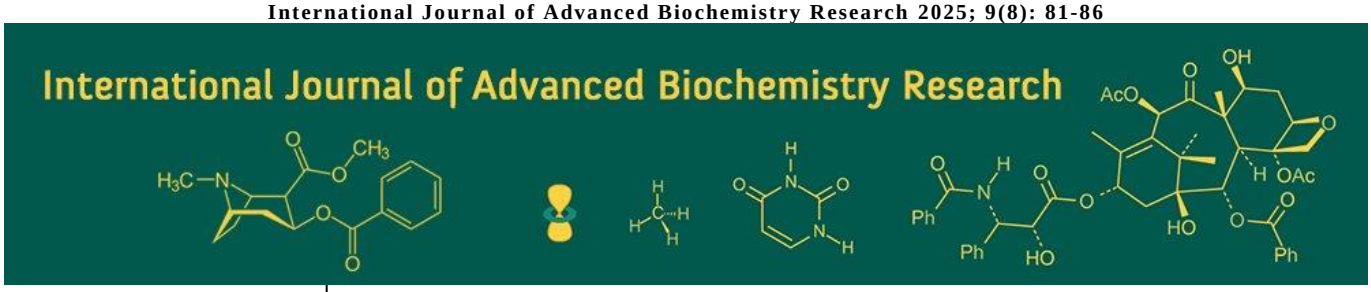

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Mineral imbalance, oxidative stress and hormonal alterations associated with Cervicovaginal prolapse in buffaloes

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Abstract
A comprehensive study was conducted to investigate the underlying causes of cervico vaginal prolapse in buffaloes, with a comparative analysis of 10 healthy pregnant buffaloes and 10 buffaloes affected by the condition. The research revealed that the affected animals exhibited no significant difference in Haemoglobin, Packed Cell Volume and DLC, however significantly lower levels of essential minerals was identified between Group I and Group II buffaloes. The results were shown as 10.34±0.45 mg/dl, 6.0±0.19 mg/dl and 164.36±5.0 µg/dl of calcium, phosphorus and zinc respectively in Group-I buffaloes and in Group-II buffaloes the calcium, phosphorus and zinc levels were found to be 8.33±0.19, 4.89±0.32 and 138.43±5.41 respectively and no significant difference was found in iron levels between Group-I (321.86±9.80 µg/dl) and Group-II (315.29±8.43 µg/dl) buffaloes. Furthermore, the study evaluated oxidative stress status and found non-significant differences in their levels between the healthy and affected buffaloes. The levels of MDA, SOD and Catalase were noted as 0.92±0.09 µ mol/l, 182.39±6.46 u/ml and 4.14±0.41 K units/litre respectively in Group-I buffaloes and in Group-II buffaloes it was noted as 1.31±0.13 µ mol/l, 116.19±6.5 u/ml and 2.41±0.28 K units/litre levels of MDA, SOD and Catalase respectively. The investigation also explored the role of estrogen hormone in the development of cervicovaginal prolapse, observing non-significant variations in its levels between Group-I (52.41±5.78 pg/ml) and Group-II (88.27±14.90 pg/ml) buffaloes. This study highlights the need for targeted supplementation, better management, and preventive measures to address mineral deficiencies, oxidative stress, and elevated estrogen levels.

Keywords: Cervicovaginal prolapse, minerals, oxidative status, estrogen

Introduction
Cervico vaginal prolapse (CVP) is a significant reproductive health issue affecting buffalo populations, leading to substantial economic losses and animal welfare concerns. The etiology of CVP is multifactorial, with nutritional, hormonal, and oxidative stress related factors contributing to its development. Minerals, such as calcium, phosphorus, and copper, are essential for tissue integrity and reproductive function. Calcium, among the all the minerals has a significant impact on pathophysiology of the genital prolapse, as it is required for the stiffness, contraction and tonicity of the muscles (Ruegg, 2012) [1]. Oxidative stress, resulting from an imbalance between free radical production and antioxidant defenses, can lead to tissue damage and inflammation, exacerbating CVP. The free radicals changes the membrane permeability which sequentially leads to the changes in the alteration of matrix proteases thus eventually causing the destruction of the collagen, a protein that provides structural support to the connective tissue (Marcu *et al.*, 2020) [2]. Estrogen hormone, crucial for maintaining reproductive tract health, has been implicated in the pathogenesis of CVP. High levels of estrogen cause relaxation of pelvic ligaments and the birth canal (Hudson 1986) [3]. Higher estradiol concentrations in the later stages of pregnancy, combined with a deficiency of certain minerals and faulty management conditions, contribute to vaginal prolapse (Nanda, 1979) [4]. Understanding the interplay between these factors is crucial for developing effective prevention and treatment strategies for CVP in buffaloes.
This article aims to record the effects of minerals, oxidative stress, and estrogen hormone on CVP in buffaloes, highlighting the complex relationships between these factors and their implications in this condition.

Materials and Methods

Selection of experimental animals

The buffaloes (n=10) in their last trimester of normal pregnancy were included in the study as control group and the buffaloes (n=10) exhibiting clinical signs of prepartum cervicovaginal prolapse were included in the experimental group. The buffaloes in their first and second trimester of pregnancy and pregnant buffaloes with systemic illness were excluded from the study. These animals were maintained by rural individual farmers under diverse nutritional and managemental conditions. These animals were allowed to graze and were supplemented with green fodder, paddy straw, rice bran, groundnut cake and concentrates.

Sample collection

Blood samples were collected once from the jugular vein of each buffalo in both groups and subsequently subjected to centrifugation to separate the serum, which was then stored at-20°C until further analysis. The blood was analysed for haemotological parameters viz haemoglobin, differential leucocytes and packed cell volume and serum samples were tested to identify the key serum minerals, oxidative stress markers and estrogen hormone.

Estimation of serum minerals

Serum concentrations of calcium, phosphorus, (M/S Excel Diagnostics Pvt.Ltd, Opp Kukatpally, JNTU, Hyderabad, India) zinc, and iron (Coral Clinical Systems, Goa) were estimated using commercial diagnostic kits following the manufacturers' protocols. Calcium was measured by the O-Cresolphthalein Complexone (OCPC) method, phosphorus by the Daly and Ertingshausen method, zinc by the colorimetric method with Nitro-PAPS, and iron by the Ferrozine/Magnesium Carbonate method. In each assay, mineral-specific reagents formed colored complexes proportional to their concentrations, which were quantified spectrophotometrically at respective wavelengths (570 nm for calcium, zinc, and iron; 340 nm for phosphorus).

Estimation of oxidative stress markers

Serum levels of malondialdehyde (MDA), superoxide dismutase (SOD), and catalase were estimated to assess oxidative stress. MDA was measured using the thiobarbituric acid reactive substances (TBARS) method (Satoh, 1978), with absorbance recorded at 535 nm. SOD activity was determined by the inhibition of pyrogallol auto-oxidation, as described by Marklund and Marklund (1974), and absorbance was measured at 420 nm.Catalase activity was estimated by the decomposition of H_2O_2 and formation of a yellow molybdate complex, with absorbance read at 374 nm.

Estimation of estrogen hormone

Serum estrogen levels were estimated using the Atellica IM Enhanced Estradiol (eE2) kit based on the radioimmunoassay (RIA) principle. The assay employs a competitive chemiluminescent format, where estradiol in the sample competes with a labeled analog for binding to anti-estradiol antibodies. The resulting chemiluminescence, inversely proportional to estradiol concentration, was measured in relative light units (RLUs) using the Atellica IM system, following the manufacturer's automated protocol.

Statistical analysis

Data from Group I and Group II were analyzed using the independent t-test (IBM SPSS Statistics 20.0). Statistical significance was defined as $p<0.05$, with $p<0.01$ considered highly significant and $p>0.05$ considered non-significant (NS).

Results and Discussion

Haemotological parameters

The Hb was reported as 12.8±0.35 g/dl and 12.4±0.84 and PCV was recorded as 37.5±1.22 and 36.7±0..83 in Group I and Group II buffaloes respectively. In differential leucocyte count, the percentage of lymphocytes, neutrophils, monocytes, basophils and eosinophils were recorded as 73.3±1.84, 22.8±1.7, 1.6±0.27, 1.5±0.37 and 0.5±0.17 in Group I and 67±4.40, 29.8±4.45, 1.9±0.40, 0.8±0.25 and 0.7±0.15 in Group II respectively. There was no significant difference in Haemoglobin, Packed Cell Volume and DLC between Group I and Group II buffaloes. The haemoglobin levels in advanced pregnant buffaloes recorded as 13.26±0.61 g/dL and 12.2±1.86 g/dL by Patel *et al.* (2016) [5] and Dhillon *et al.* (2020) [6] respectively which were in agreement with this study. In contrast Ahmed *et al.* (2005) [7] and Upadhay *et al.* (2021) [8] reported significant decrease in Hb in CVP affected buffaloes compared to pregnant buffaloes. These discrepancies might be attributed to fluid loss, dehydration or reduced erythrocyte size (Ahmed *et al.*, 2005) [7]. The decrease in PCV levels may be attributed to stress-induced increases in antidiuretic hormone (ADH), leading to fluid retention and decreased blood cell concentration (Wani and Mavi, 2020) [9]. Additionally, anorexia and toxemia may also contribute to reduced PCV levels (Ahmed *et al.*, 2005) [7].

Serum Minerals

A comparison of serum mineral levels between buffaloes with cervico vaginal prolapse (CVP) and healthy controls revealed significant lower values of calcium, phosphorus, zinc except for iron (Table 1) in CVP affected buffaloes.

Notably, serum calcium levels in affected buffaloes varied across studies, ranging from 3.99±0.11 mg/dl (Sharma *et al.*, 2015) [10] to 7.45±0.15 mg/dl (Vikas *et al.*, 2019) [11], with intermediate values reported by other studies (Ahmed *et al.*, 2005 [7] (6.42±1.05 mg/dl); Akhtar *et al.*, 2008 [12] (6.75±0.13 and 6.31±0.13 mg/dl respectively in irrigated zone and rain fed zone); Gangawar *et al.*, (2015) [13] (7.03±0.15 mg/dl)). These levels were consistently lower than those found in the present study.The recorded variations in calcium levels may be attributed to factors such as stress, hormonal fluctuations, and sudden calcium diversion, ultimately leading to impaired muscle function and contributing to the development of genital prolapse. These findings suggest that calcium deficiencies, play a crucial role in the pathogenesis of CVP in buffaloes.

Studies have reported varying serum phosphorus levels in buffaloes with cervico vaginal prolapse (CVP), with Sharma *et al.*, (2015) [10], Singh *et al.*, (2020) [14] and Molefe and Mulunda (2023) [15] finding lower levels of 2.88±0.08, 3.90±0.37, and 2.70 mg/dl, respectively, while Vikas *et al.*, (2019) [11] reported higher phosphorus levels of 6.49±0.67 mg/dl in CVP affected Murrah buffaloes. The observed hypophosphatemia in affected animals may be attributed to hormonal responses, such as the release of calcitonin, as reported by Ahmed *et al.*, (2005) [7], and parathyroid

hormone (PTH), as stated by Molefe and Mwanza (2023) [15], triggered by low calcium levels, leading to increased phosphorus loss through saliva and urine. Furthermore, stress and carbohydrate metabolism during the prepartum period may contribute to hypophosphatemia, as enhanced carbohydrate metabolism and elevated phosphorus requirements during this period may predispose to the development of hypophosphatemia, as stated by Sharma *et al.*, (2015) [10]. A deficiency in both calcium and phosphorus can have a synergistic effect, resulting in decreased genital muscle tone, excessive pelvic ligament relaxation, and increased predisposition to prolapse, ultimately highlighting the importance of maintaining optimal mineral levels, particularly calcium and phosphorus, to prevent CVP in buffaloes.

Akhtar *et al.* (2012) [16] reported serum zinc levels in CVP affected buffaloes in the irrigated zone area as 133.59±5.14 µg/dl, 128.19±5.87 µg/dl, and 117.87±5.23 µg/dl for gestation periods of less than 8 months, 8-9 months, and more than 9 months, respectively, and in the rainfed area as 133.81±5.76 µg/dl, 134.19±5.51 µg/dl, and 118.12±5.59 µg/dl, respectively, which were inconsistent with the present study's findings for buffaloes below 8 months and 8-9 months of pregnancy. In contrast, Bhatti *et al.*, (2006) [17] reported lower zinc levels (127.25±12.04 µg/dl) than the present study in CVP affected buffaloes, while Molefe and Mwanza (2023) [15] reported higher serum zinc levels (144.3 µg/dl) in CVP affected cattle. The decrease in zinc levels in affected animals may be attributed to mechanical stress, which increases serum cortisol levels (Wegner *et al.*, 1973; Dufty *et al.*, 1977; Bhatti *et al.*, 2006) [18, 19, 17] compared to healthy pregnant buffaloes. Furthermore, a negative correlation between plasma zinc status and corticosteroid levels in cattle suggests that pregnancy complications associated with hormonal imbalances may be linked to alterations in zinc levels. Although lower zinc concentrations are often associated with high calcium and phosphorus levels, which can reduce zinc absorption in the intestines (Waldner *et al.*, 2014) [20], this was not observed in the present study, as calcium and phosphorus levels were found to be low.

The iron concentration in buffaloes with prolapse was reported to be 354.66±39.75 µg/dl by Bhatti *et al.* (2006) [17], which was higher than the present study's findings. In contrast, Akhtar *et al.* (2008) [12] found a significant decrease in serum iron concentrations in buffaloes with prolapse in the 9th month of gestation compared to healthy controls. Iron imbalances in animals have been linked to compromised immune function, leading to poor overall health, decreased appetite, and diminished body condition (Kumar *et al.*, 2011) [21].

Table 1: (Mean ± SE) of concentration of minerals in the serum of normal advanced pregnant buffaloes (Group I) and CVP affected buffaloes (Group II)

Serum mineral	Group-I	Group-II	p value (0.05)
Calcium (mg/dl)	10.34±0.45^{a}	8.33±0.19^{b}	0.0015**
Phosphorus (mg/dl)	6.0±0.19^{a}	4.89±0.32^{b}	0.0084**
Iron (µg/dl)	321.86±9.80	315.29±8.43	0.6174
Zinc (µg/dl)	164.36±5.06^{a}	138.43±5.41^{b}	0.0025**

**= Significance at $p<0.01$

Values sharing different superscripts in the same row differ significantly.

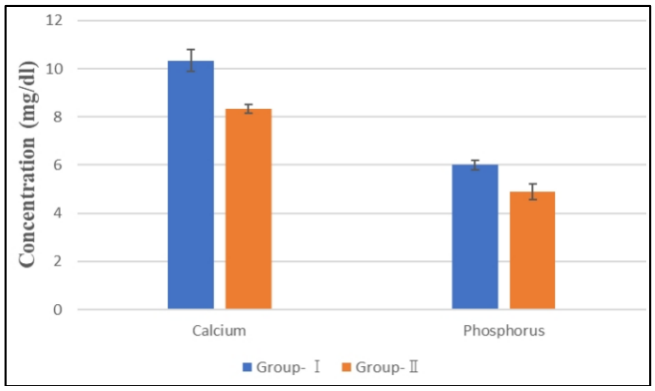

Fig 1: Mean ± SE of Calcium and Phosphorus in normal advanced pregnant buffaloes (Group-I) and CVP affected buffaloes (Group-II)

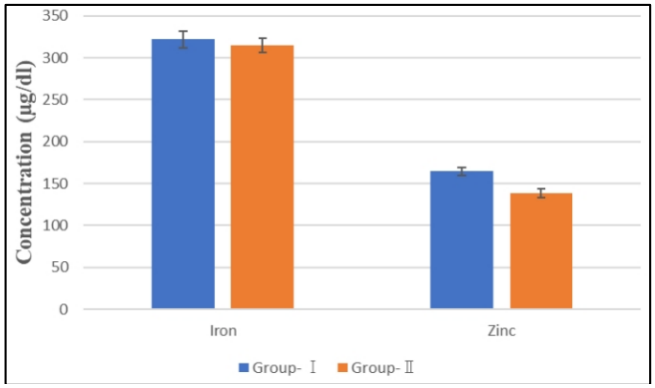

Fig 2: Mean ± SE of iron and zinc minerals in normal advanced pregnant buffaloes (Group I) and CVP affected buffaloes (Group II)

Oxidative Stress

Lipid peroxidation occurs when free radicals, particularly reactive oxygen species (ROS), engage with lipids found in cell membranes and is quantified through the assessment of malondialdehyde (MDA). Antioxidants, Superoxide dismutase (SOD), Catalase typically minimize the damage caused by these reactive species by either removing them or preventing their formation (Davies, 1995: Sies 1997) [22, 23]. In the present study significantly elevated levels of MDA and significantly low levels of SOD and catalase were reported in CVP affected buffaloes (Table No.2).

Consistent with the present study, several researchers have reported elevated malondialdehyde (MDA) levels in buffaloes experiencing reproductive disorders. Erisir *et al.*, (2006) [24] found significantly higher MDA levels (5.10±0.25) in cows with uterine prolapse. Bansal *et al.*, (2011) [25] reported higher plasma MDA levels (1.57±0.31 µmoles MDA/mg protein/ml and 2.04±0.19 µmoles MDA/mg protein/ml) in buffaloes with fetal dystocia and uterine torsion, respectively. Thangamani *et al.*, (2019) [26] also found significantly higher MDA levels (2.42±0.17 µmoles MDA/mg protein/ml) in buffaloes with maternal dystocia compared to those with normal calving (1.10±0.08 µmoles MDA/mg protein/ml). Wani and Mavi (2020) [9] reported significantly higher MDA levels (6.70±0.65 nmol/g Hb) in buffaloes that underwent fetotomy compared to those that gave birth normally (4.6±0.59 nmol/g Hb).

The elevated MDA levels observed in the present study might be attributed to physical stress, which contributes to increased lipid peroxidation. Stress enhances the generation of reactive oxygen species (ROS) and increases the levels of

noradrenaline, glucocorticoids, and adrenaline-induced pathways of aerobic energy production (Singh *et al.*, 2017) [27], leading to excessive ROS production (Freeman and Crapo, 1982) [28]. This, in turn, causes peroxidation of placental membrane lipids, particularly polyunsaturated fatty acids (PUFA), resulting in lipid peroxidation.

The observed increase in MDA levels may also be due to an imbalance between the generation and scavenging of ROS (Ahmed *et al.*, 2009) [29]. Moreover, low antioxidant enzyme activity leading to oxidative stress, combined with high energy expenditure triggering increased lipolysis and subsequent production of free radicals, may also contribute to elevated MDA levels (Nisa *et al.*, 2020) [30].

Several studies have reported varying superoxide dismutase (SOD) levels in buffaloes with reproductive disorders. Ahmed *et al.*, (2009) [29] found significantly lower SOD levels (271.0±17.39 U/ml) in buffaloes with retained placenta compared to normal placental expulsion (338.16±7.11 U/ml). Thangamani *et al.*, (2019) [26] also reported reduced SOD activity in maternal dystocia cases (6.15±1.60 units/µg protein/min) versus normal parturition (10.02±1.72 units/µg protein/min). Conversely, Wani and Mavi (2020) [9] observed higher SOD levels in fetotomy-affected buffaloes (55.77±6.69 U/mg Hb) than in those with normal calving (41.91±5.96 U/mg Hb). Variations in superoxide dismutase (SOD) concentrations may result from obstetrical complications, physiological stress, and inflammation (Jens and Ove, 2006) [31]. The calving process itself is associated with increased reactive oxygen species (ROS) production, altered oxygen metabolism, and fluctuations in antioxidant capacity (Ahmed *et al.*, 2009) [29]. Zinc deficiency is another contributing factor, as it can reduce SOD activity, compromise antioxidant defense mechanisms, and heighten susceptibility to oxidative stress (Singh *et al.*, 2017) [27]. In cases of cervicovaginal prolapse, mechanical stress promotes ROS accumulation, while the prolapsed tissues exhibit downregulation of antioxidant enzymes, leading to impaired antioxidative capacity and an imbalance between ROS production and neutralization (Li *et al.*, 2016) [32].

Catalase activity shows variable trends in reproductive disorders of bovines. Erisir *et al.* (2006) [24] found no significant change in catalase levels in Holstein cows with uterine prolapse (30.10±3.49 k/g Hb) and normal parturition cows (31.57±2.44 k/g Hb). In contrast, Ahmed *et al.* (2009) [29] reported significantly reduced catalase activity in buffaloes with retained placenta (0.88±0.15 U/ml) versus normal placental expulsion (2.28±0.04 U/ml), aligning with the present study. Conversely, Wani and Mavi (2020) [9] observed elevated catalase levels in fetotomy cases (336.69±2.83 k/mg Hb) compared to normal calving (290.79±2.39 k/mg Hb). Variations in catalase levels may reflect differences in the intensity of oxidative stress, mechanical strain, and the duration of obstetrical complications. In the peripartum period, the elevated metabolic demands in dairy cows contribute to increased reactive oxygen species (ROS) production and a consequent reduction in antioxidant defenses, including catalase activity (Gitto *et al.*, 2002) [33].

Table 2: Mean ± SE of MDA, SOD and Catalase, estimating oxidative stress in normal advanced pregnant buffaloes (Group I) and CVP affected (Group II)

Parameter	Group-I	Group-II	p value
MDA (µ mol/l)	0.92±0.09[a]	1.31±0.13[b]	0.028*
SOD (u/ml)	182.39±6.46[a]	116.19±6.5[b]	0.0001**
Catalase(K units/litre)	4.14±0.41[a]	2.41±0.28[b]	0.002**

**= Significance at $p<0.01$

* = Significance at $p<0.05$

Values sharing different superscripts in the same row differ significantly.

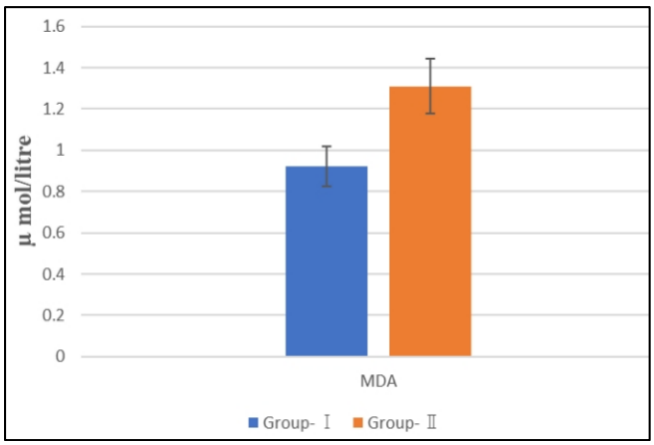

Fig 3: Mean ± SE of MDA in normal advanced pregnant buffaloes (GroupI) and CVP affected buffaloes (Group II)

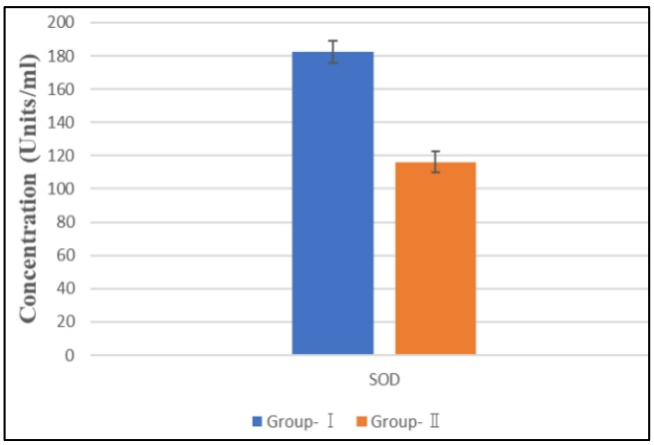

Fig 4: Mean ± SE of SOD in normal advanced pregnant buffaloes (Group I) and CVP affected buffaloes (Group II)

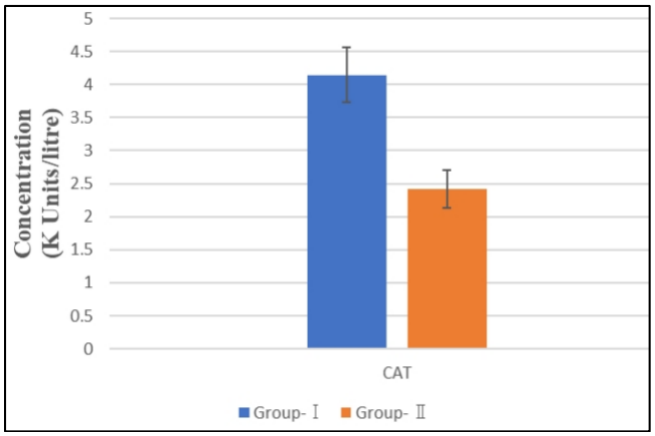

Fig 5: Mean ± SE Catalase in normal advanced pregnant buffaloes (Group I) and CVP affected buffaloes (Group II)

Estrogen

In the present study, estrogen levels in buffaloes with cervico-vaginal prolapse were significantly higher (88.27±14.90 pg/ml) compared to normal advanced pregnant animals (52.41±5.78 pg/ml). Other studies reported varying estrogen levels in affected animals, with Kumar *et al.* (2009) [34] finding levels ranging from 303.5 to 379.75 pg/ml in buffaloes, Molefe and Mwanza (2020) [35] reporting 187.94±91.44 pg/ml and Rajamanickam *et al.* (2018) [36] finding lower levels of 49.76±7.07 pg/ml in cattle. Despite the variations in estrogen levels reported across these studies, all authors consistently found significant differences in estrogen concentrations between affected animals and unaffected advanced pregnant animals.

The increased levels of estrogen causes relaxation of the pelvic ligaments (Kumar *et al.*, 2009) [34]. There was a ten fold increase in expression of estrogen receptor alpha in prolapsed animals (Kumar *et al.*, 2009 and Rajamanickam *et al.*, 2018) [34, 36] than normal pregnant animals at same stage of gestation. The expression of estrogen receptor alpha in the genital tract, increases the effect of circulating estrogen hormone (Ennen *et al.*, 2011) [37]. The ratio of estrogen and progestrerone increases the risk of vaginal prolapse as reported by Siddiquee *et al.* (2006) [38] and Akhtar *et al.* (2012) [16]. The combined effects of estrogen and relaxin also accelerate the loosening of the supportive structures of the reproductive tract, thereby compromising its integrity and contributing to the development of prolapse (Hafez and Hafez 2013) [39]. Additionally, estrogen's anabolic and mitogenic effects stimulate the production of actin and myosin, thereby increasing muscle contractility (Rajamanickam *et al.*, 2018) [36] to facilitate parturition might favour occurrence of CVP. Elevated estrogens combined with reduced muscle tone caused by hypocalcimia and hypophasphatemia work synergistically to contribute to genital prolapse (Vikas *et al.*, 2019) [11]. This suggests that estrogen plays a crucial role in the development of cervico vaginal prolapse in buffaloes.

Conclusion

It can be concluded that mineral deficiencies, increased oxidative stress, and elevated estrogen levels in CVP-affected buffaloes underscore the need for targeted supplementation, improved management practices, and preventive measures to reduce its occurrence.

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