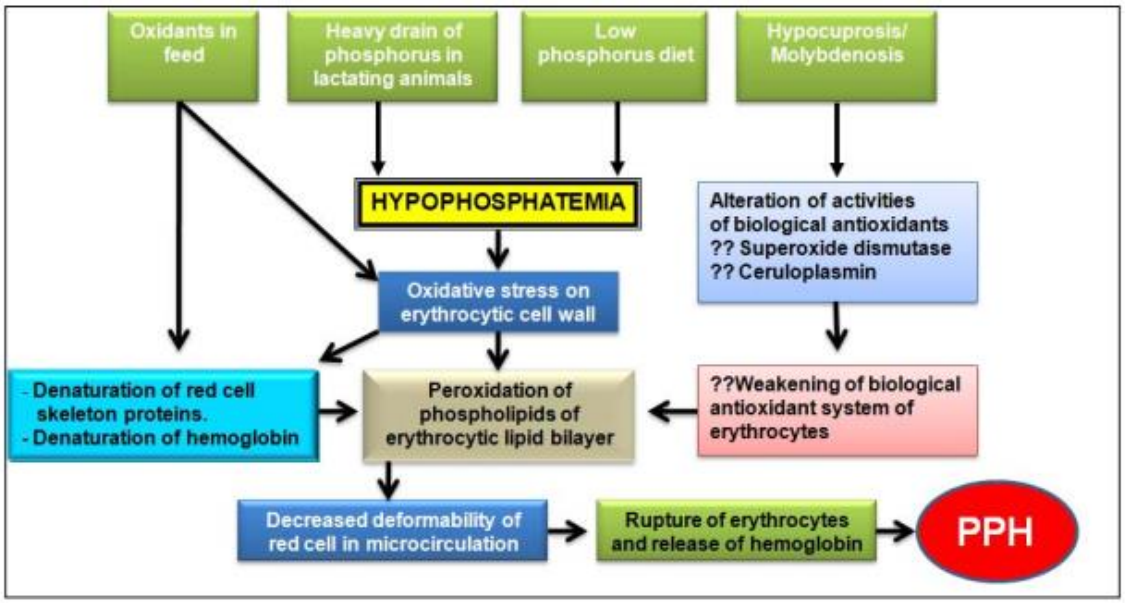

Fig. (4): Flow diagram showing probable inter-relationship of various factors leading to post-parturient hemoglobinuria (Navjot et al., 2017).

Clinical findings and lesions

Grunberg (2008) stated that acute decline of plasma inorganic phosphate is commonly the result of sudden disruption of equilibrium between P uptake and P loss. Such disequilibrium could be caused by sudden increase in milk production at the onset of lactation and decreased feed intake. While, chronic phosphorous deficiency is characterized by poor growth or loss of weight, decreased milk production, low fertility and in late state osteodystrophy with decreased bone weight and osteopenia.

Grunberg et al. (2017) mentioned that acute hypophosphatemia has been associated with anorexia, muscle weakness, muscle pain, bone pain, increase fragility of RBCs and intravascular hemolysis. Other potential effects of hypophosphatemia are neurological signs

due to alteration in energy metabolism. Also, impaired cardiac and respiratory function due to decreased contractility of striated and heart muscle. Dysfunction of WBCs and platelet also recorded. Chronic hypophosphatemia are the most commonly seen in cattle fed on phosphorous deficient diet over several months. Young animals grow slowly, developed rickets and tend to have rough hair coat whereas adult animals in early stages may become lethargic, anorectic and lose of weight, decrease milk production and fertility have been attributed to phosphorous depletion. These signs appear to be the result of decreased energy and protein intake. In later stages animals may develop pica, osteomalacia, abnormal gait, lameness and become recumbent.

Purohit et al. (2018) stated that the first notable clinical signs in the affected buffaloes with acute (severe) hypophosphatemia are red to coffee colored urine, pulse rate, temperature, respiratory rates are increased, enlarged lymph nodes, pale mucous membranes, dyspnea, weak pulse, tachycardia, anorexia and decreasing milk yield in post parturient hemoglobinuria affected buffaloes.

Zaghawa et al. (2019) reported that the most common clinical signs in hypophosphatemic infected buffaloes were dark red to coffee urine, anemia, dehydration and decreased milk production. Meanwhile a significant reduction in ruminal motility, anorexia, icterus and lameness can be observed in some cases.

Rahmati et al. (2021) recorded that the remarkable clinical signs in hypophosphatemic buffaloes and dairy cows are red to brown urine,

increasing rectal temperature, dry feaces, anorexia, paleness mucous membranes and significantly decrease in milk production.

Effect of hypophosphatemia on hematological parameters

Ogawa et al. (1989) reported that there were significant decreases in RBCs counts, hemoglobin concentration and PCV in hypophosphatemic cows when compared with normal.

Muhammed et al. (2001) recorded that erythrocytic count, hemoglobin concentration and hematocrit were significantly reduced in the post parturient hemoglobinuria affected buffaloes when compared with normal. A significant neutrophilia and lymphopenia were also recorded.

Mahmut et al. (2009) proved a significant decrease in erythrocytic count, hemoglobin concentration, packed cell volume (PCV) and mean corpuscular hemoglobin concentration (MCHC) in post parturient hemoglobinuria affected cows when compared with healthy one.

Durrani et al. (2010) reported that erythrocytic count, hemoglobin concentration and hematocrit were significantly reduced in post parturient hemoglobinuria affected buffaloes while total leukocytic counts and erythrocyte sedimentation rate were significantly elevated in comparison with normal control one.

Al-Mujalli (2010) concluded that a significant decrease in erythrocytic count, hemoglobin concentration and packed cell volume in addition to a significant neutrophilia and lymphocytopenia were

recorded in post parturient hemoglobinuria affected buffaloes when compared with normal control. Meanwhile, erythrocytic sedimentation rate (ESR) was significantly increased.

Navjot et al. (2017) recorded a significant reduction in erythrocytic count, hemoglobin concentration and packed cell volume while erythrocytic sedimentation rate was significantly higher in post parturient hemoglobinuria affected buffaloes when compared with normal control.

Ziwei et al. (2017) stated that erythrocytic count and mean corpuscular hemoglobin level (MCH) were significantly decreased while mean corpuscular volume (MCV) was significantly higher in acute hypophosphatemia affected cows when compared with normal control.

Purohit et al. (2018) mentioned that erythrocytic count, hemoglobin concentration, packed cell volume (PCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were significantly reduced with leukocytosis and neutrophilia in post parturient hemoglobinuria affected buffaloes when compared with normal control.

Kumar et al. (2019) proved a significant decrease in erythrocytic count, hemoglobin concentration, packed cell volume with leukocytosis, neutrophilia and lymphopenia in post-parturient hemoglobinuria affected buffaloes when compared with healthy normal control animals.

Rahmati et al. (2021) reported a significant decrease in RBCs count, Hb concentration, PCV and MCH. While, MCV and total leukocytic counts were significantly elevated in hemoglobinuria affected cattle.

Rashid et al. (2021) recorded a significant decrease in RBCs count, Hb concentration, PCV with a significant increase in erythrocytes sedimentation rate and neutrophil count in hypophosphatemic pregnant murrah buffaloes**.**

Biochemical changes associated with hypophosphatemia

Muhammed et al. (2001) stated a significant reduction in serum copper and erythrocytic glucose-6-phospahte dehydrogenase while serum urea, creatinine and glucose levels were significantly increased in post parturient hemoglobinuria affected buffaloes when compared with healthy control.

Mahmut et al. (2009) recorded a significant decrease in serum levels of glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and magnesium in post parturient hemoglobinuria affected cows when compared with healthy control.

Durrani et al. (2010) mentioned that serum levels of phosphorous and copper were significantly lower while serum levels of molybdenum was significantly higher in addition to highly significant increase in serum urea and creatinine levels in post-parturient hemoglobinuria affected buffaloes when compared with control.

Kurek et al. (2010) recorded a significant increase in the activities of serum of ALT, AST and ALP, beside a significant increase in the levels of serum creatinine and total bilirubin while total protein was significantly decreased in both moderate and severe hypophosphatemic cows.

Al-Mujalli (2010) reported a significant decrease in serum levels of phosphorous, total proteins, albumin and globulins, glucose and erythrocytic glucose-6-phosphate dehydrogenase activity. In addition to a significant increase in serum levels of calcium, aminotransferases (ALT, AST), urea and creatinine in post parturient hemoglobinuria affected cattle when compared with normal.

Navjot et al. (2017) reported a significant decrease in serum levels of phosphorous and copper while serum levels of urea and creatinine were significantly increased in post parturient hemoglobinuria affected buffaloes when compared with control.

Ziwei et al. (2017) recorded a significant decrease in serum phosphorous level but a significant increase in serum activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total and indirect bilirubin in acute phosphorous deficient cows when compared with control.

Purohit et al. (2018) reported a significant decrease in serum levels of phosphorous, copper, selenium and erythrocytic glucose-6-phosphate dehydrogenase while a significant increase in serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), alkaline phosphatase (ALP), total, direct and indirect bilirubin,

glucose and creatinine in post parturient hemoglobinuria affected buffaloes when compared with control.

Kumar et al. (2019) mentioned that there was a significant reduction in serum levels of inorganic phosphorous and calcium while a significant increase in serum levels of glucose, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were recorded in post parturient hemoglobinuria affected buffaloes when compared with normal control.

Zaghawa et al. (2019) reported a significant dimunation in serum phosphorus level and activity of erythrocytic glucose 6-phosphate dehydrogenase with a significant elevation in serum glucose, urea, creatinine, total bilirubin and alkaline phosphatase in acute hypophosphatemic affected buffaloes in comparison with normal control group.

Rahmati et al. (2021) recorded a significant decrease in serum phosphorous level while serum level of urea, creatinine, ALT, AST and ALP were significantly increased in hemoglobinuria affected cattle and buffaloes when compared with control.

Rashid et al. (2021) concluded a significant decrease in serum phosphorous level, total proteins, albumin and activity of glucose -6 phosphate dehydrogenase with a significant increase in serum levels of glucose, total bilirubin and activities of aminotransferases (ALT, AST) and alkaline phosphatase.

Erythrocytic oxidant enzymatic activity changes associated with hypophosphatemia

Ogawa et al. (1989) reported a significant decrease in erythrocytic, reduced glutathione value in chronic hypophosphatemia affected buffaloes when compared with control.

Mata et al. (1994) recorded a significant increase in erythrocytic malondialdehyde level in post parturient hemoglobinuria affected buffaloes when compared with normal control.

Sarma et al. (2014) reported a significant decrease in erythrocytic, reduced glutathione value while erythrocytic malondialdehyde (MDA) level was significantly increase in post parturient hemoglobinuria affected buffaloes when compared with normal control.

Ziwei et al. (2017) reported a significant decrease in erythrocytic superoxide dismutase (SOD) and glutathione peroxidase levels while a significant increase in erythrocytic malondialdehyde (MDA) level in acute phosphorous deficient cows when compared with control.

Rashid et al. (2021) concluded that several metallo enzymes such as glutathione peroxidase having se as co-factor, catalase having fe as Co-factor and super oxide dismutase having Cu, Zn and MN as co-factor play an important role in protecting constituents of RBCs from oxidative damage.

MATERIAL

AND METHODS

g) The serum level of parathyroid hormone (PTH) was estimated using automatic analyzer HITACHI 902, Germany.

h) The serum level of creatine phosphokinase (CPK) was estimated using automatic analyzer HITACHI 902, Germany.

i) Serum malondialdehyde (MDA) and glutathione peroxidase (GPx) levels were estimated using ELISA kits.

3- Chemicals

- NaOH 4%: for estimation of serum ALT activity.

- Dipotassium salt of Ethylene Diamine Tetra Acetic acid (EDTA): was used for hematological analysis (1 mg for 1ml blood).

4- Instruments

a- Automatic cell counter (Sysmx KX-2IN, Germany): for complete blood picture.

b- Automatic pipettes (finn pipette, Finland): for biochemical tests.

c- Centrifuge (MP- England): for separation of serum samples.

d- Deep freezer (-20ºC): for preservation of serum samples.

e- Spectrophotometer (Spectronic Gensys 5): for measurement of some biochemical parameters.

f- Other equipments: Eppendorf tubes, glass slides, beakers, flasks, test tubes, pipettes and graduated cylinders.

II. Methods

1- Experimental design

The study was conducted on 40 adult female water buffaloes, distributed into 3 groups. The first group was 10 apparently and clinically healthy buffaloes kept as a normal control group. The second group was 15 buffaloes suffering from all signs suggesting moderate hypophosphatemia (subclinical cases). The serum phosphorous level was moderately decreased. By clinical examination the buffaloes publicized, anorexia, weakness, emaciation and have paleness conjunctiva and vulvar mucous membranes, also the buffaloes have stiffness in gait. The third group was 15 buffaloes suffering from signs suggesting severe hypophosphatemia (clinical cases). The serum phosphorous level was severely declined. Some of these animals were in the last gestation period (6^{th} month of gestation) and the others were post-partum (3^{rd}-5^{th} week post-partum). The clinical examination revealed red urine, anorexia, weakness, pale mucous membranes of conjunctiva and vulva, laboured breathing, jugular pulsation and the buffaloes may be recumbent in late stage.

2- Blood sampling

Blood sample was collected from each animal via jugular vein puncture and divided into 2 portions. The first portion was collected in EDTA vacutainer tubes for hematolotgical studies according to standard techniques described by **Weiss et al. (2021)**. The second portion was collected in plain centrifuge tubes for separation of serum for biochemical studies.

3- Hematological studies

Red blood cells (RBCs), hemoglobin (Hb) concentration, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and total and differential leukocytic count were determined using automatic cell counter (Sysmex KX-21N).

Biochemical studies

1) Determination of serum inorganic phosphorus level according to Tietz (1990)

Principle:

The measurement of inorganic phosphorus in the serum is usually accomplished by forming a phosphomolybdate complex and in turn reducing it to a molybdenum blue color complex. Methods differ as the choice of reducing agent: stannous chloride, phenyl hydrazine, aminonepathol sulfuric acid, ascorbic acid, p-methylaminophenol sulfate, N-phenyl-p-phenyl nediamine and ferrous sulfate. These methods suffered from instability, deprotinization steps and complexity of performance. Vitro regent eliminated to the need to prepare a protein free filtrate, accelerated color production, stabilized the color and simplified the procedure.

1- Inorganic phosphorus reacts with ammonium molybdate in an acidic medium to form a phosphomolybdate complex.

2- This complex is reduced by ferrous ammonium sulfate to produce a molybdenum blue complex.

Inorganic phosphorus + ammonium molybdate

In acidic medium ↓

Non-reduced phosphomolybdate

Non-reduced phosphomolybdate + ferrous ammonium → Molybdenum blue complex.

The intensity of the color measured photometrically at 600-675 nm and its intensity is directly proportional to inorganic phosphorous concentration in the specimen.

Calculation

$$\frac{\text{Absorbance of specimen}}{\text{Absorbance of standard}} \times 5 \text{ (Standard value)} = \text{mg/dl}$$

2) Determination of serum calcium level according to Tietz (1995)

Principle:

Many colorimeteric methods have been developed for determination of calcium. The methods include calorimetric fluorescent, graximetric, ion selective, titrimetric and atomic absorption techniques.

The methods applied by using alizarin 3-sulphonate and cresolphthalein complexone whilst Gindler and King have described a method using thymol blue. There have been many subsequent modifications to those methods vitro calcium reagent is based on the cresolphthalein complex (CPC) method of Moorehead and Briggs. CPC react with calcium and magnesium in alkaline solution to form a

deep color complex 8-hydroxy quinolone is in corporated into the reagent to preferentially bind magnesium and prevent interference from this cation. CPC is in acid-base indicator necessitating the use of a strong buffer to stabilize the pH.

Calcium react with cresolphthalein to form purple color complex in alkaline medium.

$$\text{Calcium + O-cresolphthalein complexone} \xrightarrow[\text{medium}]{\text{Alkaline}} \text{Calcium-cresolphthalein complexone complex}$$

The intensity of the color measured photometrically between 450 and 600 nm with maximum absorbance at 575 nm is directly proportional to calcium concentration in specimen.

Calculation

$$\text{Calcium (mg/dl)} = \frac{\text{Absorbance of specimen}}{\text{Absorbance of standard}} \times 10 \text{ (Standard value)}$$

3) Determination of total serum proteins level according to Doumas et al., (1981)

Principle:

Proteins give an intensive violet blue complex with copper salts in an alkaline medium. Iodine is included as antioxidant. The intensity of the color formed is proportional to the total protein concentration in the samples.

Calculation:

$$\frac{\text{A sample}}{\text{A standard}} \times 6 \text{ (Standard conc.)} = \text{g/dl total proteins in the sample}$$

4) Determination of serum albumin level according to Doumas et al., (1971)

Principle:

Albumin in the presence of Bromocresol green at a slightly acid pH, produces a colour change of the indicator from yellow-green to green blue. The intensity of the colour formed is proportional to the albumin concentration in the sample.

Calculation:

$$\frac{\text{A sample}}{\text{A standard}} \times 4 \text{ (Standard conc.)} = \text{g/dl}$$

5) Determination of the activity of alanine aminotransferase (ALT) according to Reitman and Frankel (1957)

Assay principle:

The reaction involved in the assay systems as follows:

The amino group is enzymatically transferred by ALT present in the sample from alanine to carbon atom of 2-oxoglutrate yield pyruvate and L-glutamate.

$$\text{L-Alanine + 2-oxoglutrate} \xrightarrow{\text{ALT}} \text{Pyruvate + L-glutamate}$$

ALT activity is measured by monitoring the concentration of pyruvate formed with 2,4-dinitrophenyl hydrazine.

Calculation:

The absorbance measured at wave length 546 nm and ALT activity in the serum sample can be determined according to the table

Buffer (approximately 400 µl) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher and let it sit for 1~2 minutes. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 100 µl of Detection Reagent B working solution to each well. Cover with a new Plate sealer. Incubate for 45 minutes at 37ºC.

5. Repeat the aspiration/wash process for five times as conducted in step 3.

6. Add 90 µl of Substrate Solution to each well. Cover with a new Plate sealer. Incubate within 15-30 minutes at 37°C. Protect from light.

7. Add 50 µl of Stop Solution to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.

8. Determine the optical density of each well at once, using a microplate reader set to 450 nm.

Calculation

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean

absorbance for each standard on the x-axis against the concentration on the y-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the Malondialdehyde concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. It is recommended to use some related software to do this calculation, such as curve expert 1.3. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

b- Determination of glutathione peroxidase activity (GPX):

Glutathione peroxidase activity in serum was determined according the method adapted by **Paglia and Valentine (1967)**.

Principle

The microtiter plate provided in this kit has been pre-coated with an antibody specific to GPx-1. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for GPx-1 and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well. Only those wells that contain GPx-1, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The

concentration of GPx-1 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

Procedure

Allow all reagents to reach room temperature (Please do not dissolve the reagents at 37℃ directly.). All the reagents should be mixed thoroughly by gently swirling before pipetting. Avoid foaming. Keep appropriate numbers of strips for 1 experiment and remove extra strips from microtiter plate. Removed strips should be resealed and stored at 4℃ until the kits expiry date. Prepare all reagents, working standards and samples as directed in the previous sections. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.

1. Add 100 μl of Standard, Blank, or Sample per well. Cover with the Plate sealer. Incubate for 2 hours at 37˚C.
2. Remove the liquid of each well, don't wash. Add 100 μl of Detection Reagent A working solution to each well. Cover with the Plate sealer. Incubate for 1 hour at 37℃. Detection Reagent A working solution may appear cloudy. Warm to room temperature and mix gently until solution appears uniform.
3. Aspirate each well and wash, repeating the process three times for a total of three washes. Wash by filling each well with Wash Buffer (approximately 400 μl) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher and let it sit for 1~2 minutes. Complete removal of liquid at each step is essential to

good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 100 µl of Detection Reagent B working solution to each well. Cover with a new Plate sealer. Incubate for 1 hour at 37ºC.
5. Repeat the aspiration/wash process for 5 times as conducted in step 3.
6. Add 90 µl of Substrate Solution to each well. Cover with a new Plate sealer. Incubate within 15-30 minutes at 37ºC. Protect from light.
7. Add 50 µl of Stop Solution to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
8. Determine the optical density of each well at once, using a microplate reader set to 450 nm.

Calculation

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the x-axis against the concentration on the y-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the GPx-1 concentrations versus the log of the O.D. and the best fit line can be

determined by regression analysis. It is recommended to use some related software to do this calculation, such as curve expert 1.3. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Parathyroid hormone:

Serum parathyroid hormone was determined using automatic analyser (HITACHI).

Active form of vitamin D (2 hydroxycalciferol) was estimated using automatic analyser HITACHI 902, Germany.

Determination of LDH using automatic analyser HITACHI 902, Germany.

Determination of serum creatine phosphokinase (CPK) activity:

Serum creatine phosphokinase (CPK) activity was determined using automatic analyser HITACHI, 902, Germany.

Statistical analysis

All values will be presented as mean (±) standard error (SE). The significant difference between the mean of the groups will be statistically analyzed by one way ANOVA. The significance was set as ≤ 0.05 **(Tamhan and Dunlop, 2000)**.

RESULTS

RESULTS

The present study was carried out on adult female water buffaloes suffering from the clinical signs suggested to be hypophosphatemia.

(1) Clinical signs

The clinical examination of moderate cases of hypophosphatemia revealed pale mucous membrane of vulva and conjunctiva, weakness, anorexia, emaciation, laboured breathing, rough coat (Photo 1), decreasing milk production in lactating animals and stiffness in gait (Photo 2). While in severe cases the affected buffaloes were suffering from all the previous symptoms in addition to the presence of red urine (Photo 3) and the animals may be recumbent in some cases.

Photo (1): Buffalo with subclinical hypophosphatemia showing rough coat.

Photo (2): Buffalo with subclinical hypophosphatemia showing loss weight and stiffness in gait

Photo (3): Buffalo with clinical (acute) hypophosphatemia showing red urine.

Hematological changes

a) Changes in erythrogram

The results of the present work showed a significant decrease in RBCs count, Hb concentration and PCV in subclinical cases with development of normocytic normochromic anemia. Moreover, a highly significant decrease in RBCs count, Hb concentration, PCV with development of macrocytic hypochromic anemia was reported in clinical cases when compared with normal control group (Table 3 and Figs 5-10).

Table (3): Effect of subclinical and clinical hypophosphatemia on erythrogram of buffaloes (mean values ± SE)

Parameters / Groups	RBCs (10^6/µl)	Hb (g%)	PCV (%)	MCV (fl)	MCH (pg)	MCHC (%)
Control	8.02 ± 0.23^a	11.00 ± 0.31^a	33.07 ± 0.45^a	41.23 ± 2.09^b	13.70 ± 0.35^b	33.26 ±1.11^a
Subclinical cases	6.55 ± 0.12^b	9.35 ± 0.05^b	28.05 ± 0.42^b	42.18 ± 1.01^b	14.27 ± 0.56^b	33.33 ± 0.62^a
Clinical cases	4.06 ± 0.16^c	5.82 ± 0.11^c	20.46 ± 0.33^c	50.39 ± 2.40^a	14.33 ± 0.42^a	28.34 ± 1.06^b

Significant at $P \leq 0.05$

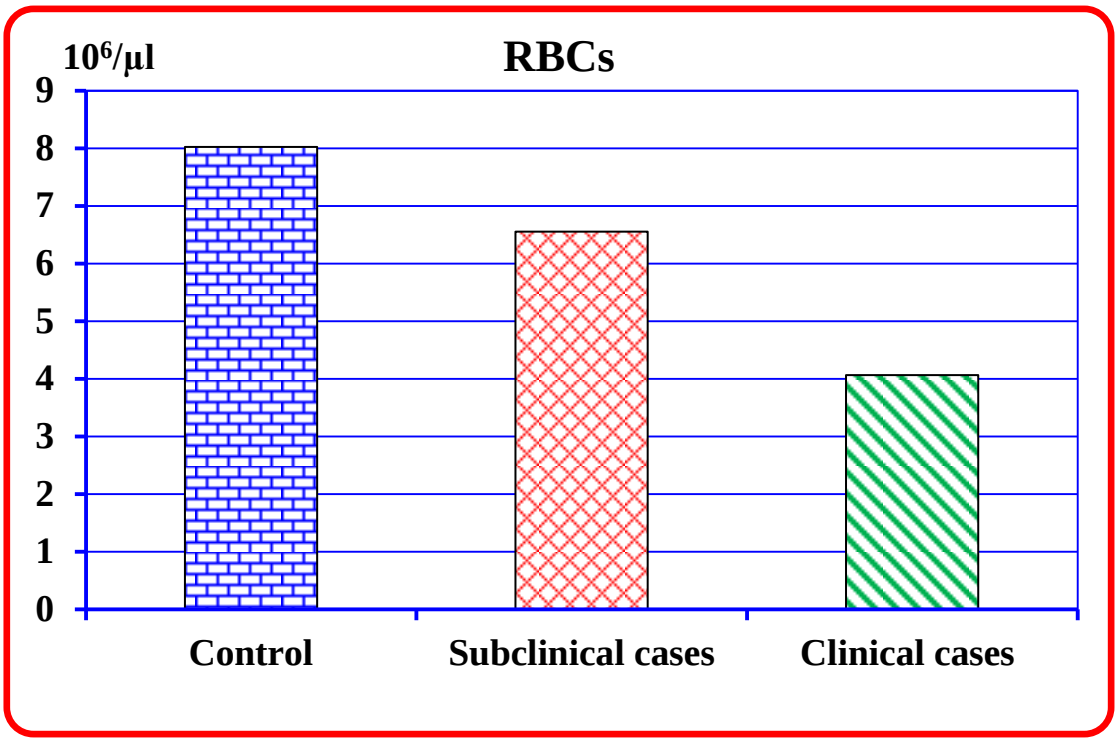

Fig. (5): Effect of subclinical and clinical hypophosphatemia on RBCs

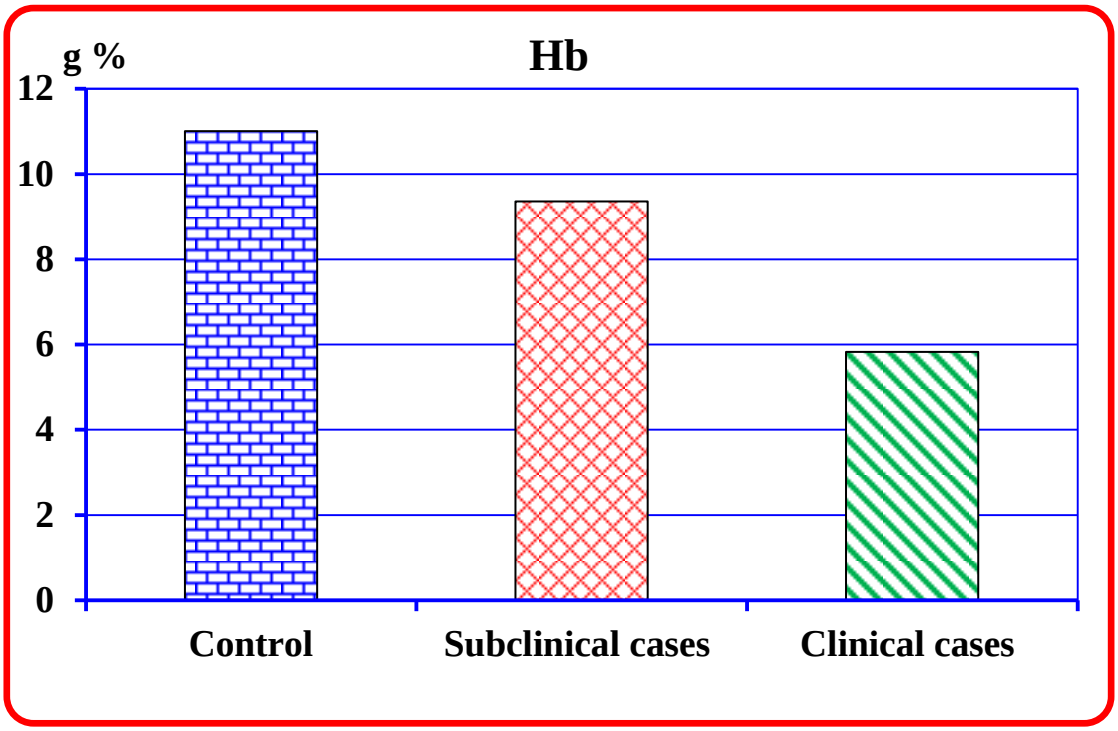

Fig. (6): Effect of subclinical and clinical hypophosphatemia on Hb concentration.

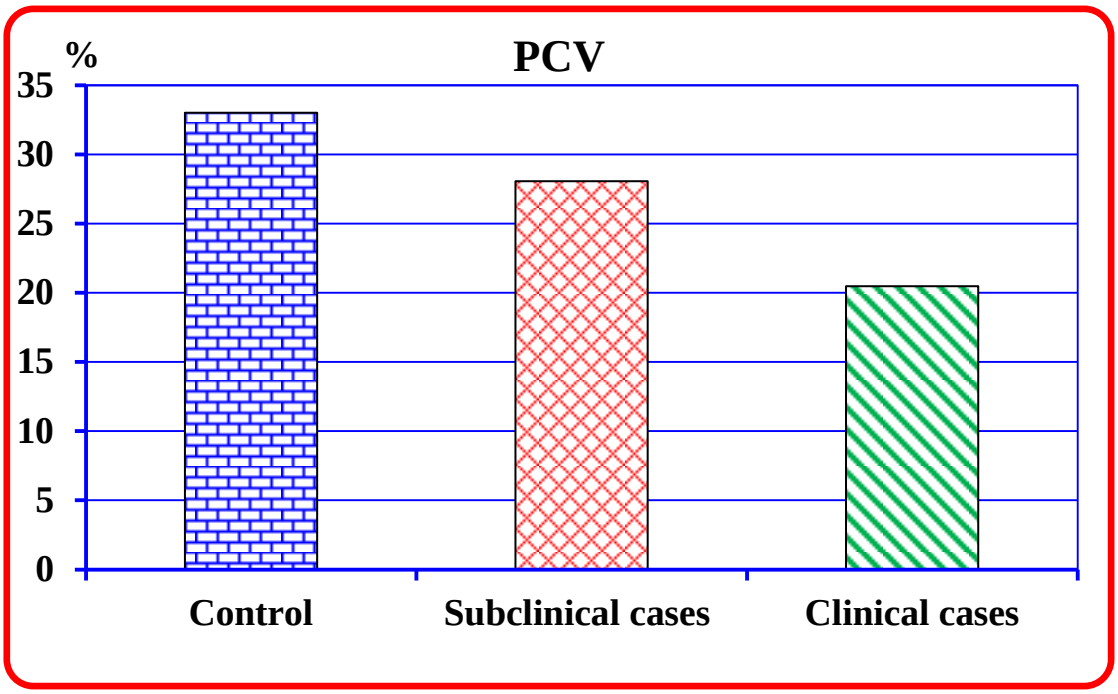

Fig. (7): Effect of subclinical and clinical hypophosphatemia on PCV

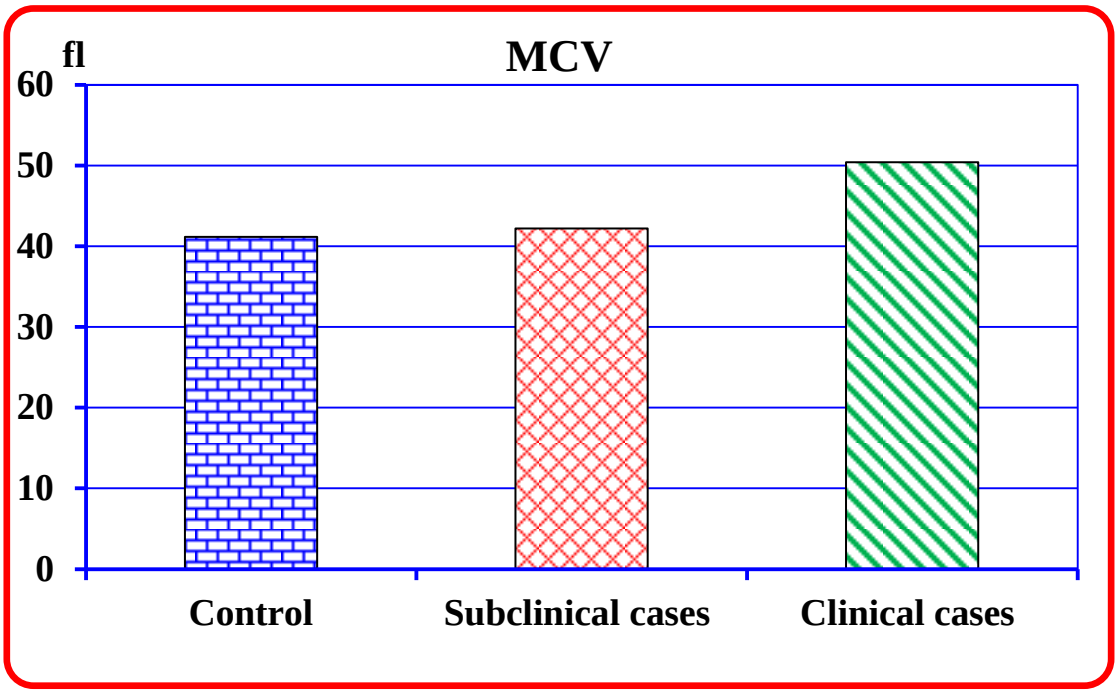

Fig. (8): Effect of subclinical and clinical hypophosphatemia on MCV

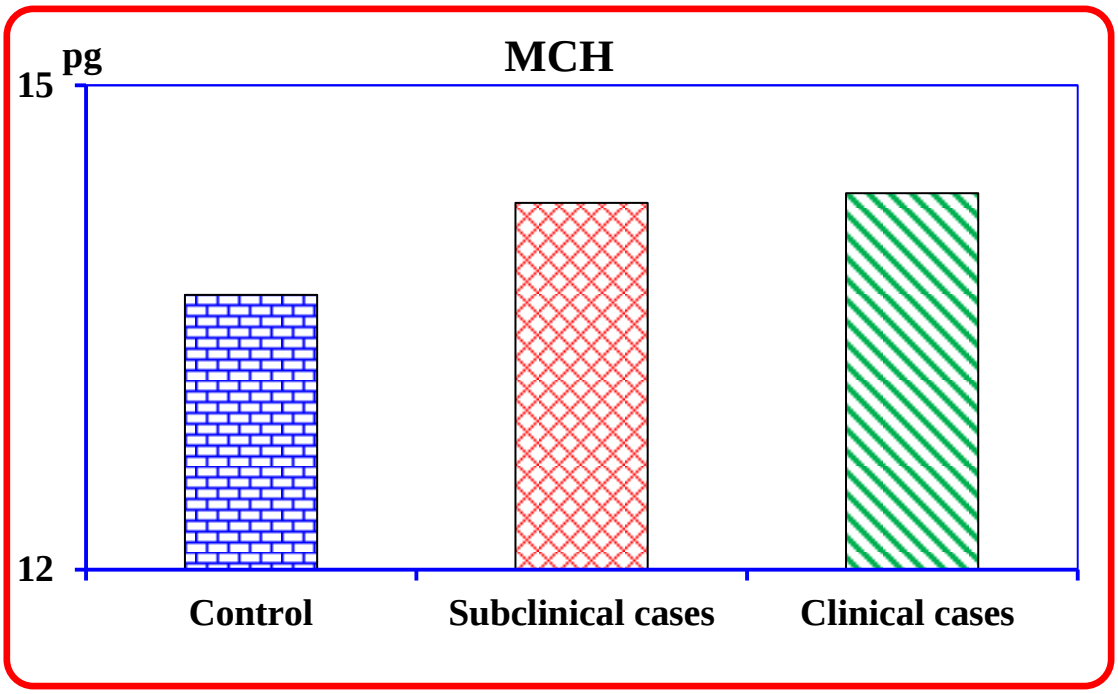

Fig. (9): Effect of subclinical and clinical hypophosphatemia on MCH

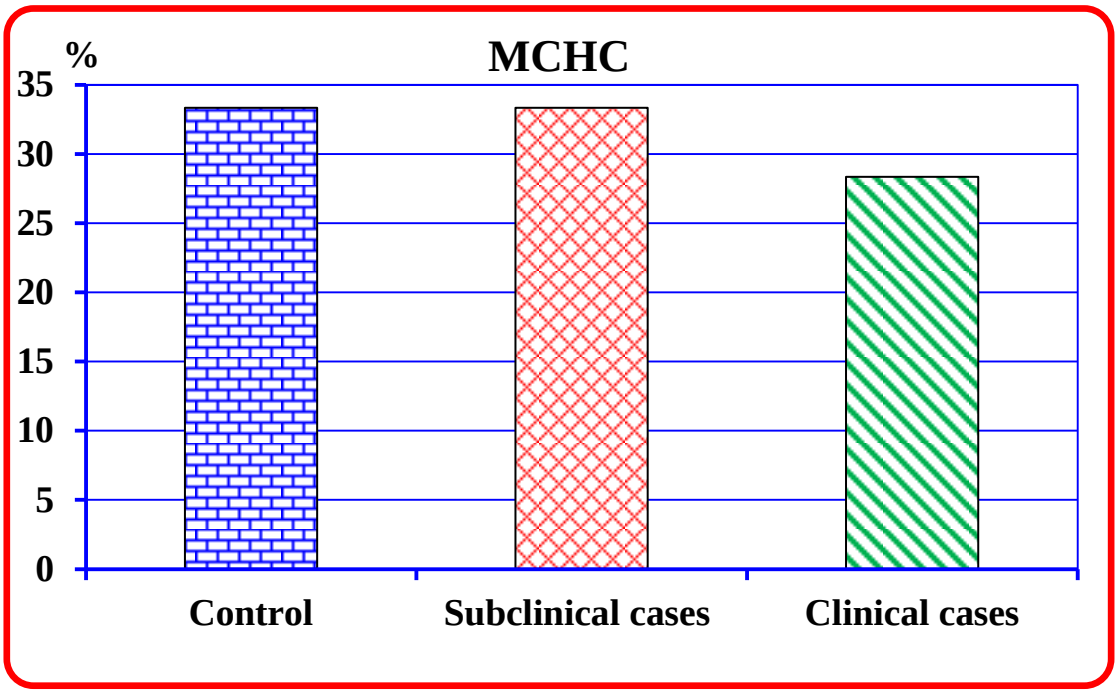

Fig. (10): Effect of subclinical and clinical hypophosphatemia on MCHC

b) Changes in leukogram

The subclinical cases showed non significant changes in total leukocytic, neutrophilic and lymphocytic counts while the clinical cases reported a significant increase in total leukocytic and neutrophilic counts, with a significant decrease in lymphocytic count. The eosinophils and monocytes showed non significant changes in subclinical and clinical cases when compared with normal control group (Table 4 and Fig. 11-15).

Table (4): Effect of subclinical and clinical hypophosphatemia on leukogram of buffaloes (mean values ± SE)

Parameters / Groups	WBCs (10^3/µl)	Neutrophils (10^3/µl)	Lymphocytes (10^3/µl)	Eosinophils (10^3/µl)	Monocytes (10^3/µl)
Control	9.23 ± 0.45[b]	3.44 ± 0.34[b]	5.30 ± 0.20[a]	0.09 ± 0.02[a]	0.40 ± 0.08[a]
Subclinical cases	9.40 ± 0.60[b]	3.83 ± 0.05[b]	5.00 ± 0.10[a]	0.07 ± 0.01[a]	0.50 ± 0.15[a]
Clinical cases	11.18 ± 0.95[a]	7.60 ± 0.05[a]	3.00 ± 0.16[a]	0.08 ± 0.01[a]	0.50 ± 0.03[a]

Significant at $P \leq 0.05$

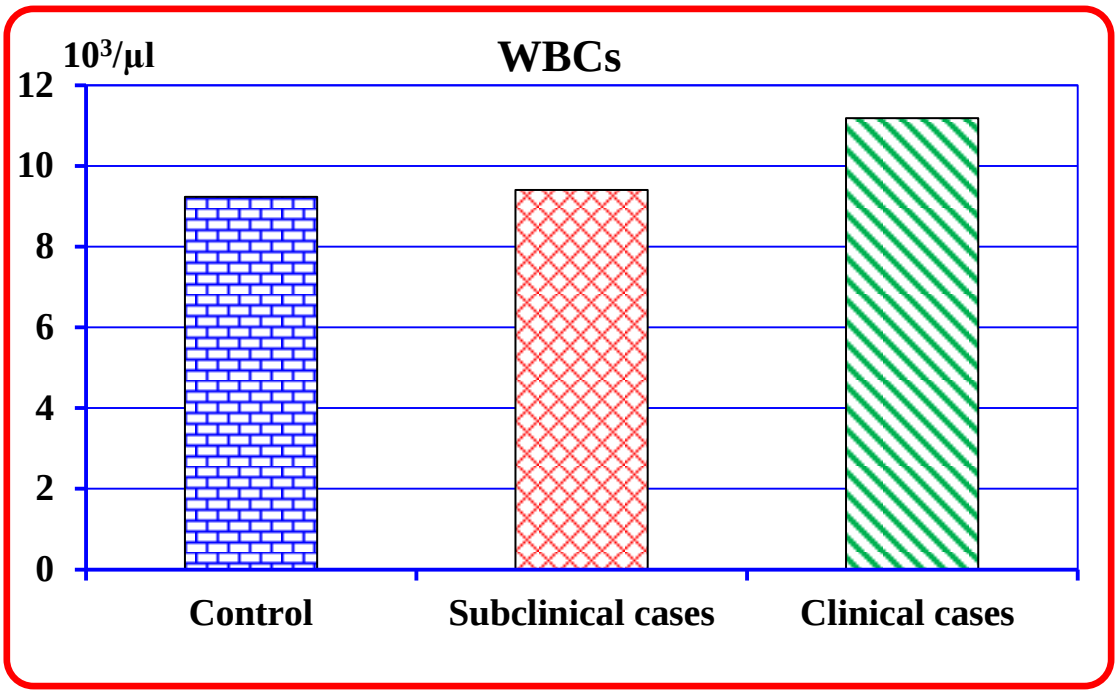

Fig. (11): Effect of subclinical and clinical hypophosphatemia on total leukocytic count

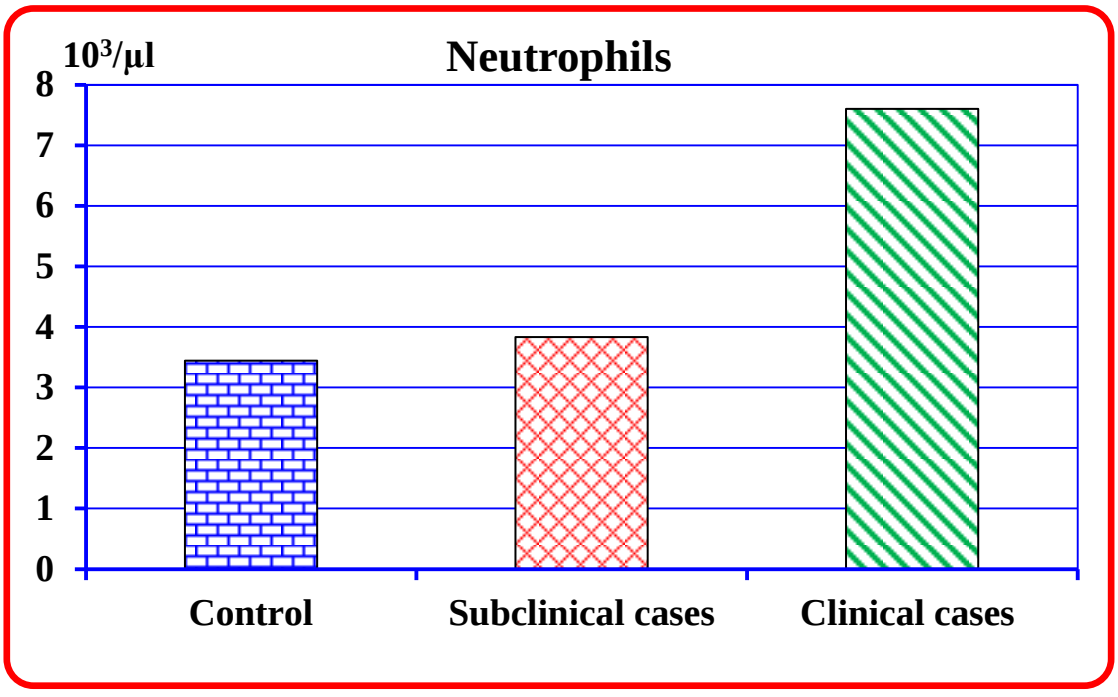

Fig. (12): Effect of subclinical and clinical hypophosphatemia on neutrophilic count

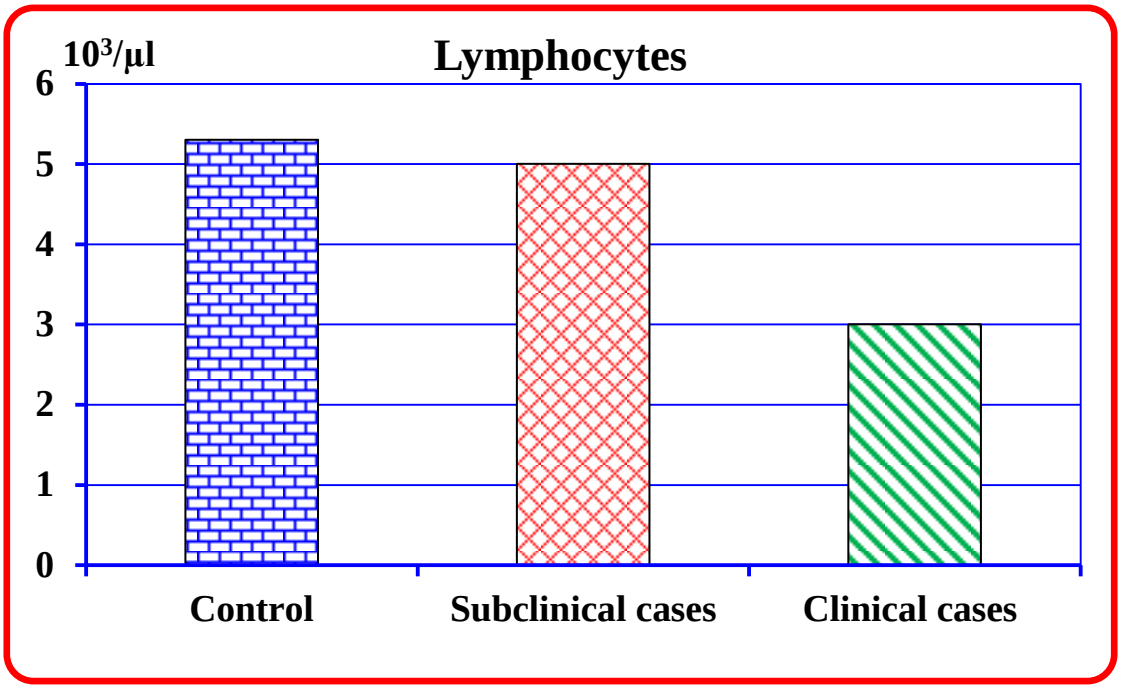

Fig. (13): Effect of subclinical and clinical hypophosphatemia on lymphocytic count

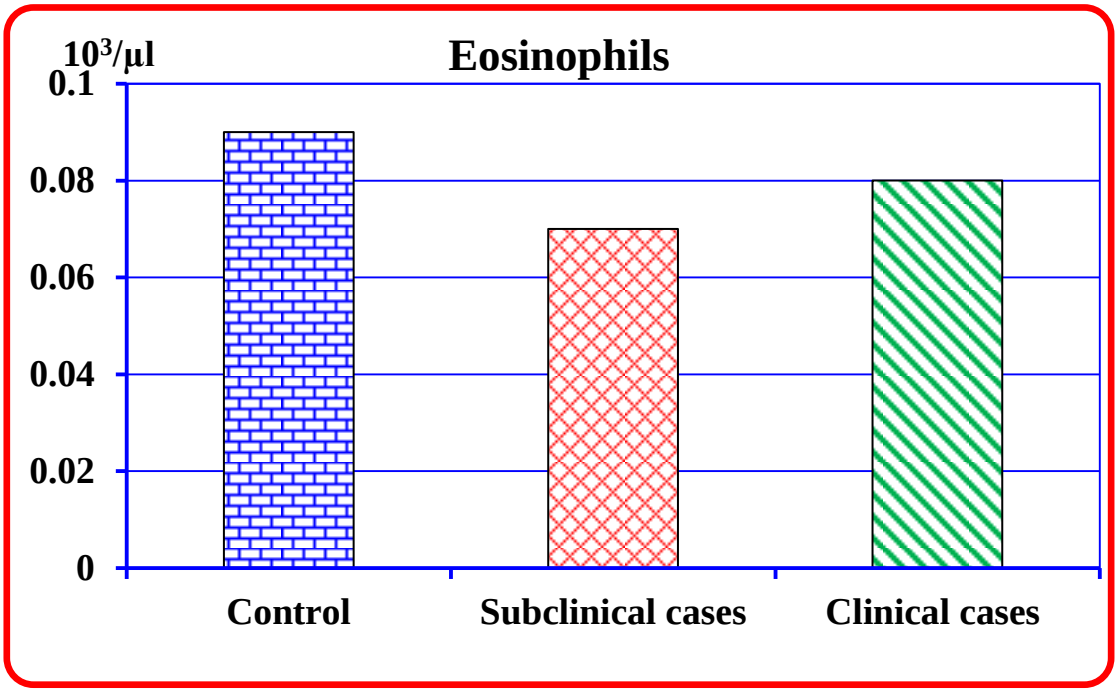

Fig. (14): Effect of subclinical and clinical hypophosphatemia on eosinophilic count

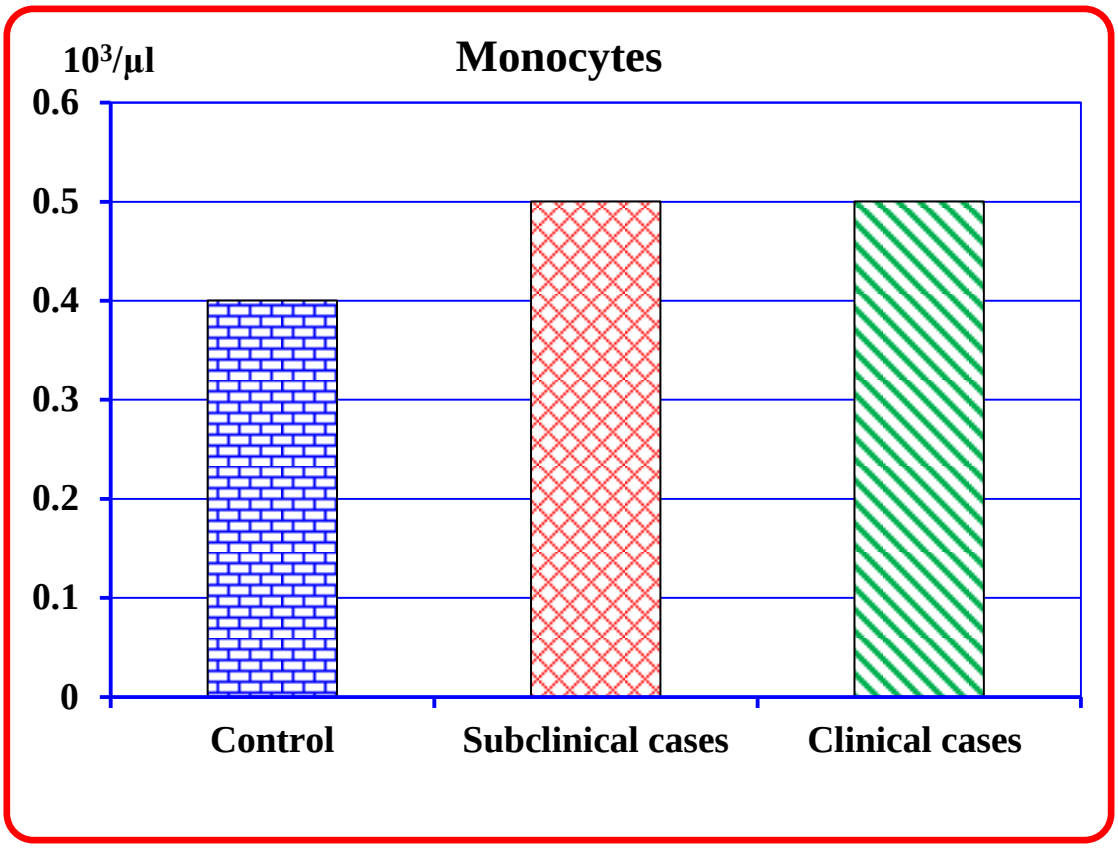

Fig. (15): Effect of subclinical and clinical hypophosphatemia on monocytic count

(3) Biochemical findings

a) Changes in some serum electrolytes

The present study revealed a significant decrease in serum phosphorous level in both subclinical and clinical cases while serum calcium level was not significantly altered in the subclinical cases but it significantly decreased in the clinical cases when compared with normal control group (Table 5 and Figs.16-17).

Table (5): Effect of subclinical and clinical hypophosphatemia on some serum minerals (mean values ± SE)

Parameters / Groups	P (mg/dl)	Ca (mg/dl)
Control	6.94 ± 0.31^{a}	10.53 ± 0.13^{a}
Subclinical cases	2.65 ± 0.14^{b}	9.75 ± 0.10^{a}
Clinical cases	1.42 ± 0.43^{c}	8.11 ± 0.46^{b}

Significant at $P \leq 0.05$

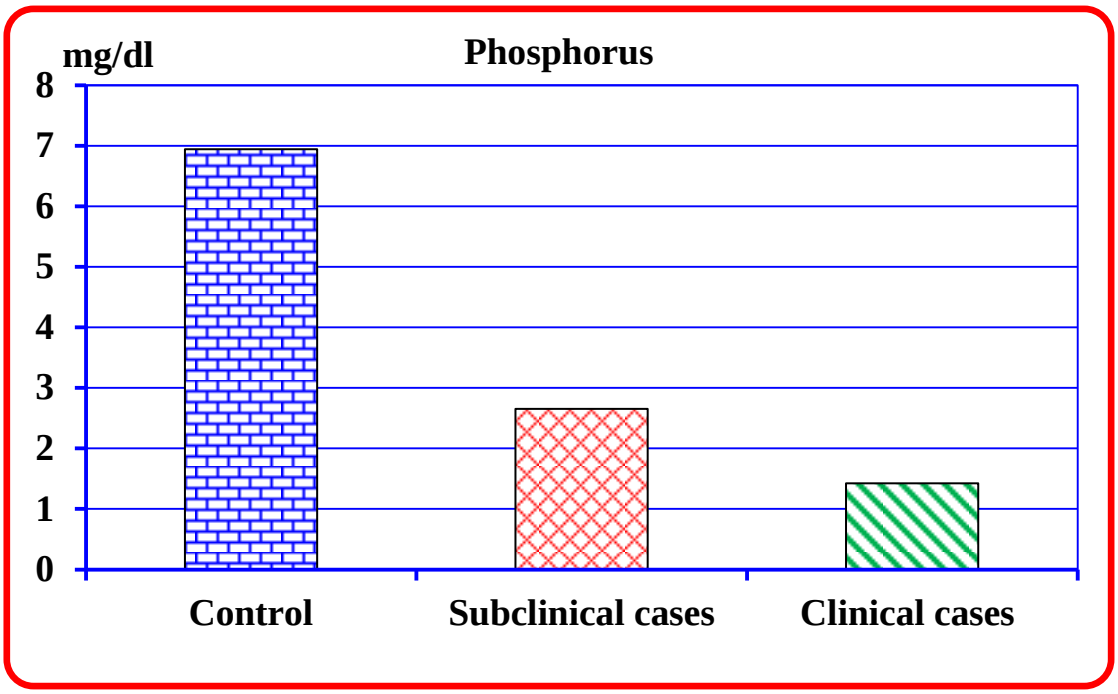

Fig. (16): Effect of subclinical and clinical hypophosphatemia on phosphorus level

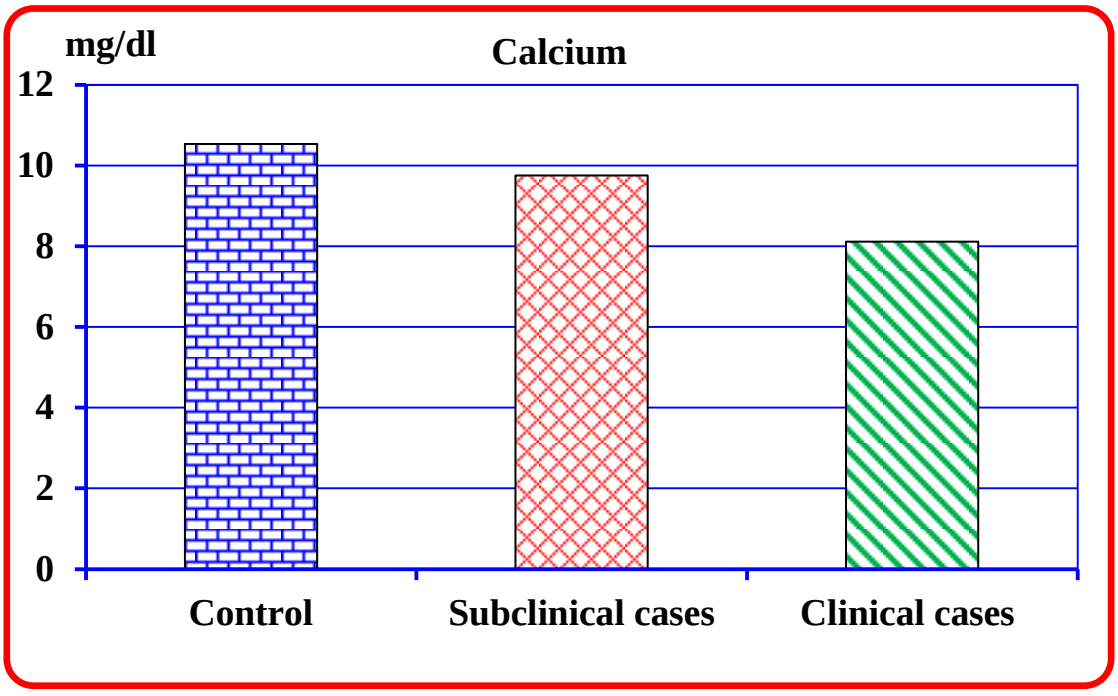

Fig. (17): Effect of subclinical and clinical hypophosphatemia on calcium level

b) Changes in proteinogram

The subclinical and clinical cases revealed a significant decrease in serum total proteins, albumin, globulins and albumin globulin ratio in paralleled with the normal control group (Table 6 and Figs. 18-21).

Table (6): Effect of subclinical and clinical hypophosphatemia on some serum proteinogram of buffaloes (mean values ± SE)

Parameters / Groups	T.P. (g/dl)	Albumin (g/dl)	Globulins (g/dl)	A/G ratio
Control	7.13 ± 015[a]	3.76 ± 0.11[a]	3.38 ± 0.13[a]	1.05 ± 0.50[a]
Subclinical cases	6.16 ± 0.14[b]	3.04 ± 0.11[b]	3.12 ± 0.10[b]	0.95 ± 0.03[b]
Clinical cases	5.88 ± 0.13[b]	2.72 ± 0.12[b]	3.16 ± 0.08[b]	0.86 ± 0.08[b]

Significant at $P \leq 0.05$

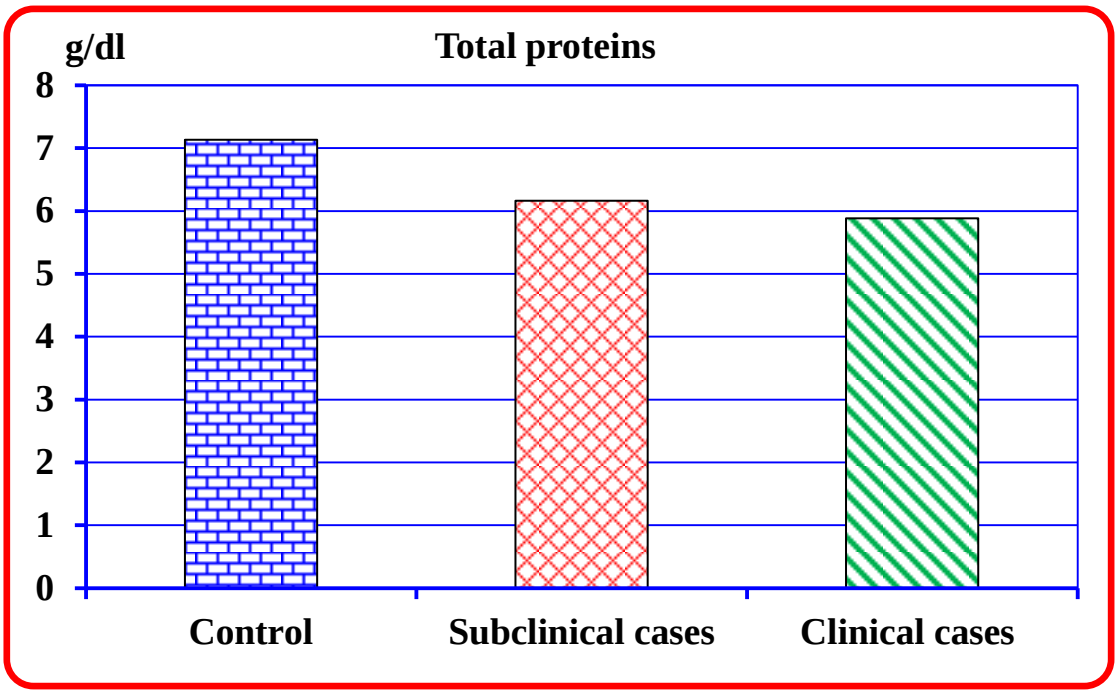

Fig. (18): Effect of subclinical and clinical hypophosphatemia on serum total protein

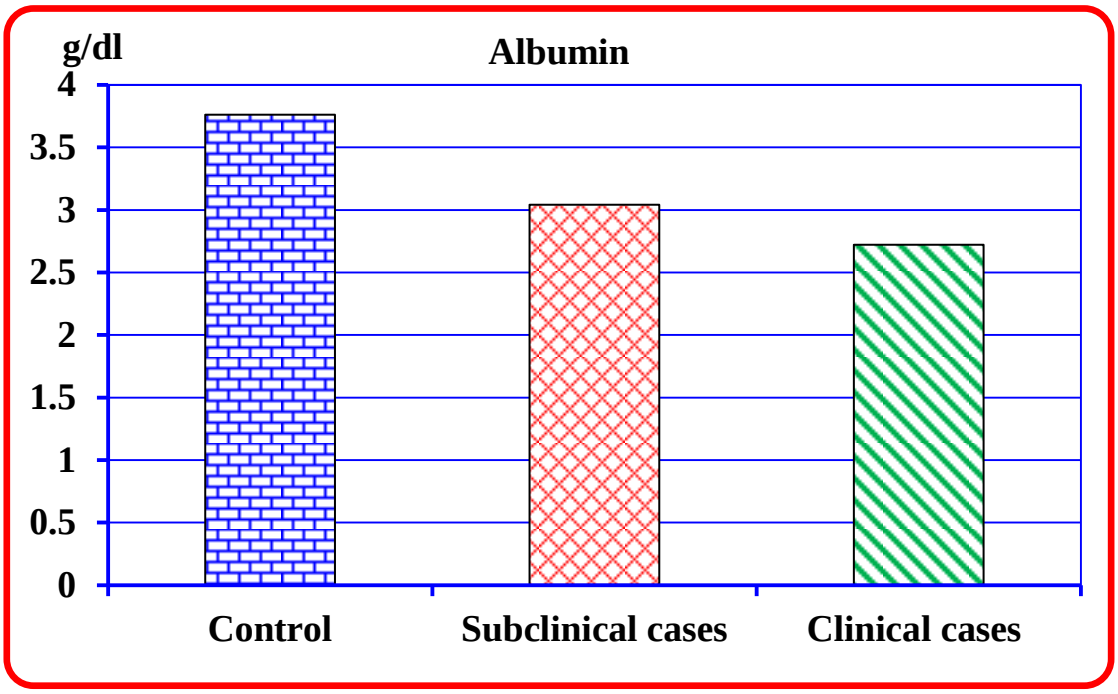

Fig. (19): Effect of subclinical and clinical hypophosphatemia on serum albumin

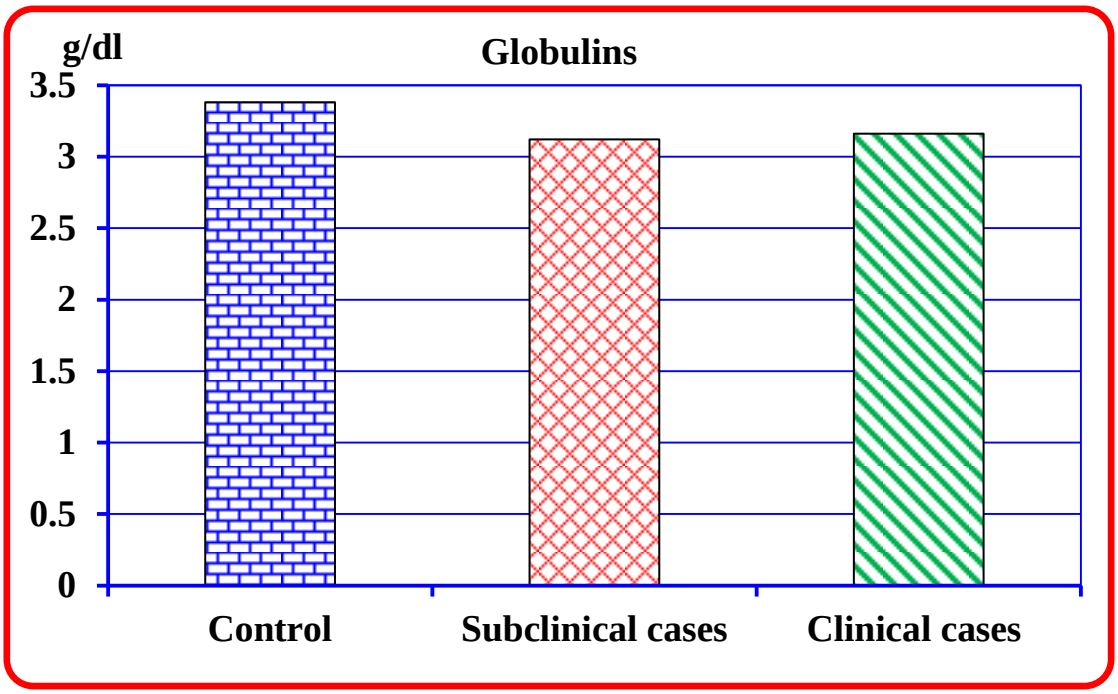

Fig. (20): Effect of subclinical and clinical hypophosphatemia on serum globulins

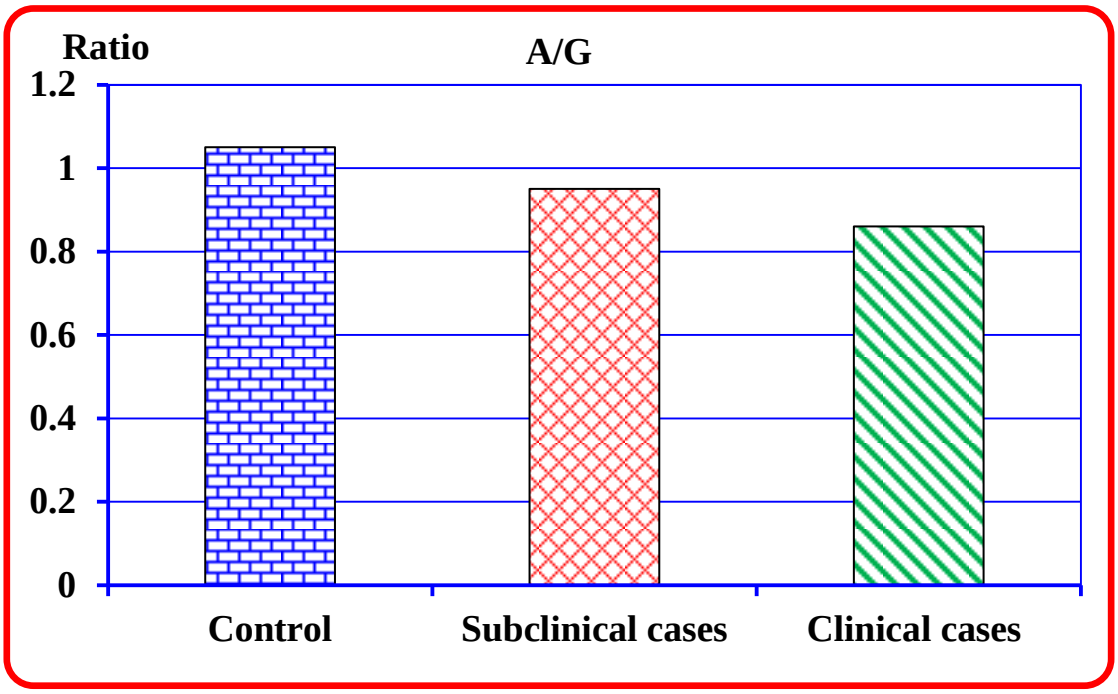

Fig. (21): Effect of subclinical and clinical hypophosphatemia on serum albumin/globulins ratio

c) Changes in serum activities of aminotransferases, alkaline phosphatase, total, direct and indirect bilirubin

Both 2nd and 3rd groups revealed a substantial increase in transferases activities (ALT and AST) and activity of alkaline phosphatase (ALP). Also, serum levels of total, direct and indirect bilirubin were significantly elevated in subclinical and clinical cases when paralleled with healthy control (Table 7 and Figs. 22-27).

Table (7): Effect of subclinical and clinical hypophosphatemia on serum activities of aminotransferases, alkaline phosphatase, total, direct and indirect bilirubin (mean values ± SE)

Parameters / Groups	ALT (U/L)	AST (U/L)	ALP (U/L)	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)	Indirect bilirubin (mg/dl)
Control	19.40 ± 0.54^{c}	66.40 ± 0.10^{c}	57.00 ± 2.12^{c}	0.30 ± 0.01^{c}	0.16 ± 0.02^{c}	0.14 ± 0.03^{c}
Subclinical cases	31.80 ± 1.44^{b}	80.29 ± 4.30^{b}	69.27 ± 5.00^{b}	1.15 ± 0.05^{b}	0.24 ± 0.02^{b}	0.91 ± 0.01^{b}
Clinical cases	47.28 ± 0.13^{a}	147.80 ± 3.70^{a}	131.00 ± 6.50^{a}	3.02 ± 0.06^{a}	0.80 ± 0.01^{a}	2.22 ± 0.02^{a}

Significant at $P \leq 0.05$

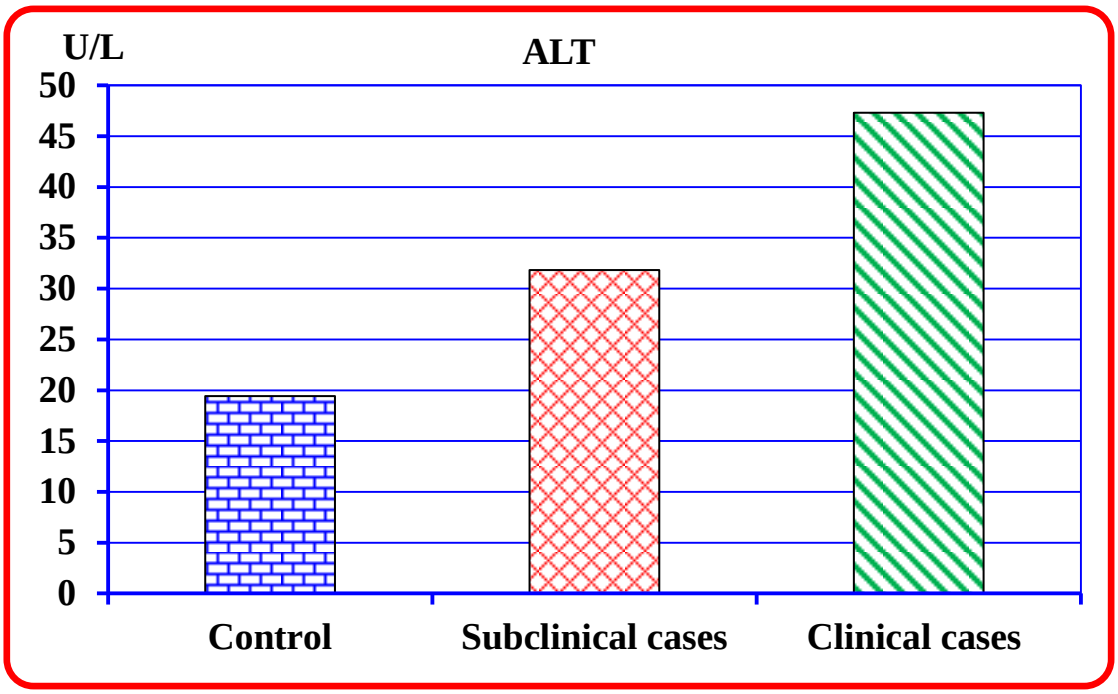

Fig. (22): Effect of subclinical and clinical hypophosphatemia on the activities of ALT

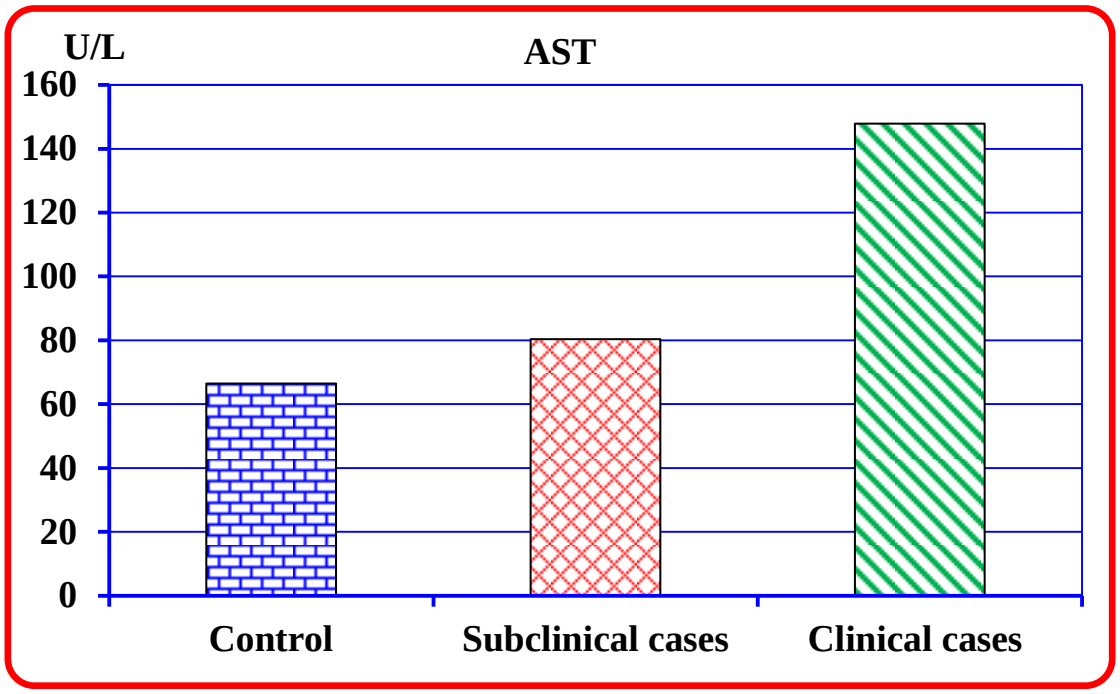

Fig. (23): Effect of subclinical and clinical hypophosphatemia on the activities of AST

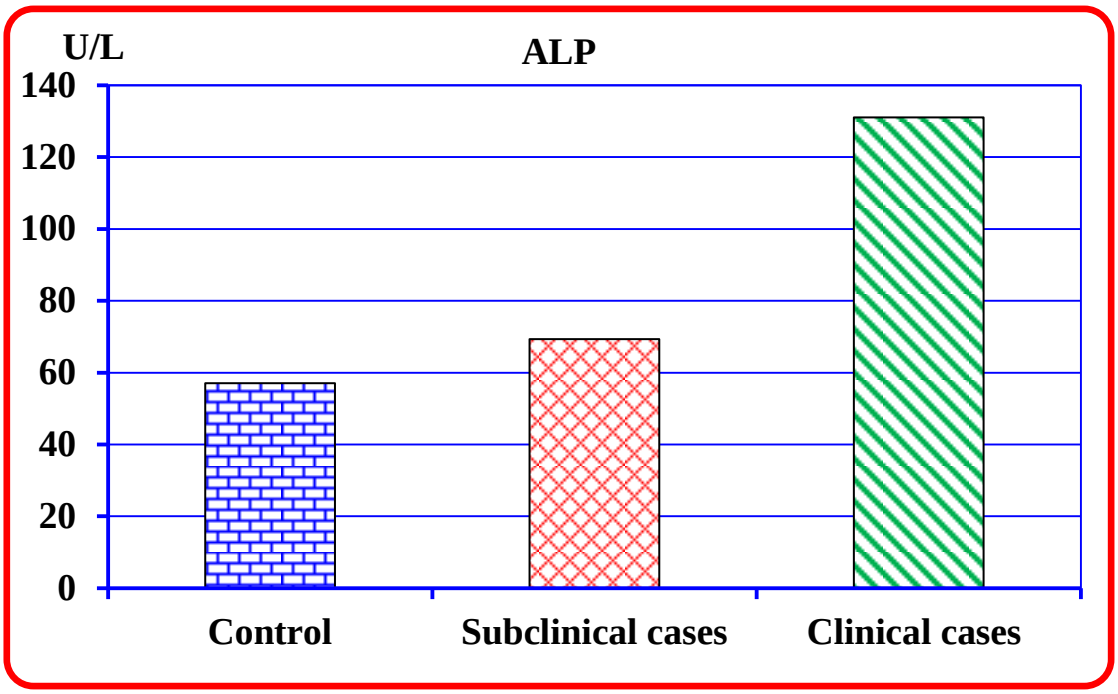

Fig. (24): Effect of subclinical and clinical hypophosphatemia on the activities of ALP

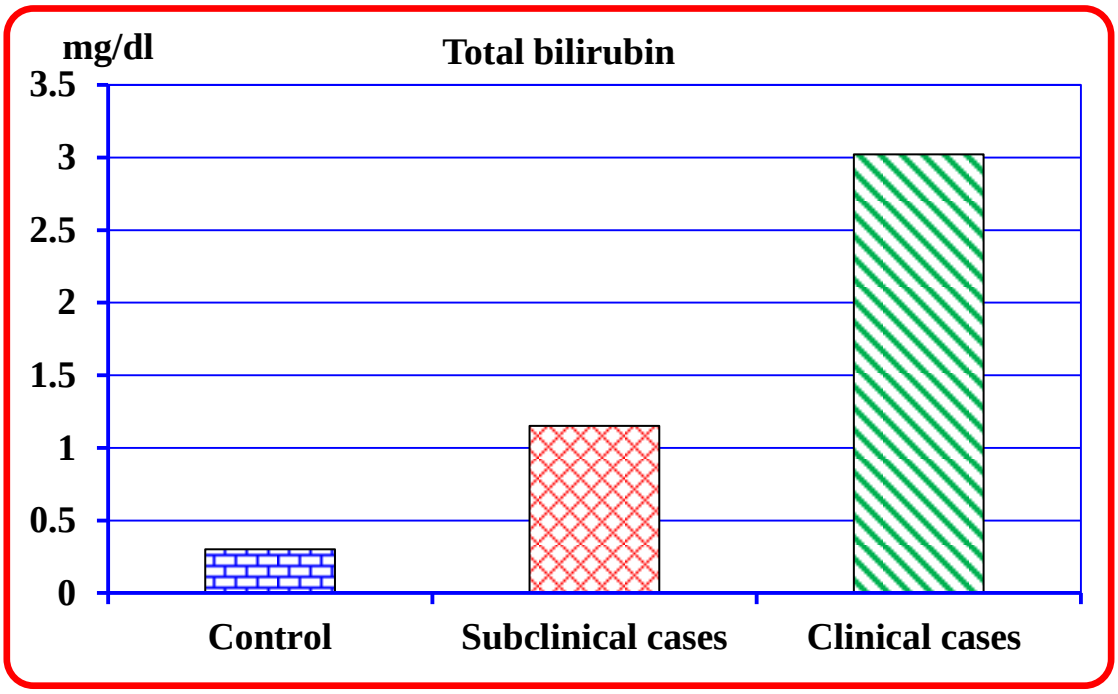

Fig. (25): Effect of subclinical and clinical hypophosphatemia on total bilirubin

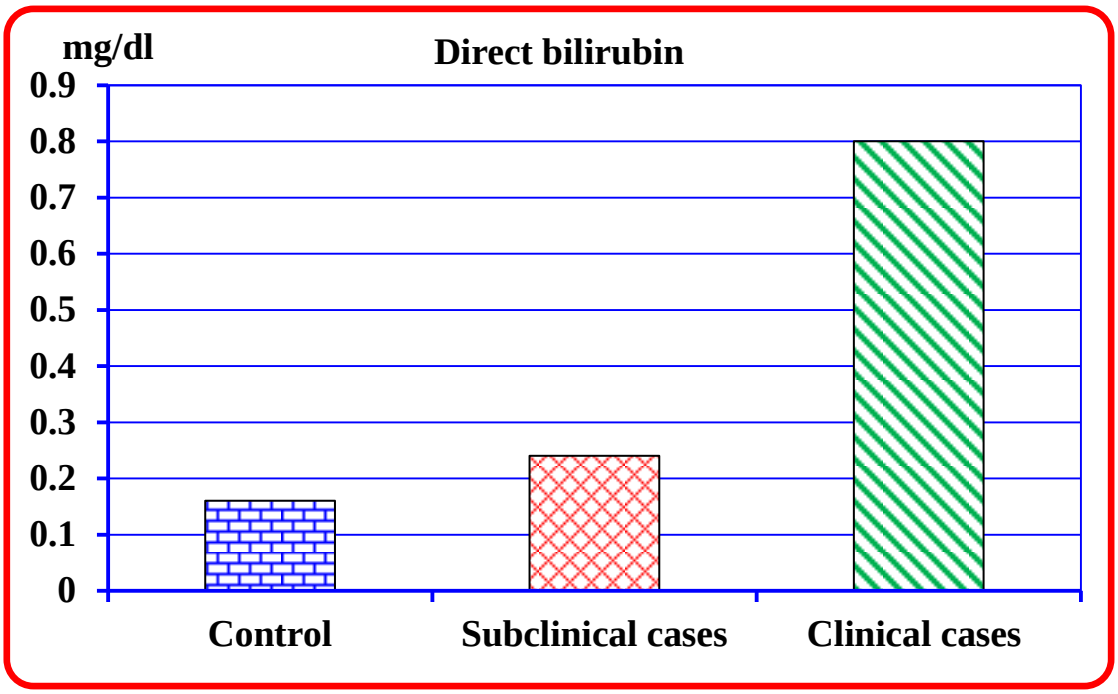

Fig. (26): Effect of subclinical and clinical hypophosphatemia on direct bilirubin

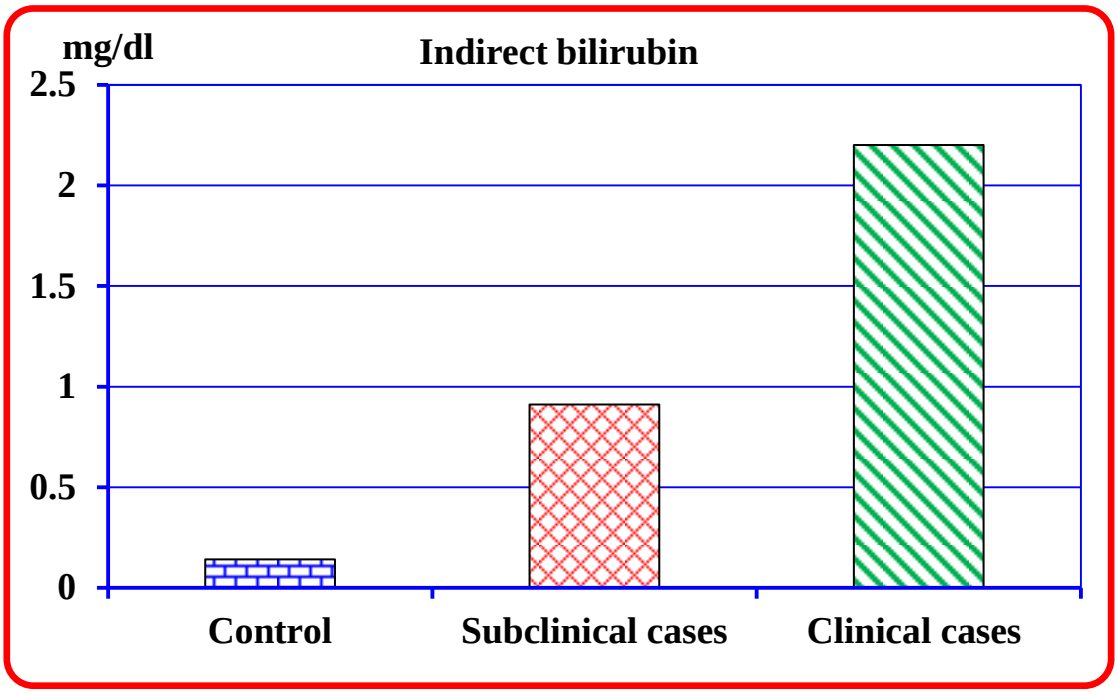

Fig. (27): Effect of subclinical and clinical hypophosphatemia on indirect bilirubin

d) Changes in some biochemical parameters

There was a significant elevation in serum glucose level in subclinical and clinical cases. On the other hand, a substantial dimunation in activity of erythrocytic glucose-6-phosphate dehydrogenase in both groups. Serum urea and creatinine levels were significantly rised in subclinical and clinical cases in paralleled with normal control group (Table 8 and Figs. 28-31).

Table (8): Effect of subclinical and clinical hypophosphatemia on some biochemical parameters (mean values ± SE)

Parameters / Groups	Glucose (mg/dl)	G-6-P (pg/ml)	Urea (mg/dl)	Creatinine (mg/dl)
Control	52.15 ± 0.24^{c}	107.97 ± 3.90^{a}	32.86 ± 0.15^{c}	1.21 ± 0.07^{c}
Subclinical cases	69.40 ± 1.10^{b}	95.00 ± 2.50^{b}	39.00 ± 1.21^{b}	1.61 ± 0.07^{b}
Clinical cases	101.45 ± 2.42^{a}	86.40 ± 2.40^{c}	48.76 ± 1.42^{a}	2.30 ± 0.23^{a}

Significant at $P \leq 0.05$

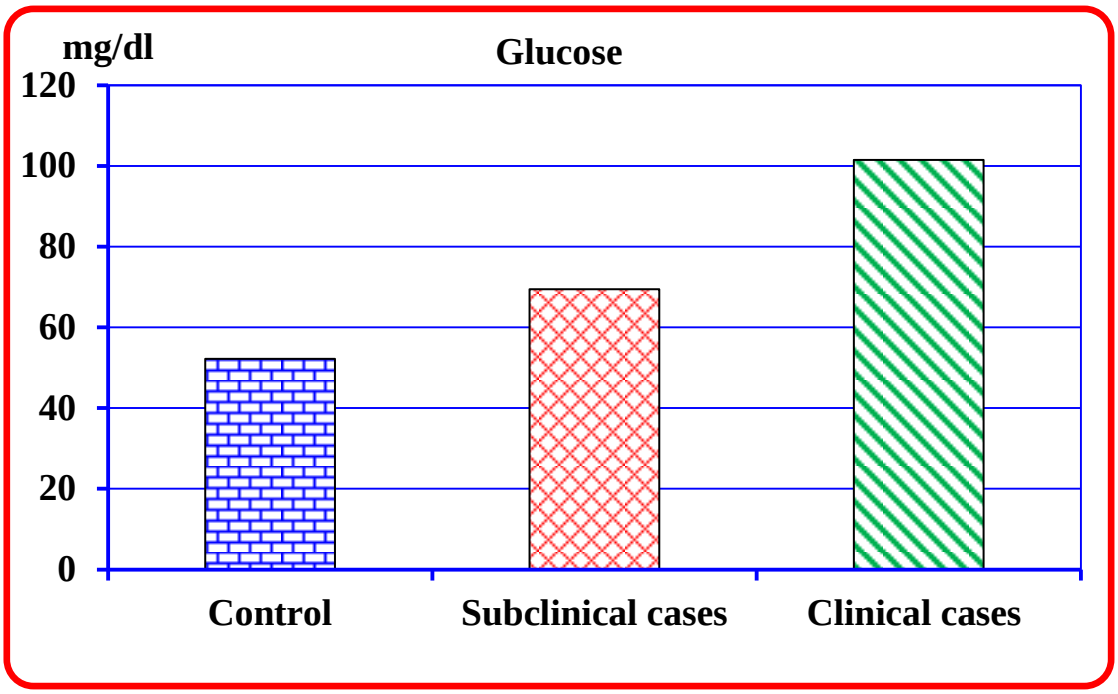

Fig. (28): Effect of subclinical and clinical hypophosphatemia on serum glucose

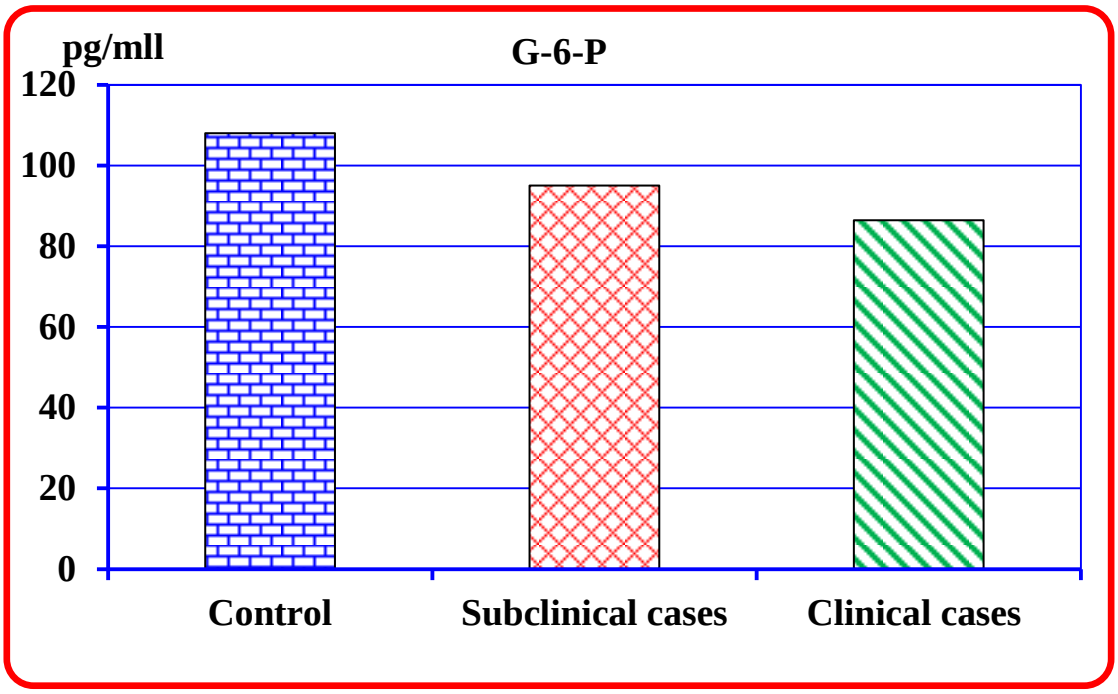

Fig. (29): Effect of subclinical and clinical hypophosphatemia on glucose-6-phosphate

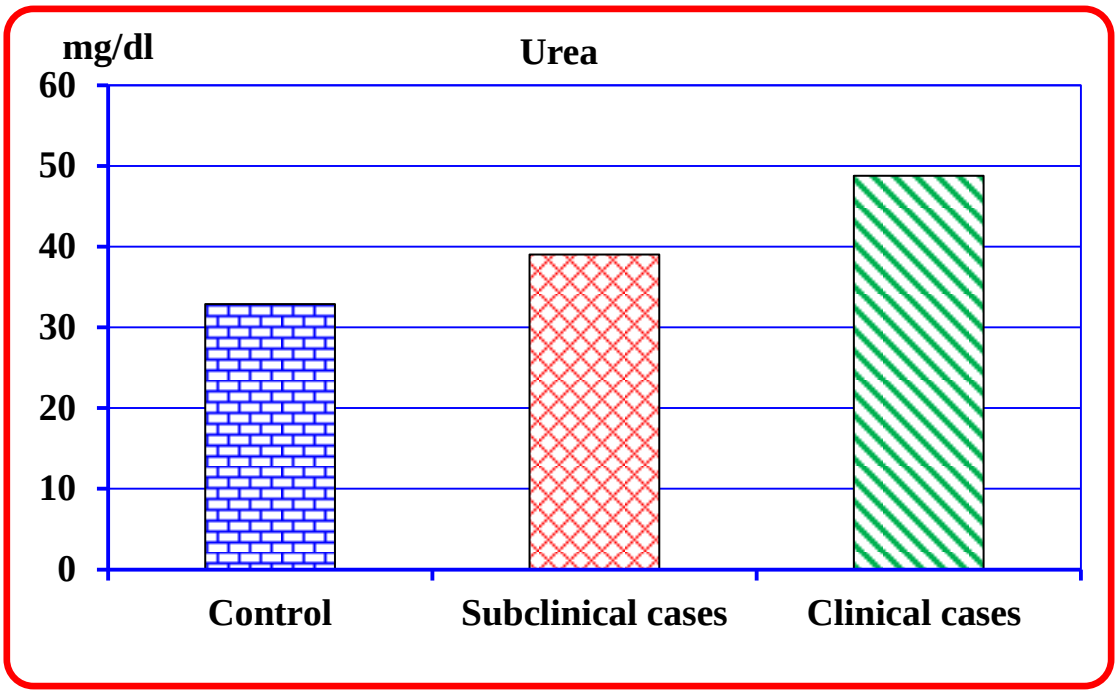

Fig. (30): Effect of subclinical and clinical hypophosphatemia on serum urea

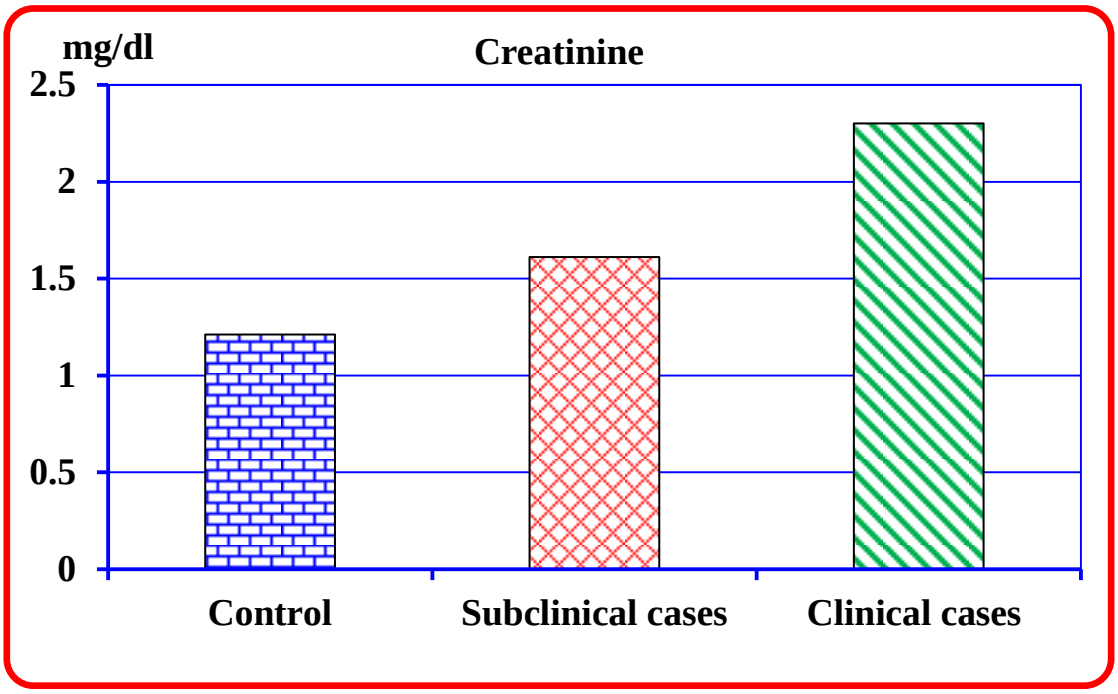

Fig. (31): Effect of subclinical and clinical hypophosphatemia on serum creatinine

e) Changes in oxidative stress markers

The subclinical and clinical cases revealed a significant elevation in serum MDA level. On the other hand, there was a significant dimunation in serum glutathione peroxidase activity (GPx) in both cases when compared with the normal control group (Table 9 and Figs. 32-33).

Table (9): Effect of subclinical and clinical hypophosphatemia on oxidative stress markers (mean values ± SE)

Parameters / Groups	MDA (nmol/ml)	GPx (U/ml)
Control	0.64 ± 0.11^{c}	202.00 ± 0.490^{a}
Subclinical cases	4.01 ± 0.64^{b}	149.00 ± 3.50^{b}
Clinical cases	6.90 ± 0.95^{a}	89.00 ± 1.20^{c}

Significant at $P \leq 0.05$

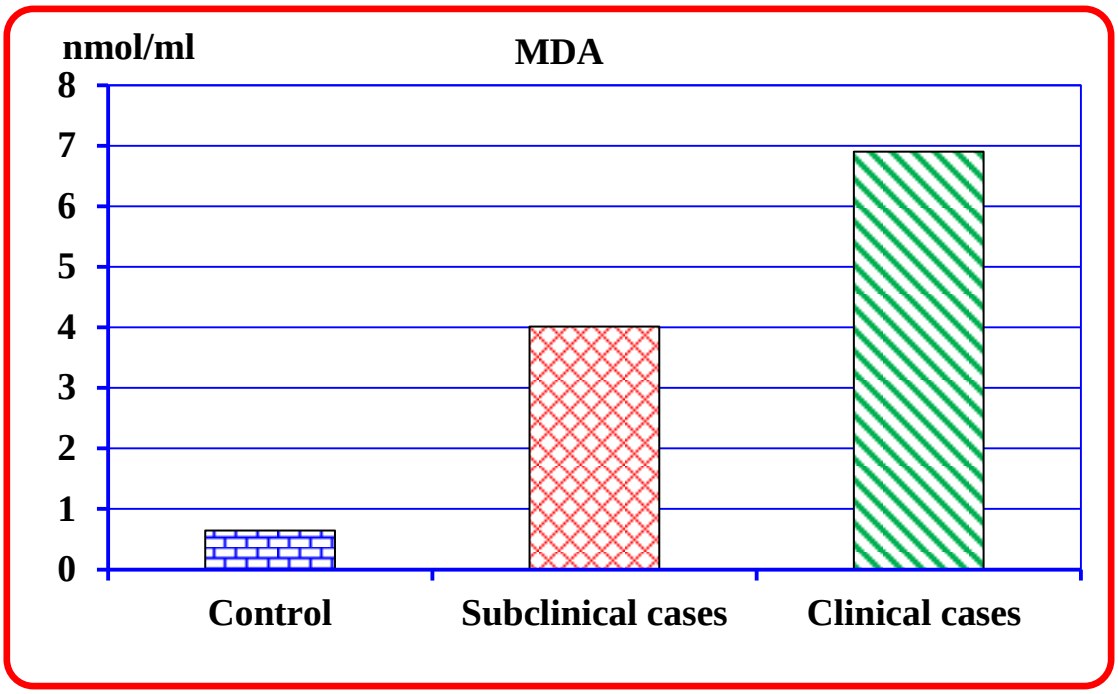

Fig. (32): Effect of subclinical and clinical hypophosphatemia on MDA

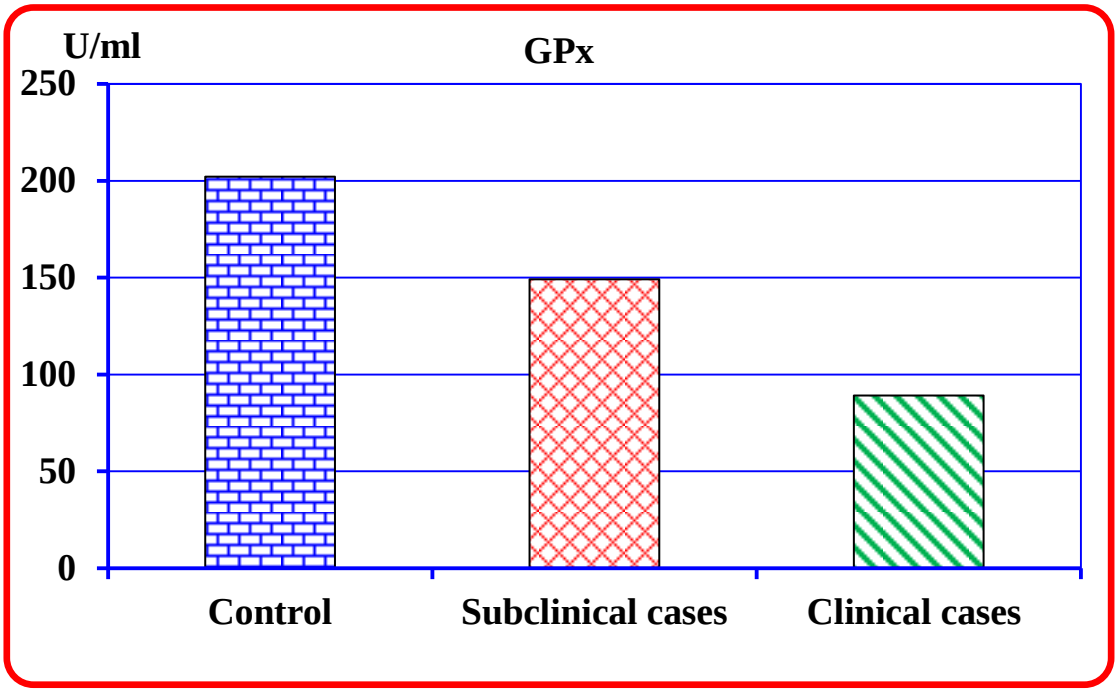

Fig. (33): Effect of subclinical and clinical hypophosphatemia on GPx

f) Changes in other biochemical parameters

The subclinical and clinical hypophosphatemic buffaloes showed a significant decrease in serum PTH level when compared with the normal control group.

Regarding to the active form of vitamin D (25 dihydroxycholecalciferol), the subclinical cases bared non significant changes while clinical cases showed a significant dimunation in serum level of (25-hydroxycholecalciferol) when at paralleled the normal control group.

The subclinical and clinical cases of hypophosphatemic buffaloes reported a significant rise in serum level of lactate dehydrogenase (LDH). Serum creatine phosphokinase (CPK) was significantly elevated in clinical cases while subclinical cases reported non significant change when compared with normal control group (Table 10 and Figs. 34-37).

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Table (10): Effect of subclinical and clinical hypophosphatemia on other biochemical parameters (mean values ± SE)

Parameters / Groups	PTH (pg/ml)	Vit. D (ng/ml)	LDH (U/L)	CPK (U/L)
Control	11.66 ± 1.03[a]	27.38 ± 1.20[a]	238.00 ± 3.20[c]	137.20 ± 3.30[b]
Subclinical cases	1.94 ± 0.23[b]	25.48 ± 1.80[a]	343.00 ± 3.80[b]	149.00 ± 4.20[b]
Clinical cases	1.32 ± 0.13[b]	8.00 ± 0.33[b]	697.00 ± 10.40[a]	360.00 ± 8.60[a]

Significant at $P \leq 0.05$

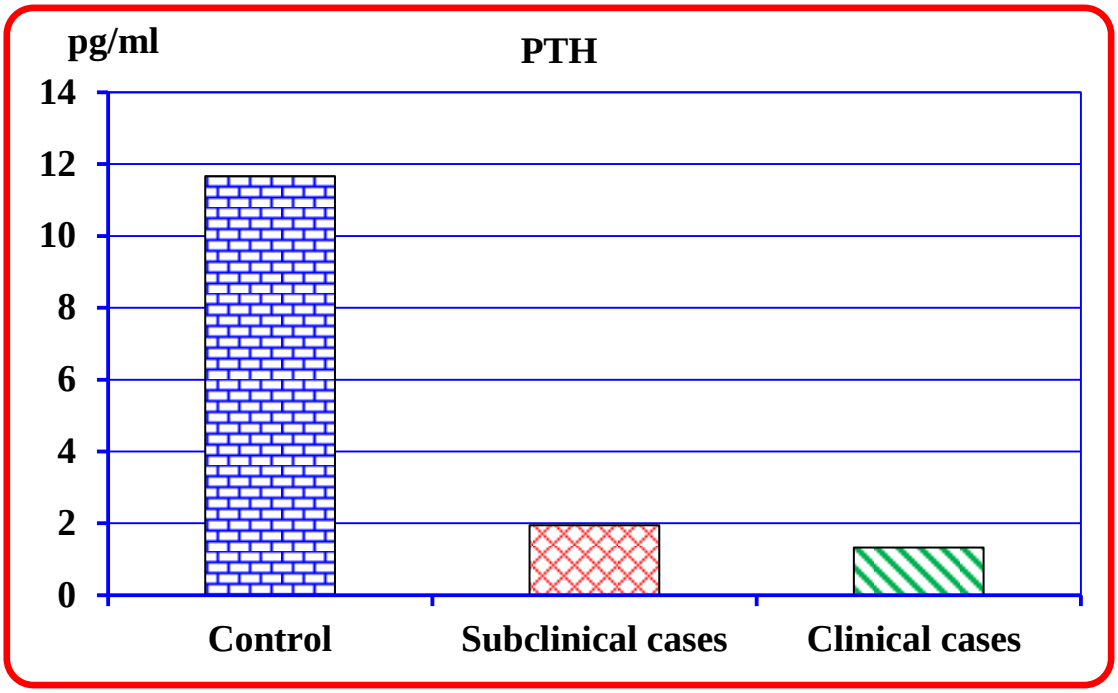

Fig. (34): Effect of subclinical and clinical hypophosphatemia on PTH

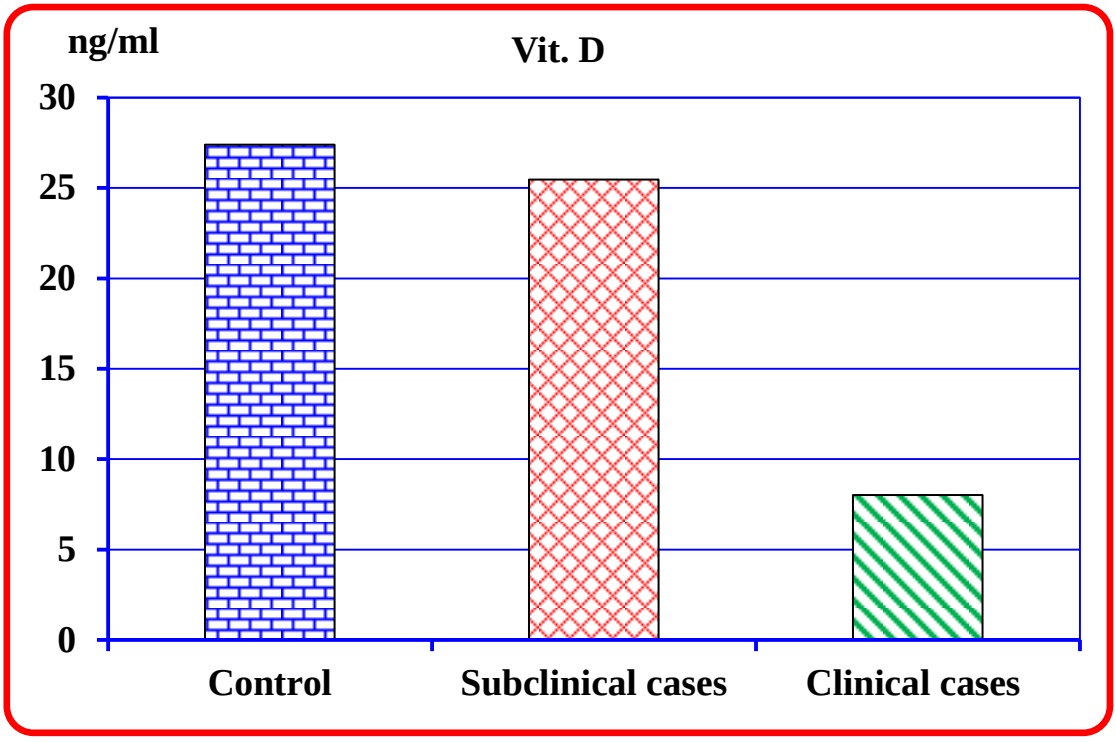

Fig. (35): Effect of subclinical and clinical hypophosphatemia on vitamin D

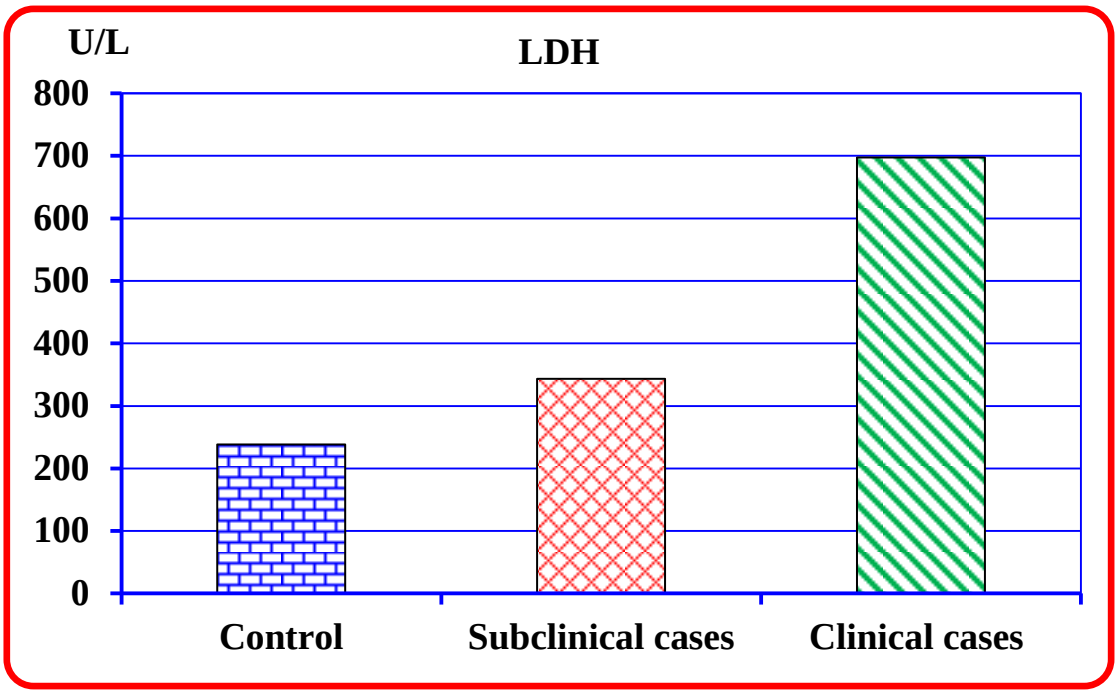

Fig. (36): Effect of subclinical and clinical hypophosphatemia on the activity of LDH

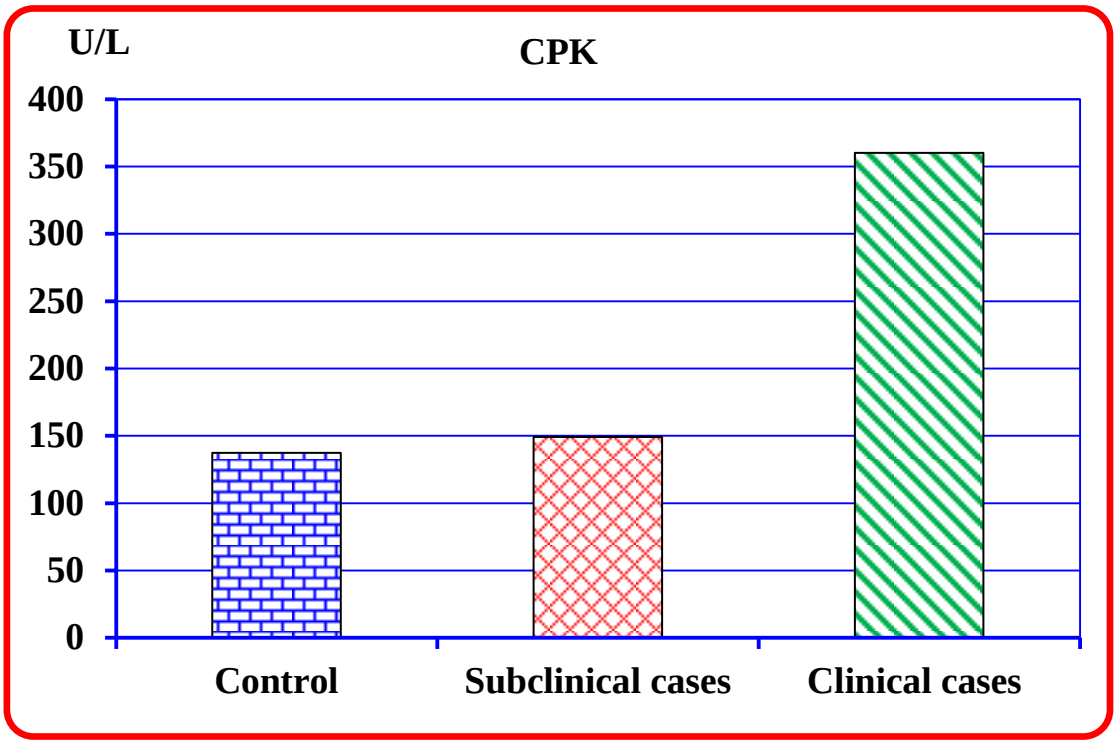

Fig. (37): Effect of subclinical and clinical hypophosphatemia on CPK

DISCUSSION

DISCUSSION

Hypophosphatemia is one of the most common metabolic disorders of bovines. It's mainly caused due to the decrease in serum inorganic phosphorous level. The most common causes for deficiency are incorrect P/Ca ratio in food, low absorption of phosphorous compounds and disorders of hormonal system **(Kurek et al., 2010)**.

Severe hypophosphatemia (clinical cases) usually affects adult buffaloes immediately after parturition (1-60 days) post-partum **(Durrani et al., 2010)**.

It's commonly seen during third to sixth lactation **(Kumar et al., 2019)** and it can be also appear during 6^{th}-8^{th} month of pregnancy **(Purohit et al., 2018)**. The disease is characterized by rapid intravascular hemolysis, hemoglobinuria, anemia, anorexia, weakness, marked decrease in milk production in lactating animals and may be obvious with recommbancy **(Mahmut et al., 2009)**.

Moderate hypophosphatemia (subclinical cases) can be seen in grazing animals in region with low phosphorous content in the soil and also feeding the animals on inadequate ration as feeding on cabbage, barseem, wheat or straw **(Purohit et al., 2018)**. the disease characterized by poor growth, loss of weight, anorexia **(Grunberg et al., 2017)**.

The present study was carried out on adult female water buffaloes suffering from the clinical signs suggested to be hypophosphatemia. The subclinical cases (moderate hypophosphatemia) revealed poor

growth, low weight, anorexia, stiffness in gait, pale vulvar and conjunctival mucous membrane, serum phosphorous level was 2.65 ±0.14 mg/dl. The same clinical signs were reported by previous authors **(Forrester and Moreland, 1989; Grunberg, 2008; Durrani et al., 2010 and Grunberg et al., 2017)**.

While in clinical cases (severe hypophosphatemia) the buffaloes were suffering from all of the previous symptoms in addition to red urine and the buffaloes may be recumbent and serum phosphorous level was 1.42 ± 0.43 mg/dl. These clinical signs were in the agreement with those reported by **Durrani et al. (2010), Al-Mujalli (2010), Navjot et al., (2017), Ziwei et al., (2017) and Purohit et al. (2018)**.

The reduction in serum inorganic phosphate level in dairy cattle is one of the most mutual metabolic disorders. This drop is considerably subsequent a sudden loss of phosphorous as it arises at the onset of lactation through mammary gland also hypophosphatemia can be a result of shifting phosphorous from extracellular fluid into intracellular fluid **(Montiel et al., 2007)**.

The present study revealed a significant decrease in serum phosphorous level in both 2nd and 3rd groups. This decrease could be ascribed to prolonged serving on phosphorous deficient diet as barseem, cereals and concentrates. Also, high calcium to phosphorous ratio diet results in declining phosphorous absorption from intestine **(Akhtar et al., 2007)**.The transition between late pregnancy and early lactation from calving till 3rd to 6th weeks post-partum **(Mahmut et**

al., 2009) lessened absorption from gut due to vitamin D deficiency and gastrointestinal tract illness **(Bhikane and. Syed, 2014)**. Also, advanced gestation may cause hypophosphatemia as more calcium and phosphorous are required by the feotus **(Digraskar et al., 1991)**. Similar results were obtained previously by **Muhammed et al. (2001), Mahmut et al. (2009). Kurek et al. (2010), Navjot et al. (2017), Kumar et al. (2019) and Rahmati et al. (2021)** compared with the normal control.

Regarding to hematological result, gp (2) revealed a significant decrease in RBCs, Hb concentration and PCV with the development of normocytic normochromic anemia early stage of hemolytic anemia. This may be attributed to inadequate dietary intake of phosphorous, decrease absorption by the intestine and disturbed metabolism of liver. The same results were obtained by **Forrester and Moreland (1989), Grunberg (2008), Kurek et al. (2010), Durrani et al. (2010) and Grunberg et al. (2017)**.

The clinical cases of hypophosphatemia (gp. 3) showed a significant decrease in RBCs count, hemoglobin concentration and PCV with the development of macrocytic hypochromic anemia as a result of late stage of hemolytic anemia. The intravascular hemolysis is due to hypophosphatemia as mammalian red blood cells depend on phosphorous, for glucose metabolism which is the main source of energy and maintaining function and structure and red cells **(Malik and Samad, 1996)**. Phosphorous depletion resulted in marked drop in adenosine triphosphate (ATP) and 2,3 diphosphoglycerate (2,3 DPG)

synthesis in RBCs **(Wang et al., 1985)**. RBCs require ATP to control cell volume and deformability, without sufficient ATP the intracellular sodium concentration rises and the cells become more rigged and rupture **(Goff, 2000)**.

Also, the reduced ATP resulted in decrease membrane phospholipids which responsible for maintaining the shape and integrity of red cells **(Rana and Bhardway, 1988)**. The same results were obtained by **Durrani et al. (2010), Al Mujalli (2010), Navjot et al. (2017), Ziewi et al., 2017) and Purohit et al. (2018)**.

The pentose phosphate pathway is important for normal red blood cells survival. It acts as catalytic action of G-6-PD enzyme in oxidizing glucose 6 phosphate. Reduction of the potential in cell in form of nicotinamide adenosine dimucleatide phosphate (NADPH) is liberated in this action by which glutathione is maintained in reduced state thus protect RBCs from oxidative stress. Due to deficiency of this enzyme increase of oxidative stress in red cells causes hemolytic anemia **(Rashid et al., 2021)**.

Concerning to leukogram, the 2nd group bared non significant change in total leukocytic, neutrophilic and lymphocytic counts while substantial increase in total leukocytic and neutrophilic in the 3rd group. The increase in total leukocytic count is due to neutrophilia (neutrophilic leukocytosis). The increase in neutrophilic count could be attributed to the endogenous release of corticosteroids as hypophosphatemia (metabolic disorder) is the source of the release of corticosteroids **(Stockdale et al., 2005)**.

Neutrophils are short live cells and replaced 2.5 times daily **(Benjamin, 1978)**. So, they leave the circulation rapidly about (9-10 hours) but under the disease condition they are retained in the circulation. Moreover, the marginal neutrophils are pooled into the circulation leads to neutrophilia.

Also, the present study showed a significant decrease in lymphocytic count in clinical cases such decrease due to corticosteroids that suppress lymphoid tissue and bone marrow resulting in lymphopenia **(Bremmer et al., 2000)**.The same results were obtained by **Durrani et al. (2010), Al-Mujalli (2010), Navjot et al. (2017), Ziwei et al. (2017) and Purohit et al. (2018)**.

The serum calcium level was not significantly altered in 2nd group. Similar results were gotten by **Al-Mujalli (2010) and Sarma et al. (2014)**. While in the 3rd group there were a significant decrease in serum calcium level when matched with normal control group. The decrease could be ascribed to decline feed intake, lessened calcium absorption from the intestine and or hypoalbuminemia. Approximately a like outcomes were obtained by **Grunberg (2008), Kurek et al. (2010) and Kumar et al. (2019)**.

The proteingram of the 2nd and 3rd groups revealed a significant decrease in serum levels of total serum proteins, albumin, globulins and albumin globulins ratio in comparison with normal control group. The decrease in albumin globulins ratio was due to the decrease in serum albumin was more than serum globulins. The hypoproteimemia could be attributed to hypoalbuminemia and hypoglobulinemia. The

hypoalbuminemia and hypoglobulinemia may be due to decrease feed intake and decrease production by damaged liver. Our results come in agreement with **Kurek et al. (2010), Al-Mujalli (2010) and Fayed et al. (2018)**.

The present study showed a substantial increase in serum activities of aminotransferases (ALT and AST) in the 2nd and 3rd groups when compared with normal control group. This could be accredited to fatty liver changes concomitant with negative energy balance that cause hepatocellular damage **(Kaneko et al., 1997)**. The same results obtained by **Kurek et al. (2010), Al-Mujalli (2010), Ziwei et al. (2017) and Kumar et al. (2019)**.

Also, there is a connotation of hypophosphatemia with liver injury instigated by hepatic lipidosis are common in highly producible dairy cows in intitial lactation and occur along side with hypophosphatemia **(Bode et al., 2004)**. Relation between hypophosphatemia and biochemical markers of disturbed liver function in early lactating dairy cattle has been reported by **Grunberg et al., 2005**.

Serum bilirubin is a specific indicator for biliary duct disease, hepatocellular damage and disorders of red blood cells **(Kurek et al., 2010)**.Our study reported a significant increase in serum total bilirubin in the second group. This may be attributed to hepatocellular damage while in the third group there was a highly significant increase in serum total bilirubin which could be attributed to the increase in indirect bilirubin that induced by hemolytic anemia. The same results

were obtained by **Kurek et al. (2010), Ziwei et al. (2017), Purohit et al. (2018) and Zaghawa et al. (2019)**.

Concerning the activity of serum alkaline phosphatase (ALP), both 2nd and 3rd groups showed a significant elevation in paralleled with normal control group. This could be attributed to anemia that creats generalized hypoxia which, cause hepatocellular damage resulting in leakage of ALP into the circulation **(Cornelius, 1980)**. The same result was obtained by **Mahmut et al. (2009), Kurek et al. (2010) Purohit et al. (2018) and Zaghawa et al. (2019)**.

Serum glucose level showed a significant rise in the 2nd and 3rd groups in comparison with normal control group. The hyperglycemia may be due to increasing stress in the disease condition that leading to increase cortisol level **(Marik and Belloma, 2013)**. Also, hyperglycemia may be due to hypocalcemia in the 3rd group as calcium ions are required for insulin secretion from pancreas **(Kaneko et al., 1997)**.Our results come in agreement with **Muhammed et al. (2001), Purohit et al. (2018) and Kumar et al. (2019)**. In contrary, **Mahmut et al. (2009) and Al Mujalli (2010)** reported a significant decrease in serum glucose level.

The present study revealed a significant diminution in activity of erythrocytic glucose-6-phosphate dehydrogenase in both 2nd and 3rd groups when compared with healthy control group. This diminution could be owed to the mutation which is the utmost mutual reason for enzymatic abnormalities **(Agar and Board, 1983)**. Also, low activity of glucose-6-phosphate dehydrogenase may be ascribed to the

increase in ALT and AST activity **(Singari et al., 1991)**. Similar results were recorded by **Muhammed et al. (2001), Al-Mujalli (2010) and Purohit et al. (2018)**.

Serum urea and creatinine levels showed a significant increase in subclinical and clinical cases. This could be attributed to improper kidney function **(Latimer et al., 2003)**. This improper function resulted from the endogenous release of corticosteroids and tubular epithelial necrosis which resulted in decreasing renal perfusion and so on decreasing glomerular filtration resulting in increasing blood urea and creatinine levels **(Benjamin, 1978, Stockdale et al., 2005)**. The same results were obtained by **Muhammed et al. (2001), Durrani et al. (2010), Al Mujalli (2010)**, **and Navjot et al. (2017)**.

About the oxidative stress markers there was a significant dimunation in serum glutathione peroxidase (GPx) activity in both subclinical and clinical hypophosphatemic buffaloes in comparison with normal control group. This may be ascribed to decline activity of erythrocytic glucose-6-phosphate dehydrogenase which, liable for decreasing reduced glutathione and so instigating oxidative stress to erythrocytes leads to hemolytic syndrome **(Singari et al., 1991)**. Nearly similar results were obtained by **Ogawa et al. (1989), Sarma et al. (2014) and Ziwei et al. (2017)**.

The present study showed a significant increase in serum malondialdehyde (MDA) in subclinical and clinical cases when paralled with normal control group. This rise could be attributed to lessening serum phosphorous level which estop ATP production cause

oxidative stress as a consequence of high creation of reactive oxygen species that damage to RBCs structure **(Ogawa et al., 1989)**. The same results were obtained by **Ogawa et al. (1989), Mata et al. (1994), Sarma et al. (2014) and Ziewi et al. (2017)**.

A propose to serum level of parathyroid hormone both subclinical and clinical cases showed a significant decrease in serum level of PTH when compared with normal control group. This decrease in serum PTH level could be attributed to the decrease in serum phosphorous to which parathyroid gland respond by decreasing PTH mRNA level, decreasing gene expression and cell proliferation of the gland. Also, decreasing serum phosphorous level resulted in decreasing production of arachidonic acid in parathyroid gland that decreasing PTH secretion **(Silver and Naveh, 2009)**. Nearly similar results were obtained by **Silver and Naveh (2009) and Bergwtiz and Juppner (2011)** who reported that the decrease in serum phosphorous level found to decrease serum PTH level.

Regarding to the active form of vitamin D, the subclinical cases in the present study bared non significant change in serum level of 25 hydroxycholecalciferol at paralled with normal control group. While the clinical cases showed a significant diminution in serum level of 25 hydroxycholecalciferol.

This lessening might be due to the decrease in serum PTH level which stimulate the conversion of vitamin D to its active form in the kidney **(Martin et al., 2012)**. Also, it may be attributed to liver damage which occasioned in decrease production of 25

hydroxycholecalciferol that converted to the active form 1,25 dihydroxycholecalciferol in the kidney **(Knochel, 1985)**. Also, the decrease in serum calcium level may occasioned vitamin D lessening.

Creatine kinase (CPK) is the utmost specific and sensitive indicator of muscular impairment and necrosis **(Kaneko et al., 1989**). Our study reported non significant change in serum CPK level in subclinical group when compared with normal control group. While severe (clinical) cases showed a significant increase in serum CPK level when compared with normal control group.The same results were reported by **Forrester and Moreland (1989) and Knochel (1985)**.

The elevation in the activity of transferase especially AST can be associated with the alterations of skeletal or cardiac muscles and hepatic cells **(Jayanithi et al., 1997)**. So, the increase in serum level of CPK together with AST indicate muscle damage and necrosis.

Lactate dehydrogenase (LDH) is an enzyme originate in the most nucleated and non nucleated cells including skeletal and cardiac muscles, liver and erythrocytes **(Bhagavan and Chung, 2015)**. The present work showed that significant raise in LDH activity in subclinical cases while the clinical cases revealed a highly significant increase in activity of LDH. The escalation in LDH activity in equivalent with increasing AST and ALT activities can sustenance liver dystrophy instigated by severe hypophosphatemia **(Dzoyrm and Eloff, 2014)**. The increase in the activity of LDH together with AST activity and CPK is an indicator for necrosis of skeletal muscle. Also,

the increase in LDH may be escorted with the hemolysis of RBCs leads to outflow of LDH into the circulation **(Bhagavan and Chung, 2015)**.

SUMMARY

SUMMARY

The present study was performed to investigate the effect of subclinical and clinical hypophosphatemia on hematological, biochemical, oxidative status, skeletal muscle and appraise the interrelation between phosphorous deficiency and vitamin D.

The experiment was carried out on 40 adult female water buffaloes belonging to different localities in Sharkia governorate, Egypt. These animals were divided into 3 groups as follow:

Group (1) was ten apparently clinically healthy female water buffaloes and kept as a normal control.

Group (2) was fifteen buffaloes suffered from moderate signs of phosphorous deficiency. The signs included anorexia, emaciation, weakness, paleness mucous membranes and stiffness in gait. Phosphorous level displayed a significant decline.

Group (3) was fifteen buffaloes suffered from severe signs of phosphorous deficiency. The signs included all the above signs in addition to red urine and the animal might be recumbent. Phosphorous level displayed a highly significant decrease.

Via jugular vein puncture, two blood samples were collected from each buffaloe. The first blood samples were taken into dipotassium salt of EDTA tube for hematological examination. The second samples were drawn in clean tubes without anticoagulant for serum separation to be used in biochemical assessment.

Hematological findings:

The present study showed a significant decrease in RBCs count, Hb concentration and PCV with the development of normocytic normochromic anemia in subclinical cases. Moreover, a highly significant decrease in RBCs count, Hb concentration, PCV with the development of macrocytic hypochromic anemia in clinical cases when compared with normal control group.

The subclinical cases showed non significant changes in total leukocytic, neutrophilic and lymphocytic counts. While the clinical cases reported a significant increase in total leukocytic and neutrophilic counts with a significant decrease in lymphocytic count. There were non significant changes in eosinophilic and monocytic counts in both subclinical and clinical cases when compared with normal control group.

Changes in some serum electrolytes

The present study revealed a significant decrease in serum phosphorous level in both subclinical and clinical cases while serum calcium level was not significantly altered in the subclinical cases but it significantly decreased in the third group when compared with normal control group.

Changes in some proteinogram

The subclinical and clinical cases showed a significant decrease in serum total proteins, albumin, globulins and albumin globulins ratio in paralleled with normal control group.

Changes in serum activities of aminotransferases, alkaline phosphatase, total, direct and indirect bilirubin

Both 2nd and 3rd groups revealed a substantial increase in transferases activities (ALT and AST) and activity of alkaline phosphatase (ALP). Also, serum levels of total, direct and indirect bilirubin were significantly elevated in subclinical and clinical cases when paralleled with healthy control group.

Changes in some biochemical parameters

There was a significant elevation in serum glucose level in subclinical and clinical cases. On the other hand, a substantial dimunation in activity of erythrocytic glucose-6-phosphate dehydrogenase in both 2nd and 3rd group. Serum urea and creatinine levels were significantly rise in both subclinical and clinical cases in paralleled with normal control group.

Changes in oxidative stress markers

The subclinical and clinical cases revealed a significant elevation in serum MDA level. On the other hand, there was a significant dimunation in serum glutathione peroxidase activity (GPx) in both subclinical and clinical cases when compared with the normal control group.

Changes in other biochemical parameters

Both subclinical and clinical hypophosphatemic buffaloes showed a significant decrease in serum PTH level when compared with the normal control group.

Regarding to the active form of vitamin D (25 dihydroxycholecalciferol), the subclinical cases bared non significant changes while clinical cases showed a significant dimunation in serum level of (25-hydroxycholecalciferol) when at paralleled the normal control group.

The subclinical and clinical cases of hypophosphatemic buffaloes reported a significant rise in serum level of lactate dehydrogenase (LDH). Serum creatine phosphokinase (CPK) was significantly elevated in clinical cases while subclinical cases reported non significant change when compared with normal control group.

تأثير الاصابة بنقص الفوسفور على بعض العناصر البيوكيميائية:

بتحليل مصل الدم أظهرت النتائج زيادة معنوية فى نسبة الجلوكوز واليوريا والكرياتينين فى الحيوانات متوسطة وشديدة النقص فى الفوسفور بينما أظهر انزيم الجلوكوز-٦-فوسفات ديهيدروجينيز نقص معنوى فى الحالات المتوسطة والشديدة الاصابة بنقص الفوسفور عندما قورنت بالحيوانات الضابطة.

تأثير الاصابة بنقص الفوسفور على الحالة التأكسدية للحيوان:

أثبتت الدراسة وجود اجهاد تأكسدى فى الحيوانات متوسطة وشديدة النقص فى الفوسفور حيث وجدت زيادة معنوية فى نسبة المالوندالدهيد ونقص معنوى فى نسبة الجلوتاثيون بيرأوكسيديز فى الحالات المتوسطة والشديدة الاصابة بنقص الفوسفور مقارنة بالمجموعة الضابطة.

تأثير الاصابة بنقص الفوسفور على هرمون الغدة الجار درقية وفيتامين د3 وكفاءة العضلات:

أثبتت الدراسة وجود نقص معنوى فى هرمون الغدة الجار درقية فى كلاً من الحيوانات متوسطة وشديدة الاصابة بنقص الفوسفور بينما نسبة فيتامين $د_3$ لم تتغير فى الحيوانات متوسطة الاصابة ونقصت نقص معنوى فى الحيوانات شديدة الاصابة بنقص الفوسفور مقارنة بالحيوانات الضابطة.

بفحص مصل الدم أثبتت الدراسة وجود زيادة معنوية فى اللاكتات ديهيدروجينيز فى الحالات المتوسطة والشديدة النقص فى الفوسفور. بينما أثبتت الدراسة وجود زيادة معنوية فى نسبة الكرياتين كينيز فى الحالات شديدة النقص فى الفوسفور ولم تتغير فى الحيوانات متوسطة الاصابة عندما قورنت بالحيوانات الضابطة.

مما سبق نستخلص إنه يوصي بالحفاظ علي مستوي الفسفور في الدم وذلك من خلال الامتداد بالفسفور خاصه في أواخر الحمل وبداية فتره الرضاعة. أيضا ينصح الامتداد بفيتامين د ومضادات الأكسدة خلال فترة العلاج.