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Variations in blood mineral profile and conception rate of Sahiwal cows treated with clomiphene citrate, co-synch protocol and mineral mixture supplementation

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Abstract
Thirty anestrus Sahiwal cattle aged between 3.5- 4.5 years, were used to study the effect of Clomiphene citrate, Co-synch protocol and Mineral supplementation on estrus induction and fertility of cattle. Group 1, the animals (n=6) were fed ration containing 0% mineral mixture. Group 2, the animals (n=6) were fed ration containing clomiphene citrate (300mg). In Group 3, the animals (n=6) were subjected to co-synch protocol. Group 4, the animals (n=6) were fed ration containing mineral mixture 1% of ration and clomiphene citrate (300mg). In Group 5, the animals (n=6) were fed ration containing mineral mixture 1% of ration and subjected to co-synch protocol. The mean concentration of serum calcium, phosphorus, magnesium, copper, zinc, cobalt, selenium and iron from day 0, 21, 42, and 60 in Control group-1 and treatment groups-2, 3, 4 and 5 were recorded, where calcium, phosphorus, magnesium, selenium and iron differ significantly in days as well as in groups and zinc differ significantly in groups. Deficiencies of calcium, phosphorus, magnesium, zinc and selenium either singly or in combination could be responsible for anestrus condition in cattle and by improving the nutritional status the animal fertility can be improved. Therefore, in present study co-synch protocol along with mineral mixture improved the reproductive status of anestrus Sahiwal cows.

Keywords: clomiphene citrate, co-synch protocol, mineral mixture, reproductive status, mineral concentration

1. Introduction
The dairy and livestock sector plays a very important role in national economy of India by contributing close to one third of gross income of rural households and nearly half of gross income in case of those without land (Bhasin, 2016) [7]. The contribution of livestock sector in national economy in terms of GDP was 4.11% during 2018-2019. As per the report of National Accounts Statistics, 2019, the percentage contribution of livestock in terms of gross value added (GVA) in agriculture was 17.1%. India possesses 192.49 million cattle and contributes around 35.92% of the total livestock population (20th Livestock census).
Among the indigenous breeds Sahiwal is known to be the best milch breed of India and had shown an excellent adaptability to the agro-climatic condition of Madhya Pradesh state, with an average milk production potential of 2100 liter in 300 days of lactation length.
It has been noticed that the normal productive and reproductive behavior in domestic animals is closely associated with interaction between hormonal and nutritional status of the animal system (Reddy and Reddy, 1988) [48]. In this regard, minerals particularly some major and trace elements play a very crucial role. Mineral deficiency in animal may be related either to low intake or to antagonistic effect among different minerals (Humphries *et al.*, 1983) [20]. Deficiency of single and combined minerals and their imbalances may cause various reproductive failures such as poor conception and anestrus (Hidiroglou, 1979) [18]. Singh *et al.* (1981) [56] have reported that the incidence of anestrus (39.01%) has been the highest of the total reproductive disorders. Various minerals can influence reproductive performance of ruminants including Cu, Co, Se, Mn, Zn and Iodine (Hidiroglou, 1979) [18]. However, Olivieri *et al.* (1979) [38] stated that iron and zinc level were not related to postpartum anestrus period and fertility in cows. Concomitant infertility in cattle with trace element deficiencies is believed to be associated with enzymatic dysfunction (Leonhard, 2000) [32].
Keeping in view the major problem of anestrus in cows which are maintained at field level and use of Clomiphene citrate and Co-synch protocol in anestrus with importance of mineral

and hormonal profile values for impairing fertility, the present research was planned to study fertility evaluation after Clomiphene citrate and Co-synch protocol treatment in anestrus Sahiwal cows under field conditions.

2. Materials and Methods

The present investigation was carried out in the Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science & Animal Husbandry, Rewa (M.P.) and different villages in and around Rewa (M.P.). The present study was carried out by selection of 30 anestrus cows and randomly divided in four Groups-1, 2, 3, 4 and 5. Group-1 is control group, Group-2 is treated with clomiphene citrate, Group-3 is subjected to co-synch protocol and AI done with second GnRH, Group-4 is given combination of clomiphene citrate along with mineral mixture supplementation and Group-5 is subjected to co-synch protocol along with mineral mixture then comparison of conception rate was done. Different parameters were used to study effect of clomiphene citrate, co-synch protocol and mineral mixture on anestrus cattle.

Blood samples were collected by jugular venipuncture on day pre feeding (day 0) and on day of 21, 42 and 60. Major elements (Ca, P and Mg) and trace elements (Fe, Cu, Se, Zn and Co) for animals were estimated on the basis of analysis of blood samples. Finally, the mineral availability of individual animal was compared with the mineral requirements given in feeding standards (Kearl, 1982; ICAR, 1998) [26, 21] to work out the mineral deficits/ excess/ imbalance. Zinc, iron, copper, cobalt and selenium were estimated by Atomic Absorption Spectrophotometer, whereas calcium, phosphorus and magnesium were estimated by colorimetric method using standardized kits.

Serum sample were digested as per the procedure described by Kolmer *et al.,* (1951) [27]. Two ml serum with equal volume of concentrated HNO_3 was mixed in the digestion tube. The sample were kept overnight at room temperature followed by digestion on low heat (70-80 ^{0}C) using heating bench (digestion bench) until their volume got reduce to about 1 ml to this, 2 ml of di-acid mixture (3 parts concentrated nitric acid and 1 part 70% perchloric acid) was added and low heat digestion continued until the digested sample become watery clear and white fumes are emitted. The addition of 2 ml di-acid mixture followed by low heat digestion was repeated a couple of times. Further heating was continued to reduce the volume to approximately 0.5 ml. Digested samples were diluted with triple distilled water and filtered with Whatman's filter paper No.1. Repeated washing of tubes and filter paper were done with triple glass distilled water. Final volume of the filtrate was made up to 25 ml with de-ionized triple glass distilled water after Luke warming of the solution.

Fully Automatic Atomic Absorption Spectrophotometer (AAS); Model AA 8000, (Labindia Analytical Instruments Ltd, India) was used for the estimation of minerals except phosphorus. Separate hollow cathode lamps for each mineral were used. Air/acetylene flame was used as a fuel. Sample analysis was done by attached computer and the concentrations of minerals contained in the sample were expressed in ppm. Calibration of instrument was done with three standards of known concentrations prior to analysis of unknown sample.

Stock solution for different minerals was prepared by taking readymade standard of Merck co. (Merck KGaA, 2005). The standard solution of zinc metal in 40 ml of 5N HCl. It was diluted to one liter to obtain stock solution having 1000 µg zinc/ml. This stock solution was used to prepare working standard of 0.4, 1.0 and 1.5 ppm. One gram of iron metal was dissolved in mixture containing 20 ml of 5N HCl and 5 ml of 6N HNO_3. It was diluted to one liter to get 1000 µg iron/ml stock solution. This solution was used to prepare working standard of 1.0, 5.0 and 10.0 ppm. The standard solution of copper was prepared by dissolving 1 g of copper metal in 50 ml of 6N HNO_3. It was diluted to one liter to get 1000 µg copper/ml stock solution. This stock solution was used to prepare working standard of 1.0, 2.5 and 5.0 ppm. One gram of cobalt metal was dissolved in 6N HNO_3 and diluted to one liter to get 1000 µg cobalt/ml stock solution. This stock solution was used to prepare working standard of 0.5, 1.0 and 2.5 ppm. The standard solution of selenium was prepared by dissolving 1 g of selenium metal in minimum 20 ml of aqua regia. It was diluted to one liter to get 1000 µg selenium/ml stock solution. This stock solution was used to prepare working standard of 0.5, 1.0 and 1.5 ppm.

The data was analyzed using standard statistical procedure as per (Snedecor and Cochran, 1994) [57] using IBM® SPSS software, version 20 statistical packages.

3. Results and Discussion

3.1 Mineral Assay

3.1.1 Mean serum Calcium concentration of five groups

The mean concentration of serum Calcium from day 0 to 60 in control Group-1 and treatment Groups (2, 3, 4 and 5) have been presented in Table-1. The mean serum Calcium (mg/dl) concentration in Group-1 were recorded as 7.70±0.08, 7.75±0.07, 7.8±0.06 and 7.83±0.05 on day 0, 21, 42 and 60, respectively. The Calcium level remain relatively constant throughout study. In Group-2, serum Calcium (mg/dl) were recorded as 7.78±0.05, 8.035±0.04, 8.16±0.04 and 8.31±0.02 on day 0, 21, 42 and 60, respectively. In Group-3 serum Calcium (mg/dl) were recorded as 7.90±0.02, 8.011±0.05, 8.17±0.06 and 8.32±0.07 on day 0, 21, 42 and 60, respectively. In Group-4 serum Calcium (mg/dl) were recorded as 7.77±0.14, 8.33±0.03, 8.49±0.04 and 8.8±0.05 on day 0, 21, 42 and 60, respectively. The Calcium level significantly ($P \leq 0.05$) varies between day 0, 21 and 60. In Group-5 serum Calcium (mg/dl) were recorded as 7.89±0.02, 8.25±0.03, 8.63±0.06 and 9.04±0.08 on day 0, 21, 42 and 60, respectively. The Calcium level significantly ($P \leq 0.05$) varies between day 0 and 60. Supplemented Groups-4 and 5 have more calcium levels comparison to other groups.

The findings of this study were lower than Umesh *et al.* (1995) [60] and Ramakrishna (1997) [47]. They observed that the serum calcium concentrations were non-significant difference between anestrus and cyclic cows. However, the level of calcium observed in anestrus cow by Das *et al.* (2002) [11], Singh *et al.* (2005) [55] and Dutta *et al.* (2001) [12] as 8.5 mg/dl, 8.33±0.15 mg/dl and 9.54 ± 0.22 mg/dl respectively, were found to be higher than the findings of present study. The present study shows that the better results obtained if animals were supplemented with mineral mixture and has better Ca value than control Group. The deficiency of calcium affects the GnRH stimulation of LH release from pituitary gland as it involves a calcium dependent mechanism. Free calcium ions are essential for contractibility of smooth and stripped muscles by a sequence known as excitation-contraction coupling (Radostitis *et al.*, 1994) [46]. Purohit and Bishnoi (1989) [43] reported that the serum calcium levels significantly increased at estrus than prior to treatment in anestrus cows

treated with Clomiphene citrate. They further observed that there was no variation in serum calcium levels at induced estrus than obtained at estrus in normal cycling animals. Hence, they concluded that there was no relation of rise in serum calcium levels with Clomiphene citrate treatment.

Table 1: Calcium (mg/dl) estimation during estrus induction by Clomiphene citrate and Co-synch protocol in anestrus cattle (Mean±SE).

Group	Days			
	0	21	42	60
G1	7.70±0.08	7.75±0.07^{a}	7.8±0.06^{a}	7.83±0.05^{a}
G2	7.78±0.05	8.035±0.04^{bc}	8.16±0.04^{b}	8.31±0.02^{b}
G3	7.9±0.02	8.011±0.05^{b}	8.17±0.06^{b}	8.32±0.07^{b}
G4	7.77±0.14^{A}	8.33±0.03^{Db}	8.49±0.04^{cBC}	8.8±0.05^{cC}
G5	7.89±0.02^{A}	8.25±0.03^{cdB}	8.63±0.06^{cC}	9.04±0.08^{cD}

Values with different superscripts in small letter (a, b, c, d) in a column and capital letter (A, B, C, D) in a row differ significantly (P≤0.05).

3.1.2 Mean serum Phosphorus concentration of five groups

The mean concentration of serum Phosphorous from day 0 to 60 in control Group-1 and treatment Groups (2, 3, 4 and 5) have been presented in Table-2. The mean serum Phosphorus (mg/dl) concentration in Group-1 were recorded as 4.42±0.1, 4.55±0.1, 4.74±0.14 and 4.92±0.16 on day 0, 21, 42 and 60, respectively. In Group-2 serum Phosphorus (mg/dl) were recorded as 4.52±0.13, 4.83±0.15, 5.13±0.15 and 5.45±0.17 on day 0, 21, 42 and 60, respectively. The phosphorus level significantly (P≤0.05) varies between on 60th day. In Group-3 serum Phosphorus (mg/dl) were recorded as 4.58±0.11, 4.87±0.16, 5.19±0.16 and 5.70±0.16 on day 0, 21, 42 and 60, respectively. The phosphorus level significantly (P≤0.05) varies between 0 and 60 days. In Group-4 serum Phosphorus (mg/dl) were recorded as 4.59±0.17, 5.08±0.15, 5.44±0.12 and 6.05±0.07 on day 0, 21, 42 and 60, respectively. The phosphorus level significantly (P≤0.05) varies between 0, 42 and 60 days. In Group-5 serum Phosphorus (mg/dl) were recorded as 4.61±0.12, 5.21±0.09, 5.88±0.14 and 6.32±0.09 on day 0, 21, 42 and 60, respectively. The phosphorus level significantly (P≤0.05) varies between day 0, 21 and 42. Supplemented Groups-4 and 5 have more Phosphorus levels comparison to other Groups.

The higher level of phosphorus than the present study in anestrus condition was observed by Venugopal and Ramamohan (1982) [62], Prasad *et al.* (1984), Vadnere and Singh (1989) [61], Kumar and Sharma (1991), Shrivastava and Kadu (1995) [54] and Singh *et al.* (2005) [55] as 4.65 ± 1.08 mg/dl, 6.84 mg%, 6.22 ± 0.02 mg/dl, 4.98 ± 0.08 mg/dl, 5.57 ± 0.14 mg/dl and 5.50 ± 0.22 mg/dl, respectively. The serum phosphorus level in anestrus cows were reported by Sharma *et al.* (1984) [53], Aminudeen and Pareek (1984) [3], Dutta *et al.* (1988) [13], Umesh *et al.* (1995) [60] and Das *et al.* (2002) [11] as 2.97 ± 0.23 mg/dl, 3.02 ± 0.04 mg/dl, 2.77 ± 0.61 mg/dl, 2.03 ± 0.09 mg% and 3.86 mg/dl, respectively. These findings were lower than the findings of present study. Impaired fertility has been reported in phosphorus deficient cattle. Salisbury and Van Demark (1961) [50] suggested that possible role of phosphorus in reproduction is due to its key position in the energy exchange mechanism. Phosphorus deficiency induces lowered conception rate, irregular estrus, anestrus and decreased ovarian activity (Morrow, 1980) [37].

Table 2: Phosphorus (mg/dl) estimation during estrus induction by Clomiphene citrate and Co-synch protocol in anestrus cattle (Mean±SE).

Group	Days			
	0	21	42	60
G1	4.42±0.1	4.55±0.1^{a}	4.74±0.14^{a}	4.92±0.16^{a}
G2	4.52±0.13	4.83±0.15^{ab}	5.13±0.15^{ab}	5.45±0.17^{ab}
G3	4.58±0.11	4.87±0.16^{ab}	5.19±0.16^{ab}	5.70±0.16^{bc}
G4	4.59±0.17^{A}	5.08±0.15^{abAB}	5.44±0.12^{bcB}	6.05±0.07^{cdC}
G5	4.61±0.12^{A}	5.21±0.09^{bB}	5.88±0.14^{cC}	6.32±0.09^{dC}

Values with different superscripts in small letter (a, b, c, d) in a column and capital letter (A, B, C, D) in a row differ significantly (P≤0.05).

3.1.3 Mean serum Magnesium concentration of five groups

The mean concentration of serum Magnesium from day 0 to 60 in control Group-1 and treatment Groups-2, 3, 4 and 5 have been presented in Table-3. The mean serum Magnesium (µg/ml) concentration in Group-1 were recorded as 0.163±0.002, 0.165±0.003, 0.168±0.004 and 0.170±0.004 on day 0, 21, 42 and 60, respectively. In Group-2 serum Magnesium (µg/ml) were recorded as 0.169±0.002, 0.167±0.002, 0.170±0.002 and 0.174±0.002 on day 0, 21, 42 and 60, respectively. In Group-3 serum Magnesium (µg/ml) were recorded as 0.171±0.002, 0.175±0.002, 0.177±0.002 and 0.179±0.002 on day 0, 21, 42 and 60, respectively. In Group-4 serum Magnesium (µg/ml) were recorded as 0.173±0.001, 0.176±0.002, 0.179±0.002 and 0.183±0.001 on day 0, 21, 42 and 60, respectively. The magnesium level significantly (P≤0.05) varies between day 0 and 60. In Group-5 serum Magnesium (µg/ml) were recorded as 0.175±0.001, 0.179±0.001, 0.182±0.001 and 0.186±0.001 on day 0, 21, 42 and 60, respectively. The magnesium level significantly (P≤0.05) varies between day 0, 21 and 60. Group-4 and 5 have slightly more Magnesium levels comparison to other groups.

The values were within normal range and in close agreement with Das *et al.* (2002) [11], Gowda *et al.* (2001) [16] and Tiwary *et al.* (2007) [59] reported normal level of magnesium in blood serum of animals in different parts of country. The slightly higher level of magnesium than the present study in anestrus condition was observed by Umesh *et al.* (1995) [60] and Dutta *et al.* (2001) [12] as 2.016±0.044 mg % and 2.34±0.14 mg %.

Table 3: Magnesium (µg/ml) estimation during estrus induction by Clomiphene citrate and Co-synch protocol in anestrus cattle (Mean±SE).

Group	Days			
	0	21	42	60
G1	0.163±0.002	0.165±0.003^{a}	0.168±0.004^{a}	0.170±0.004^{a}
G2	0.169±0.002	0.167±0.002^{ab}	0.170±0.002^{ab}	0.174±0.002^{ab}
G3	0.171±0.002	0.175±0.002^{ab}	0.177±0.002^{ab}	0.179±0.002^{ab}
G4	0.173±0.001^{A}	0.176±0.002^{bAB}	0.179±0.002^{bAB}	0.183±0.001^{bB}
G5	0.175±0.001^{A}	0.179±0.001^{bB}	0.182±0.001^{bBC}	0.186±0.001^{bC}

Values with different superscripts in small letter (a, b, c, d) in a column and capital letter (A, B, C, D) in a row differ significantly (P≤0.05).

3.1.4 Mean serum Copper concentration of five groups

The mean concentration of serum Copper from day 0 to 60 in control Group-1 and treatment Groups (2, 3, 4 and 5) have been presented in Table-4. The mean serum Copper (µg/ml)

concentration in Group-1 were recorded as 0.648±0.009, 0.651±0.009, 0.653±0.009 and 0.656±0.009 on day 0, 21, 42 and 60, respectively. In Group-2 serum Copper (µg/ml) were recorded as 0.642±0.009, 0.646±0.01, 0.651±0.009 and 0.657±0.009 on day 0, 21, 42 and 60, respectively. In Group-3 serum Copper (µg/ml) were recorded as 0.646±0.009, 0.652±0.009, 0.655±0.009 and 0.661±0.009 on day 0, 21, 42 and 60, respectively. In Group-4 serum Copper (µg/ml) were recorded as 0.657±0.007, 0.663±0.007, 0.669±0.006 and 0.677±0.006 on day 0, 21, 42 and 60, respectively. In Group-5 serum Copper (µg/ml) were recorded as 0.666±0.009, 0.670±0.008, 0.675±0.008 and 0.683±0.007 on day 0, 21, 42 and 60, respectively. Statistically there was non-significant difference in a level serum copper but Group-5 has slightly more Copper levels comparison to other groups.

The plasma copper levels in anestrus cows were observed by Jain, (1994) [22] (0.98 µg/ml) and Umesh *et al.* (1995) [60] (80.60±8.40 µg/dl). These findings were similar to the findings of present study. However, the level of plasma copper concentrations in anestrus cows were observed by Samanta *et al*. (1995) [51], Tambe *et al.* (1996) [58], Das *et al*. (2002) [11] and Singh *et al*. (2005) [55] as 51.66±5.47 µg/dl, 63.48±2.72 µg/dl, 0.62 ppm and 0.59±0.03 µg/ml respectively. These findings were found to be lower than the findings of present study.

Table 4: Copper (µg/ml) estimation during estrus induction by Clomiphene citrate and Co-synch protocol in anestrus cattle (Mean±SE).

Group	Days			
	0	21	42	60
G1	0.648±0.009	0.651±0.009	0.653±0.009	0.656±0.009
G2	0.642±0.009	0.646±0.01	0.651±0.009	0.657±0.009
G3	0.646±0.009	0.652±0.009	0.655±0.009	0.661±0.009
G4	0.657±0.007	0.663±0.007	0.669±0.006	0.677±0.006
G5	0.666±0.009	0.670±0.008	0.675±0.008	0.683±0.007

3.1.5 Mean serum Zinc concentration of five groups

The mean concentration of serum Zinc from day 0 to 60 in control Group-1 and treatment Groups (2, 3, 4 and 5) have been presented in Table-5. The mean serum Zinc (µg/ml) concentration in Group-1 were recorded as 1.130±0.02, 1.14±0.01, 1.156±0.01 and 1.157±0.01 on day 0, 21, 42 and 60, respectively. In Group-2 serum Zinc (µg/ml) were recorded as 1.146±0.02, 1.165±0.01, 1.171±0.01 and 1.182±0.01 on day 0, 21, 42 and 60, respectively. In Group-3 serum Zinc (µg/ml) were recorded as 1.149±0.02, 1.167±0.01, 1.175±0.01 and 1.183±0.01 on day 0, 21, 42 and 60, respectively. In Group-4 serum Zinc (µg/ml) were recorded as 1.148±0.009, 1.171±0.01, 1.185±0.01 and 1.20±0.003 on day 0, 21, 42 and 60, respectively. The zinc level significantly ($P \leq 0.05$) varies between 0 and 60 days. In Group-5 serum Zinc (µg/ml) were recorded as 1.147±0.02, 1.19±0.007, 1.2±0.002 and 1.22±0.006 on day 0, 21, 42 and 60, respectively. The zinc level significantly ($P \leq 0.05$) varies between 0 and 60 days. Group-4 and 5 have slightly more Zinc levels in comparison to other groups.

The present study corroborates with the finding of Das *et al.* (2002) [11] Statistically, there was non-significant difference in the level of plasma zinc in cyclic and anestrus cows. The findings of this study were in agreement with Dabas *et al.* (1987) [10], Joy and Nair (1995) [23] and Singh *et al.* (2005) [55], they also observed non- significant difference between anestrus and cyclic cows. Chandolia and Verma (1987) [9] reported nearly similar findings in anestrus buffaloes and Jain, (1994) [22] (1.45 µg/ml) in anestrus buffaloes and crossbred cows. Dutta *et al.* (1988) [13], George and Nair (1995) [15], Pradhan *et al.* (1995) [40] and Sharma (1996) [52] reported significantly higher plasma zinc values in anoestrus heifers than in normal cycling cows. He further stated that excess zinc concentration may be considered as cause of infertility in these heifers.

Table 5: Zinc (µg/ml) estimation during estrus induction by Clomiphene citrate and Co-synch protocol in anestrus cattle (Mean±SE).

Group	Days			
	0	21	42	60
G1	1.130±0.02	1.14±0.01	1.156±0.01	1.157 ± 0.01^{a}
G2	1.146±0.02	1.165±0.01	1.171±0.01	1.182 ± 0.01^{ab}
G3	1.149±0.02	1.167±0.01	1.175±0.01	1.183 ± 0.01^{ab}
G4	1.148 ± 0.009^{A}	1.171 ± 0.01^{AB}	1.185 ± 0.01^{AB}	1.20 ± 0.003^{abB}
G5	1.147 ± 0.02^{A}	1.19 ± 0.007^{AB}	1.2 ± 0.002^{B}	1.22 ± 0.006^{bB}

Values with different superscripts in small letter (a, b, c, d) in a column and capital letter in a row differ significantly ($P \leq 0.05$).

3.1.6 Mean serum Cobalt concentration of five groups

The mean concentration of serum Cobalt from day 0 to 60 in control Group-1 and treatment Groups (2, 3, 4 and 5) have been presented in Table-6. The mean serum Cobalt (µg/ml) concentration in Group-1 were recorded as 0.481±0.01, 0.484±0.01, 0.487±0.01 and 0.498±0.01 on day 0, 21, 42 and 60, respectively. In Group-2 serum Cobalt (µg/ml) were recorded as 0.491±0.02, 0.494±0.02, 0.496±0.02 and 0.505±0.02 on day 0, 21, 42 and 60, respectively. In Group-3 serum Cobalt (µg/ml) were recorded as 0.490±0.02, 0.499±0.02, 0.519±0.02 and 0.514±0 on day 0, 21, 42 and 60, respectively. In Group-4 serum Cobalt (µg/ml) were recorded as 0.483±0.01, 0.495±0.01, 0.513±0.01 and 0.533±0.01 on day 0, 21, 42 and 60, respectively. In Group-5 serum Cobalt (µg/ml) were recorded as 0.480±0.01, 0.499±0.01, 0.525±0.01 and 0.560±0.001 on day 0, 21, 42 and 60, respectively. The cobalt level significantly ($P \leq 0.05$) varies between 0 and 60 days.

The serum cobalt concentration in anestrus condition was observed by Vohra *et al.* (1995) [63] and Tambe *et al.* (1996) [58], who reported the cobalt level as 56.67 ± 6.6 µg/dl and 40.0 ± 1.9 µg/dl, respectively. Prasad *et al.* (1989) [41] determined serum levels of cobalt in prolonged post-partum anestrus cows as 1.05 ± 0.3 µg/ml and 0.5 ± 0.1 µg/ml in acyclic cows (smooth ovaries). These findings were found to be lower than the findings of present study. However, in the present study the level of cobalt was lower in anestrus as compare to cyclic heifers (although, to statistically non-significant level) might possibly be a factor for anestrus.

Table 6: Cobalt (µg/ml) estimation during estrus induction by Clomiphene citrate and Co-synch protocol in anestrus cattle (Mean±SE).

Group	Days			
	0	21	42	60
G1	0.481±0.01	0.484±0.01	0.487±0.01	0.498±0.01
G2	0.491±0.02	0.494±0.02	0.496±0.02	0.505±0.02
G3	0.490±0.02	0.495±0.02	0.513±0.02	0.514±0.02
G4	0.483±0.01	0.497±0.01	0.519±0.01	0.533±0.01
G5	0.480 ± 0.01^{A}	0.499 ± 0.01^{A}	0.525 ± 0.01^{AB}	0.560 ± 0.001^{B}

Values with different superscripts in small letter (a, b, c, d) in a column and capital letter in a row differ significantly ($P \leq 0.05$).

3.1.7 Mean serum Selenium concentration of five groups

The mean concentration of serum Selenium from day 0 to 60 in control Group-1 and treatment Groups (2, 3, 4 and 5) have been presented in Table-7. The mean serum Selenium (ppm) concentration in Group-1 were recorded as 0.067±0.01, 0.068±0.012, 0.078±0.01 and 0.081±0.008 on day 0, 21, 42 and 60, respectively. In Group-2 serum Selenium (ppm) were recorded as 0.075±0.01, 0.08±0.01, 0.082±0.01 and 0.84±0.01 on day 0, 21, 42 and 60, respectively. In Group-3 serum Selenium (ppm) were recorded as 0.069±0.01, 0.078±0.01, 0.083±0.01 and 0.091±0.01 on day 0, 21, 42 and 60, respectively. In Group-4 serum Selenium (ppm) were recorded as 0.078±0.01, 0.088±0.01, 0.094±0.01 and 0.12±0.01 on day 0, 21, 42 and 60, respectively. In Group-5 serum Selenium (ppm) were recorded as 0.103±0.01, 0.11±0.01, 0.12±0.005 and 0.13±0.005 on day 0, 21, 42 and 60, respectively. The Selenium level significantly ($P \leq 0.05$) varies between day 0 and 60. On day 60 selenium level significantly ($P \leq 0.05$) varies between groups where Group-5 has high selenium level and Group-1 has low selenium level.

In cattle and sheep selenium deficiency is associated with reduced fertility (Hidiroglou, 1979) [18] and high selenium concentration reduces the incidence of anestrus (Harrison *et al.*, 1984) [17]. (Kommisrud *et al*., 2005) [28] who reported that heifers and dry period cows in Norway are low in blood Se content and there seems to be a positive association between increased blood Se concentration prepartum and decreased incidence of anestrus postpartum. Brasche, (2015) [8] who reported that heifers exhibited no difference in their response to synchronization due to trace mineral containing selenium (Se). Moreover, Makkawi *et al*. (2014) [34] reported that there was no difference in their response to synchronization due to injection vitamin E and selenium in ewe. On other hand, koyuncu and yerlikaya (2007) [29] found that selenium and selenium plus vitamin E treatments had a significant beneficial effect on estrus response in ewes compared to control.

Table 7: Selenium (ppm) estimation during estrus induction by Clomiphene citrate and Co-synch protocol in anestrus cattle (Mean±SE).

Group	Days			
	0	21	42	60
G1	0.067±0.01	0.068±0.01	0.078±0.01	0.081±0.008^{a}
G2	0.075±0.01	0.08±0.01	0.082±0.01	0.084±0.01^{ab}
G3	0.069±0.01	0.078±0.01	0.083±0.01	0.091±0.01^{ab}
G4	0.078±0.01	0.088±0.01	0.094±0.01	0.12±0.01^{ab}
G5	0.103±0.01^{A}	0.11±0.01^{AB}	0.12±0.005^{AB}	0.13±0.005^{bB}

Values with different superscripts in small letter (a, b, c, d) in a column and capital letter (A, B, C, D) in a row differ significantly ($P \leq 0.05$).

3.1.8 Mean serum Iron concentration of five groups

The mean concentration of serum iron from day 0 to 60 in control Group-1 and treatment Groups (2, 3, 4, and 5) have been presented in Table-8. The mean serum Iron (ppm) concentration in Group-1 were recorded as 1.46±0.12, 1.42±0.21, 1.47±0.08, 1.53±0.12 on day 0, 21, 42 and 60, respectively. In Group-2 serum Iron (ppm) were recorded as 1.37±0.17, 1.43±0.12, 1.49±0.11 and 1.61±0.07 on day 0, 21, 42 and 60, respectively. In Group-3 serum Iron (ppm) were recorded as 1.43±0.13, 1.51±0.16, 1.58±0.13 and 1.65±0.17 on day 0, 21, 42 and 60, respectively. In Group-4 serum Iron (ppm) were recorded as 1.41±0.09, 1.65±0.17, 1.78±0.12 and 2.01±0.14 on day 0, 21, 42 and 60, respectively. The iron level significantly ($P \leq 0.05$) varies on day 0 and 60. In Group-5 serum Iron (mcmol/l) were recorded as 1.39±0.15, 1.68±0.11, 1.89±0.15 and 2.15±0.15 on day 0, 21, 42 and 60, respectively. The Iron level significantly ($P \leq 0.05$) varies on day 0 and 60.

The higher plasma iron concentration in anestrus cows than the level observed in present study was reported by Tambe *et al.* (1996) [58], Kalita *et al.* (1999) and Dutta *et al.* (2001b) [12] as 399.0 ± 24.29 µg/dl, 3.34 ± 0.27 ppm and 5.85 ± 0.17 ppm, respectively. However, lower level of plasma iron concentration in anestrus cows was observed by Vadnere and Singh (1989) [61], Yessein *et al.* (1994), Vohra *et al.* (1995) [63] and Ramakrishna (1997) [47] as 194.30 ± 8.35 54 mg/dl, 198.57 ± 7.58 mg/dl, 175.83 ± 7.71 mg/dl and 86.33 ± 6.27 mg/dl, respectively.

Table 8: Iron (ppm) estimation during estrus induction by Clomiphene citrate and Co-synch protocol in anestrus cattle (Mean±SE).

Group	Days			
	0	21	42	60
G1	1.46±0.12	1.42±0.21	1.47±0.08^{a}	1.53±0.12^{a}
G2	1.37±0.17	1.43±0.12	1.49±0.11^{a}	1.61±0.07^{a}
G3	1.43±0.13	1.51±0.16	1.58±0.13^{ab}	1.65±0.17^{ab}
G4	1.41±0.09^{A}	1.65±0.17^{AB}	1.78±0.12^{bAB}	2.01±0.14^{bB}
G5	1.39±0.15^{A}	1.68±0.11^{AB}	1.89±0.15^{bAB}	2.15±0.15^{bB}

Values with different superscripts in small letter (a, b, c, d) in a column and capital letter (A, B, C, D) in a row differ significantly ($P \leq 0.05$).

3.2 Estrus detection and AI

The onset of estrus in cattle was detected by cervico-vaginal discharge and per-rectal examination. In Group-1 (control) out of six, one cow exhibited estrus. In Group-2 out of six, two cows exhibited estrus. In Group-3 out of six, four cows exhibited estrus. In Group-4 out of six, three exhibited estrus. Where as in Group-5 out of six, five cows exhibited estrus. Cows in estrus were artificially inseminated.

3.3 Pregnancy diagnosis

Pregnancy diagnosis in cows of all groups was performed on day 60 post-insemination by per-rectal examination. In Group 1, no animals were found pregnant; in Group 2, one cow was found pregnant; in Group 3, two cows were found pregnant; in Group 4, one cow was found pregnant and in Group 5, three cows were found pregnant.

3.4 Conception Rate

The conception rate of control Group-1 and treatment Groups-2, 3, 4 and 5 during experimental period have been presented in Table no.9. Conception rate was 0% in Group-1. In Group-2 conception rate was 50%, in Group-3 conception rate was 50%, in Group-4 conception rate was 33.33% and in Group-5 conception rate was 60%. Conception rate was higher in Group-5 compared to Groups-1, 2, 3 and 4. The present findings in Clomiphene citrate treated group were lower than the findings recorded by Purohit and Bishnoi (1993) [42] who reported conception rate of 71.42% in anestrus Rathi heifers. While Kurien and Madhavan (1985) [31] and More (2013) [36] reported 42.11 and 60% conception rate in cross bred cow, respectively. Whereas, Kurien and Madhavan (1985) [31] recorded lower conception rate of 25 and 30.30%, respectively than the present study in delayed pubertal cross bred heifers. However, Hukeri *et al.* (1979) [19], Banerjee and Roychoudhary (1989) [4], Kadu and Chede (1992) [24] and

Reddy *et al*. (1994) [48] recorded 80.00, 75, 84.10 and 87.50% conception rate, respectively in anestrus buffaloes treated with clomiphene citrate which was higher than present study.

The conception rate observed by different researchers with co-synch protocol viz. Barolia *et al*. (2016) [5] 66.66%, Amle *et al*. (2015) [2] 60%, Geary *et al*. (1998) [14] 59.0%, Pursley *et al*. (1995) 50.0%, Pursley *et al*. (1997) [44] 35.0% and Melendez *et al*. (2006) found conception rate of 22.7%. The variation in pregnancy rate might be due to age, nutritional status, method of heat detection, type of breeding technique used for heat detection, season etc. In present study, in co-synch protocol the conception rate is somewhat higher than reported earlier by Barolia *et al*. 2016 [5] (66.66%) and similarly reported by Neglia *et al*. (2003) [38] & Baruselli *et al*. (2001) [6].

Table 9: Percent conception rate following AI at induced estrus and over all pregnant animals of Group-1, Group-2, Group-3, Group-4 and Group-5.

Group	No. of animals	No. of animal comes in Estrus	AI is done in animals	Pregnant animals	Conception rate (%)
G1	6	1	1	0	0
G2	6	2	2	1	50%
G3	6	4	4	2	50%
G4	6	3	3	1	33.33%
G5	6	5	5	3	60%

4. Conclusion

Deficiencies of calcium, phosphorous, zinc, copper and selenium either singly or in combination could be responsible for anestrus in cow. By improving the nutritional status, the animal fertility can be improved. Co-synch protocol has higher conception rate (60%) compared to clomiphene citrate (50%). Co-synch protocol along with mineral mixture improved the reproductive status of anestrus Sahiwal cows.

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