www.ThePharmaJournal.com

The Pharma Innovation

Patel N
College of Veterinary Science,
Assam Agricultural University,
Khanapara, Guwahati, Assam,
India

Baishya BC
College of Veterinary Science,
Assam Agricultural University,
Khanapara, Guwahati, Assam,
India

Phukan A
College of Veterinary Science,
Assam Agricultural University,
Khanapara, Guwahati, Assam,
India

Mahato G
College of Veterinary Science,
Assam Agricultural University,
Khanapara, Guwahati, Assam,
India

Goswami S
College of Veterinary Science,
Assam Agricultural University,
Khanapara, Guwahati, Assam,
India

Tamuly S
College of Veterinary Science,
Assam Agricultural University,
Khanapara, Guwahati, Assam,
India

Biochemical changes associated with subclinical hypocalcaemia in high producing crossbred dairy cows 15 days before the expected day of calving

Patel N, Baishya BC, Phukan A, Mahato G, Goswami S and Tamuly S

Abstract
The present study was undertaken to study the biochemical changes occurring due to subclinial hypocalcaemia in high producing crossbred dairy cows 15 days before the expected day of calving. A total number of 20 cows were divided into 2 groups – Group A/Healthy control (n=10) and Group B/Hypocalcaemic cows (n=10). The biochemical parameters considered were serum calcium, phosphorus, magnesium, blood urea nitrogen (BUN), creatinine and cholecalciferol (prohormone). It was recorded that there was a significant decrease ($P<0.01$) in the levels of serum calcium in animals of group B when compared to the levels in animals in group A. A significant increase ($P<0.01$) was recorded in the levels of serum BUN, creatinine and cholecalciferol in animals of group B as opposed to the levels in animals of group A. There was no significant difference ($P>0.01$) in the levels of serum phosphorus and magnesium between the 2 group.

Keywords: Subclinical hypocalcaemia, crossbred cows, biochemical changes, pre-partum period

Introduction
Peri-parturient hypocalcaemia is a metabolic disorder of high producing dairy cows occurring during the transition period. This period hugely challenges the cow to maintain calcium homeostasis [6]. The peri-parturient period, from 3 weeks pre-partum to 3 weeks post-partum [9], has an increased demand for nutrition and hormones like parathormone (PTH) and cholecalciferol. Calcium homeostasis is a complex endocrinological process involving calcium, phosphorus, magnesium, parathormone, calcitonin and vitamin-D metabolites.
Calcium mobilization from bone reserves is regulated dominantly by PTH [7]. It also stimulates the final hydroxylation of vitamin-D metabolites into their active form (1, 25-dihydroxycholecalciferol), in the kidneys [5]. Magnesium plays an important role in influencing tissue sensitivity to PTH. Hypomagnesaemia decreases tissue sensitivity to PTH [27] by reducing the integrity of interaction between PTH and its receptors [20]. Phosphorus concentration is not as tightly regulated as the calcium concentration. A high phosphorus concentration is known to inhibit the activity of 25-hydroxycholecalcifero-1-α-hydroxylase enzyme in the kidneys which is needed in the final hydroxylation of 25-hydroxycholecalciferol to 1, 25-dihydroxycholecalciferol [6]. Elevated levels of blood urea nitrogen (BUN) and creatinine indicate metabolic alkalosis. It has been suggested that metabolic alkalosis causes lowered tissue PTH response [14] which leads to lowered PTH secretion, inhibiting calcium ionization causing poor gut calcium absorption. Keeping in view the pathophysiology of hypocalcaemia and its associated biochemical changes in the late gestation period, the present study was undertaken.

Materials and Methods
The present study was conducted in and around Khanapara, Guwahati, Kamrup (Metropolitan) district in the state of Assam. A total of 20 crossbred pregnant dairy cows 15 days before expected day of calving, belonging to organized and unorganized dairy farms, were included in the study. All animals were purely stall fed.
About 5 ml blood was collected from each animal by venipuncture from the jugular vein in properly labeled sterilized vials without anticoagulant. The vials were kept in slanting position for half an hour. The vials were transported to the laboratory and centrifuged to separate the serum which was then utilized for biochemical investigations or stored at -20˚C till further use.

Corresponding Author:
Patel N
College of Veterinary Science,
Assam Agricultural University,
Khanapara, Guwahati, Assam,
India

Serum calcium, phosphorus and magnesium were estimated using commercial kits in a UV-VIS spectrophotometer 117 (Systronics ®). Serum calcium was estimated using a protocol involving arsenazo-III reagent provided by Liquizyme Code S22 (Beacon Diagnostics Pvt. Ltd.).

Serum phosphorus was estimated using the protocol involving molybdate reagent (UV Molybdate method) provided by Diatek. Serum magnesium was estimated using the protocol involving xylidyl blue reagent (Monotest kit) provided by Diatek. The same kits were used throughout the study.

Serum creatinine and BUN were estimated using a semi-automated serum analyser (Benesphera ®). Serum creatinine was estimated by the Jaffe's liquid stable method and serum BUN was calculated from the serum urea value which was estimated by the Berthlot method. The same methods were used throughout the study.

Serum vitamin D_3 (Cholecalciferol) was estimated using a standard sandwich enzyme-linked immunosorbent assay (ELISA) protocol provided as a commercially available kit and components (Figure 1) from Chongqing Biospes Co. Ltd. (Bovine Vitamin D_3 ELISA kit; Catalogue no. BYEK3513). The stored serum samples were thawed at room temperature and centrifuged at 2000-3000 RPM for 20 min and the supernatant was collected. The wash buffer was diluted in 1:30 ratio with distilled water (20 ml concentrated wash buffer in 580 ml distilled water). Five Eppendorf tubes were labeled as 200 ng/ml, 100 ng/ml, 50 ng/ml, 25 ng/ml and 12.5 ng/ml, respectfully.

One hundred micro liter standard diluent was added in the 1st tube and mixed thoroughly. Serial dilution was then done by transferring 100 µl from the 1st tube to the 2nd tube, mixing the contents and transferring 100 micro liters from the 2nd tube to the 3rd tube, and so on from the 3rd tube to the 4th tube, from the 4th tube to the 5th tube, and then finally discarding 100 micro liters from the 5th tube.

The kit components were equilibrated for 15-30 minutes. Fifty micro liters of diluted standards (200 ng/ml, 100 ng/ml, 50 ng/ml, 25 ng/ml and 12.5 ng/ml) were added in the standard wells on the anti-bovine vitamin D_3 antibodies coated ELISA plate (Figure 2) and marked. Control (Zero) well was marked and 50 µl standard buffer was added in it. For each test well, 40 µl of the sample buffer was added along with 10 µl of the serum sample. After shaking the plate well to facilitate proper mixing of the components, the plate was sealed with a plate sealer and incubated at 37°C for 30 minutes.

After 30 minutes, the plate sealer was removed and washed manually with wash buffer (1x) for a total of 5 number of washes.

Fifty micro liters of HRP conjugated anti-vitamin D_3 antibodies was added then in all the wells except the control well. The plate was sealed and incubated again at 37°C for 30 minutes.

The sealer was removed after 30 minutes and washed again as before.

Fifty micro liters of TMB substrate A and 50 µl of TMB substrate B were added in each well. The plate was vortexed gently on an ELISA shaker for 30 seconds, then incubated for at 37°C for 15 minutes in the dark. Shades of blue colour could be observed in the plates (Figure 3).

Fifty micro liters of stop solution was added into each well and mixed thoroughly by shaking. The colour changed to yellow immediately (Figure 4).

The absorbance was read at 450 nm in a microplate reader within 15 minutes after added the stop solution.

For calculation –
Relative OD_{450} = OD_{450} of each well – OD_{450} of zero well

The standard curve was plotted and the relative OD_{450} of each standard solution (Y) vs. the respective concentration of standard solution (X). The bovine vitamin D_3 concentrations of the samples were interpolated from the standard curve (Figure 5).

The data was collected diligently and analyzed using SAS system ('Local', X64_7PRO), Chi-square test and Student's t test.

Fig 1: ELISA test kit components

Fig 2: ELISA vitamin-D_3 antibody coated plate

Fig 3: ELISA colour reaction before adding stop solution

Fig 4: ELISA colour reaction after adding stop solution

Results and Discussion

In the present study, the Mean ± SE values for serum calcium in group A and group B were 9.29 ± 0.28 mg/dl and 7.38 ±0.07 mg/dl, respectively. There was significant difference (P<0.01) in the values between both groups. This was similar to the findings of Martin-Tereso and Martens [23] (2014) who proposed threshold levels of subclinical hypocalcaemia as 8 mg/dl. The findings were also similar to the findings of Jawor et al. [17] (2012), who associated serum calcium levels less than 7.6 mg/dl with subclinical hypocalcaemia related behavioural and production changes. Subclinical hypocalcaemia in animals of group B might be due to poor management and imbalanced feeding practices [22] and reduced stores of available calcium in the animal's body [10].

The Mean ± SE values of serum phosphorus for group A and group B were recorded as 4.31 ± 0.37 mg/dl and 4.92 ± 0.17 mg/dl, respectively. No significant difference was observed in the values between the two groups. These values were considered to be in the normal range and were similar to the findings of Goff [6] (2000) and Chetia and Sarma [3] (2017) who reported normal phosphorus levels as 4-8 mg/dl and 4-7 mg/dl, respectively. However, their values were considered to be higher in group B when compared to the values of calcium in the same group. Phosphorus levels in the body aren't as tightly regulated as calcium levels [6] and so, changes in phosphorus levels need not always accompany changes in calcium levels in cows with subclinical hypocalcaemia.

In the present study, the Mean ± SE values of serum magnesium in cows of group A and group B were 2.17 ± 007 mg/dl and 2.35 ± 0.09 mg/dl, respectively. There was no significant difference in the values between the two groups. Similar findings were recorded by Hodnett et al. [11] (1992) and Hove and Kristiansen [15] (1982). Although magnesium plays a role in hypocalcaemia, it is not always observed in subclinical hypocalcaemia [29].

The Mean ± SE values of serum BUN in group A and group B were recorded as 17.82 ± 0.7 mg/dl and 26.64 ± 0.83 ±, respectively. Values of both groups differed significantly (P<0.01) from each other. Similarly, Ismail et al. [16] (2011) reported elevated BUN levels in parturient paretic cows. Elevated BUN levels might be due to feeding higher quantities of paddy straw [1], as per the feeding practices in the farms included in the study, and feeding of a protein rich diet [30] and excessive carbohydrates in the last 8 weeks of gestation [18].

The Mean ± SE values for serum creatinine for group A and group B were recorded as 1.28 ± 0.09 mg/dl and 1.74 ± 0.08 mg/dl, respectively, in the present study. There was significant difference (P<0.01) in the serum creatinine values of both groups. Elevations in serum creatinine levels in parturient paretic cows were also reported by Ismail et al. [16] (2011). These higher values in hypocalcaemic cows might be due to high protein diet leading to higher creatinine production [25], increased renal activity causing increased protein metabolism[4] and also due to dehydration caused by lowered appetite [16].

In the present study, the Mean ± SE values of vitamin-D (Cholecalciferol; prohormone) in group A and group B were recorded as 87.37 ± 5.51 ng/ml and 124.9 ± 1.20 ng/ml, respectively. There was a significant difference (P<0.01) in the values of both groups. Similar elevations in the values of cholecalciferol were also reported by Horst et al [12, 13]. (1978, 1997), Jorgensen et al [19]. (1974) and Mayer et al [24] (1969). This might be due to the failure of the prohormone to convert to its active metabolites. High levels of serum BUN and creatinine observed in the study also indicate a state of metabolic alkalosis. Metabolic alkalosis is a known cause causing failure of conversion of cholecalciferol [8, 7, 6, 1, 25, 29]. Increased blood pH reduces receptor sensitivity to PTH which causes decreased production f 1-α-hydroxylase enzyme[26]. This enzyme is needed for the hydroxylation of cholecalciferol to 1, 25-dihydroxycholecalciferol. In the present study, though the phosphorus levels were within normal range, they were elevated in comparison to the existing calcium levels, i.e. the normal ration of Ca:P of 2.3:1 [1], 1.6:1 [2] and 2:1 [21] was increased to 1.48:1in group B as opposed to the normal ration of 2.13:1 in group A. High phosphorus levels inhibit the activity of 25-hydroxy-1-α-hydroxylase enzyme [28], which too is an enzyme of utmost importance in the final hydroxylation of 25-hydroxycholecalciferol to 1, 25-dihydroxycholecalciferol in the kidneys.

Table 1: Values of biochemical parameter

Parameter (in serum)	Group A (n=10)	Group B (n=10)
Calcium (mg/dl)	9.29 ± 0.27[a]	7.38 ± 0.07[b]
Phosphorus (mg/dl)	4.31 ± 0.37[a]	4.92 ± 0.17[a]
Magnesium (mg/dl)	2.17 ± 0.07[a]	2.35 ± 0.09[a]
Creatinine (mg/dl)	17.82 ± 0.70[b]	26.64 ± 0.83[a]
BUN (mg/dl)	1.28 ± 0.09[b]	1.74 ± 0.08[a]
Vitamin-D_3 (ng/ml)	87.37 ± 5.51[b]	124.9 ± 1.20[a]

Means bearing similar subscript (a, b) in a row do not differ significantly

Summary

The present study was undertaken to record the biochemical changes occurring in subclinical hypocalcaemia in high producing crossbred dairy cows 15 days before expected day of calving. It was observed that there was a significant decrease (P<0.01) in the levels of serum calcium and a significant increase (P<0.01) in the values of serum BUN, creatinine and cholecalciferol in the hypocalcaemic cows of group B when compared to the healthy control cows of group A. No significant difference was observed in the levels of serum phosphorus and magnesium between the two groups. Though serum phosphorus was elevated when compared to the existing levels of serum calcium in the hypocalcaemic cows, the values were considered to be within normal range.
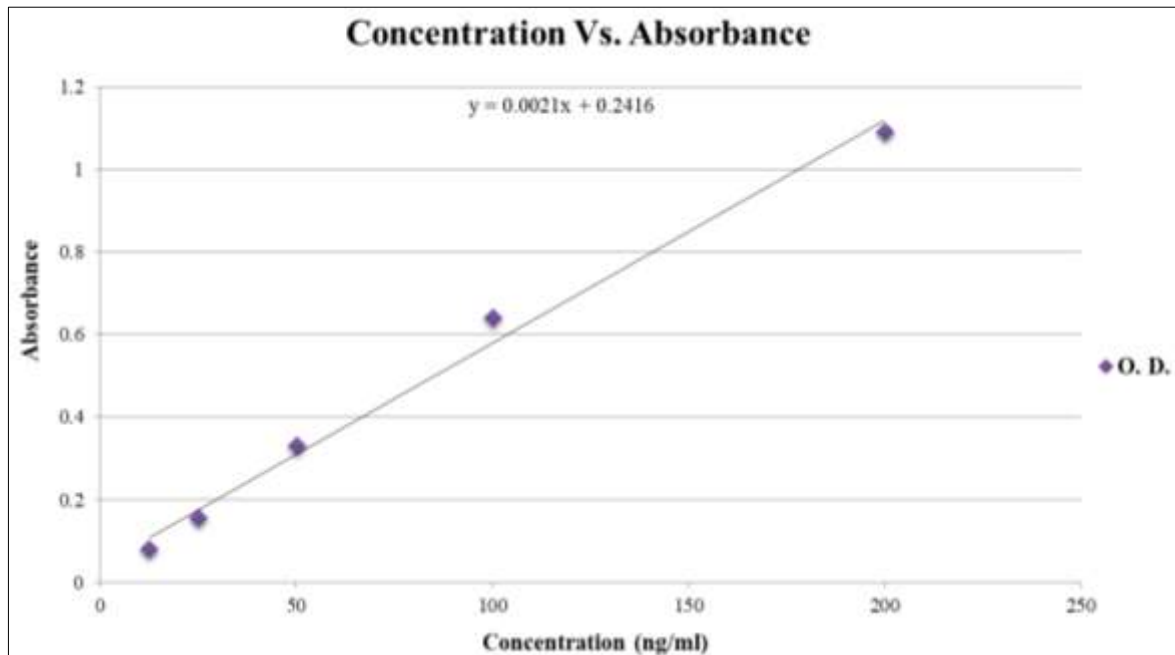
Fig 5: Concentration vs. Absorbance chart representing the standard curve for vitamin-D (Cholecalciferol) estimation using sandwich ELISA

References

1. Chakrabarti A. Textbook of Clinical Veterinary Medicine 2014, 638-648.
2. Chase L, Buchanan-Smith J, Leeson S, De Lange CFM. Phosphorus in animal nutrition. Better crops 1999;83(1):32-33.
3. Chetia M, Sarma S. Investigation of minerals in the dairy cattle (*Bos Taurus*) in different seasons in Assam, India. *J. Ent. Zoology Stud* 2017;5(2):242-244.
4. Cozzi G, Ravarotto L, Gottardo F, Stefani AL, Contiero B, Moro L *et al*. Short communication: Reference values for blood parameters in Holstein dairy cows: Effects of parity, stage of lactation, and season of production. J. Dairy Sci 2011;94:3895-3901.
5. DeGaris PJ, Lean IJ. Milk fever in dairy cows: A review of pathophysiology and control principles. Vet. J 2009;176:58-69.
6. Goff JP. Pathophysiology of calcium and phosphorus disorders. Vet. Clin. North Am. Food Anim. Pract 2000;16(2):319-337.
7. Goff JP. The monitoring, prevention and treatment of milk fever and sunclinical hypocalcaemia in dairy cows. *Vet. J* 2008;176:50-57.
8. Goff JP. Calcium and magnesium disorders. Vet. Clin. Food Anim 2014;30:359-381.
9. Grummer GR. Impact of changes in organic nutrient metabolism on feeding transition cow. J. Anim. Sci 1995;73:2820-33.
10. Hibbs JW, Krauss WE, Pounden WD, Monroe CF, Stutton TS. Studies on milk fever in dairy cows. II. The effect of vitamin D on some of the blood changes in normal and milk fever cows at parturition, 1946.
11. Hodnett DW, Jorgensen NA, DeLuca HF. 1α-hydroxyvitamin D_3 reduces parturient paresis in dairy cows fed high dietary calcium. J. Dairy Sci 1992;75:485-491.
12. Horst RL, DeLuca HF, Jorgensen NA. The effect of age on calcium absorption and accumulation of 1, 25-dihydroxyvitamin D_3 in intestinal mucosa of rats. Metab.Bone. Dis. Relat. Res 1978;1:29.
13. Horst RL, Goff JP, Reinhardt TA, Buxton DR. Strategies for preventing milk fever in dairy cows. J. Dairy Sci 1997;80(7):1269-1280.
14. Horst RL, Goff JP, Reinhardt TA. Role of vitamin D in calcium homeostasis and its use in prevention of bovine parturient paresis. Acta. Vet. Scand 2003;97:35-50.
15. Hove K, Kristiansen T. Prevention of parturient hypocalcaemia: Effect of a single oral dose of 1, 25-dihydroxyvitamin D_3. J. Dairy Sci 1982;65:1934-1940.
16. Ismail B, Zuhair A, Alzghoul, Moh'D B. Haematology, plasma biochemistry, and urinary excretion of glucose and minerals in dairy cows affected with parturient paresis. *Comp.* Clin. Pathol 2011;20:631-634.
17. Jawor PE, Huzzey JM, LeBlanc SJ, von Keyserlingk MAG. Associations of subclinical hypocalcaemia at calving with milk yield and feeding, drinking, and standing behaviours at parturition in Holstein cows. J Dairy Sci 2012**;**95:1240-1248.
18. Jonsson G, Pehrson B. The effect of overfeeding in the dry period on the health of dairy cows. Proceedings.Fifth international conference on production disease in farm animals. Uppsala, Sweden 1983, 300-303, 10-12.
19. Jorgensen NA, Horst RL, DeLuca HF, Ogilvie ML. 25-hydroxycholecalciferol for the prevention of "milk fever" in dairy cows. Vet. Rec 1978;103(7):136-138.
20. Kichura TS, Horst RL, Beitz DC, Littledike T. Relationships between prepartal dietary calcium and phosphorus, vitamin D metabolism, and parturient paresis in dairy cows. Aviat. Space Environ. Med 1995;66(6):537-541.
21. Kirk WG, Davis GK. Blood components of range cattle: Phosphorus, calcium, haemoglobin, and haematocrit. Journal of Range Management 1970;23(4).
22. Kronfeld DS. Parturient paresis in bovine medicine and surgery and herd health management. Amer. Vet. Pub. Inc 1970;3(1):383.
23. Martin-Tereso J, Martens H. Calcium and magnesium Physiology and nutrition in relation of prevention of milk fever and tetany (Dietary management of microminerals in preventing disease). Vet. Clin. Food Anim 2014, 1-29.

24. Mayer GP, Ramber CFJR, Kronfeld DS, Buckle RM, Sherwood LM, Aurbach GD *et al*. Plasma parathyroid hormone concentration in hypocalcaemic parturient cows. Am. J. Vet. Res 1969;30:1587-1597.

25. Radostits OM, Gay CC, Hinchcliff KW, Constable PD. Veterinary Medicine: A textbook of the diseases of cattle, horses, sheep, pigs and goats. 10th edition 2006.

26. Rasmussen H, Goodman DBP. Relationships between calcium and cyclic nucleotides in cell activation. Physiol. Rev 1977;57:421.

27. Rude RK, Oldham SB, Sharp CFJR *et al*. Parathyroid hormone secretion in magnesium deficiency. J. Clin. Endocrinol.Metab 1978;47(4):800-6.

28. Tanaka Y, DeLuca HF. Stimulation of 25, 25-dihydroxyvitamin D_3 production by 1, 25-dihydroxyvitamin D_3. S*cience* 1974;183(130):1198-1200.

29. Thilsing-Hansen T, Jorgensen RJ, Ostergaard S. Milk fever control principles: A review. Acta. Vet. Scand 2002;43(1):1-19.

30. Westwood CT, Lean IJ, Kellaway RC. Indications and implications for testing milk urea in dairy cattle: a quantitative review. Part 1. Dietary protein sources and metabolism. N. Z. Vet. J 1998;46(3):87-96.