Original Research

Whether Different Estrus Synchronization Protocols Modulate Fertility Plasma Progesterone and Metabolic Profile in Repeat Breeder Buffaloes?

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Abstract

This study was undertaken on 50 cyclic repeat breeder buffaloes under field conditions to evaluate the efficacy of four estrus synchronization protocols, viz. Doublesynch, Estradoublesynch, Ovsynch and Ovsynch Plus (10 animals in each protocol, and in control group). The object was to study the conception rates at induced estrus with fix timed AI and to monitor the plasma progesterone, protein and cholesterol profile at different time intervals during treatment and day 12 post-AI. All the animals received pre-synchronization treatment, i.e., Inj. 100 mg ivermectin s/c, Inj. tono-vita 20 ml, single shot Inj. enrofloxacin 40 ml i/m, and multi-minerals bolus 1 daily for 7 days. The conception rates achieved at induced estrus (FTAI) were 40.0, 50.0, 60.0 and 60.0 % with Doublesynch, Estradoublesynch, Ovsynch and Ovsynch Plus protocol, respectively, and 20% in control group. The plasma progesterone concentrations monitored on day 0 (start of treatment), 7/9 ($PGF_2\alpha$ injection), 10/12 (FTAI) and on day 12 post-AI revealed significant ($p<0.01$) effect of sampling days in all four protocols with higher values on day of $PGF_2\alpha$ injection and on day 12 post-AI compared to other days. Moreover, the plasma progesterone concentrations were significantly ($p<0.05$) higher in conceived than the non-conceived buffaloes on day 12 post-AI in all four protocols. The mean plasma protein and cholesterol profile neither varied significantly between days in any of the protocols nor between the protocols, except that the animals under Doublesynch protocol had significantly higher pooled protein value as compared to Ovsynch and Ovsynch Plus protocols. The conceived buffaloes had significantly ($p<0.05$) lower mean values of plasma protein under Ovsynch protocol (5.83±0.10 vs. 6.28±0.09 g/dl) and cholesterol under Estradoublesynch protocol (63.47±1.86 vs. 73.52±2.34 mg/dl) than their counterparts. It was concluded that all four hormonal protocols modulated plasma progesterone profile and improved conception rates in cyclic buffaloes without altering plasma protein and cholesterol profile, the maximal benefit was with Ovsynch and Ovsynch Plus protocols.

Key words: Buffalo, Conception Rate, Estrus Synchronization, Plasma Profile, Repeat Breeder

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Introduction

Repeat breeding (RB) is a challenging and frustrating reproductive disorder in dairy animals. Number of hormonal and non-hormonal strategies has been tested to handle this problem in cattle and buffaloes (Dhami *et al.*, 2009; Savalia *et al.*, 2014; Butani *et al.*, 2016). Periodic rectal palpation of genitalia, use of ultrasonography, cultural examination of vaginal mucus discharge, uterine biopsy and plasma progesterone (P4) assay may delineate some of the unapparent etiological factors of RB (Patel *et al.*, 2010). Inefficient estrus detection, ovulatory disorders, wrong time and site of insemination in relation to ovulation or compromised semen quality could be the important causes of this problem in bovines (Dhami *et al.*, 2009; Savalia *et al.*, 2014). The females with silent ovulation, irregular cycles or embryonic mortality will show irregular levels of P4. The level of nutrition and steroidogenic precursors such as protein and cholesterol may also alter the reproductive efficiency. Very few studies are available in literature, wherein mid-cycle prostaglandin F2 alpha (PGF2α) or estrus synchronization protocols with fixed time AI have been used successfully as a means of treating RB in bovines (Savalia *et al.*, 2014; Parmar *et al.*, 2015; Dhami *et al.*, 2015; Prajapati *et al.*, 2018[a,b]). Hence, this study was planned to evaluate the efficacy of different estrus synchronization protocols for improving conception rates together with monitoring the plasma P4 and biochemical profile in RB buffaloes under field conditions.

Material and Methods

The investigation was carried out during August 2016 to February 2017 on 50 cyclic (RB) buffaloes (>120 days postpartum) under field conditions of Anand as well as Mahisagar districts in Gujarat, following approval of protocols by the Institutional Animal Ethics Committee (IAEC). The animals were confirmed to be repeat breeders on the basis of history of failure of conception with regular cyclicity and quality AI for 3 or more times, without visible or palpable genital abnormality. All these infertile animals were given pre-synchronization treatment once with i/m Inj. Ivermectin, organic phosphorus, multivitamins AD_3E and a single shot injection of Enrofloxacin with multi-minerals bolus 1 daily for 7 days. The animals were then randomly allotted to 5 equal groups (n=10 each) and were subjected to four ovulation synchronization protocols, viz., Ovsynch, Ovsynch Plus, Doublesynch, and Estradoublesynch, keeping one group as untreated control (Prajapati *et al.*, 2018[a]) as detailed below.

In Doublesynch protocol, 10 cyclic RB buffaloes were administered with i/m Inj. of 500 µg $PGF_2\alpha$ analogue, i.e. Cloprostenol sodium (Estrumate, 2 ml, MSD) on day 0, Inj. of 20 µg GnRH analogue, i.e. Buserelin acetate (Receptal, 5 ml, MSD) on day 2, 500 µg $PGF_2\alpha$ analogue on day 9 and second Inj. 10 µg GnRH on day 11, followed by fix timed AI (FTAI) twice at 16 and 24 hrs later. In Estradoublesynch protocol, injection of estradiol benzoate 1 mg (Sigma, USA) on day 10 replaced the second GnRH on day 11 of Doublesynch protocol, and animals were inseminated twice at 48 and 60 hrs post-estradiol injection. In Ovsynch protocol,

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10 cyclic RB buffaloes were administered with i/m Inj. of 20 µg GnRH analogue (Receptal, 5 ml) on day 0, Inj. of 500 µg $PGF_2\alpha$ on day 7, and second Inj. of 10 µg GnRH on day 9, followed by FTAI twice at 0 and 24 hrs later, while in Ovsynch Plus protocol, an additional i/m Inj. of PMSG 500 IU (Folligon, MSD) was given 2 days before followed by standard Ovsynch protocol. Ten cyclic RB buffaloes each given pre-synchronization treatment as above, but without any hormonal intervention, and followed for spontaneous estrus and insemination, served as untreated controls. All the animals inseminated at induced or spontaneous estrus, if not returned to estrus, were followed for pregnancy diagnosis per-rectum 60 days post-AI. Animals found pregnant with FTAI were considered as conceived and those repeated to estrus or found non-pregnant on palpation were considered non-conceived and their blood profile was compared.

Blood sampling was carried out in heparinized vacutainers just before treatment (day 0), at the time of $PGF_2\alpha$ administration (day 7/9 as per protocol), induced estrus/FTAI (day 10/12) and on day 12 post-AI for determination of plasma P4 concentration by RIA technique of Kubasic *et al.* (1984) and plasma total protein and cholesterol concentrations by Biuret and CHOD/PAP methods, respectively, using assay kits of Crest Bio-system, Goa with the help of chemistry analyzer. The data on plasma profiles were analyzed statistically using standard statistical procedure, i.e. ANOVA and DMRT for comparing the mean differences between periods and between protocols, and t-test was used for comparing conceived-nonconceived groups on IBM SPSS software version 20.00 (Snedecor and Cochran, 1986).

Results and Discussion

Conception Rates at Induced Estrus/FTAI

Repeat breeder buffaloes, 10 each, subjected to Doublesynch, Estradoublesynch, Ovsynch and Ovsynch Plus protocols exhibited behavioural estrus within 48 to 80 hrs from last $PGF_2\alpha$ injection with estrus response of 90, 100, 100 and 100 %, respectively. The results with Doublesynch and Estradoublesynch protocols concurred with the earlier reports of Miramahmoudi and Prakash (2012), Miramahmoudi *et al.* (2014) and Parida *et al.* (2015) in cyclic buffaloes. The estrus response with Ovsynch and Ovsynch Plus protocols were in harmony with the results of Tiwari *et al.* (2005); Karena and Darwish (2010); Savalia *et al.* (2014); Parmar *et al.* (2016) and Patel *et al.* (2018) in cyclic/acyclic buffaloes.

The conception rates achieved at induced estrus/FTAI in cyclic buffaloes following use of Doublesynch, Estradoublesynch, Ovsynch and Ovsynch plus protocols were 40, 50, 60 and 60%, respectively, while in untreated control group it was only 20%. The present conception rate by FTAI with Doublesynch protocol in repeat breeder buffaloes is far higher than earlier report of 28.57 % by Abubaker *et al.* (2013) in lactating crossbred cows. Mirmahmoudi and Prakash (2012), Mirmahmoudi *et al.* (2014) and Parida *et al.* (2015), however, obtained conception rates of 55-62 % at FTAI in cyclic buffaloes with this protocol, which are higher than our findings. The present conception rate of 50 % obtained at FTAI following Estradoublesynch

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protocol was quite similar with earlier results of 60-62 % in cyclic buffaloes by Mirmahmoudi *et al* (2014). The conception rate obtained under Ovsynch protocol (60 %) was quite higher than the results of 21.42 to 40.00 % obtained by Tiwari *et al.* (2005), Savalia *et al.* (2014), Dhami *et al.* (2015) and Parmar *et al.* (2015) in repeat breeder buffaloes. Similarly, the conception rate following Ovsynch Plus protocol concurred with Patel *et al.* (2018) and Prajapati *et al.* (2018[a]) in cyclic/acyclic buffaloes. The results in general were better with Ovsynch and Ovsynch Plus protocols than Doublesynch and Estradoublesynch protocols in repeat breeder buffaloes.

Plasma Progesterone Profile

The plasma P4 levels on the day of initiation (day 0) of Doublesynch, Estradoublesynch, Ovsynch and Ovsynch plus protocols in cyclic RB buffaloes showed luteal activity of varying degrees in all groups. These levels further rose significantly ($p<0.01$) to the peak values on day 7/9, i.e. just before $PGF_2\alpha$ injection. The levels also differed significantly with lower ($p<0.05$) value in Ovsynch Plus and higher in Estradoublesynch protocol, while the values of other two protocols were intermediary (Table 1). Thereafter the mean P4 concentration dropped suddenly and significantly within 48 h to the basal values coincident to induced estrus, when FTAIs were carried out. The concentrations again increased significantly ($p<0.01$) on day 12 post-AI in all the groups with values of 3.75±0.42, 3.82±0.52, 3.95±0.66 and 3.03±0.42 ng/ml with development and maintenance of CL suggestive of ovulatory induced estrus in treated animals (Table 1).

Table 1: Plasma progesterone concentrations (ng/ml) in repeat breeding buffaloes during different days of various estrus synchronization protocols and on day 12 post-AI

Estrus Synchronization Protocol	Status	No.	Plasma progesterone on days from treatment/AI			
			D-0	Day of $PGF_2\alpha$ Inj.	Day FTAI	D-12 post-AI
Doublesynch	Conceived	4	0.78±0.67	2.67±0.748	0.20±0.07	4.97±0.31^{y}
	Non-concd	6	2.33±0.71	1.46±0.19	0.30±0.18	2.93±0.41^{x}
	Overall	**10**	**1.71±0.54^{q}**	**1.95±0.35^{qab}**	**0.26±0.10^{p}**	**3.75±0.42^{r}**
Estra-doublesynch	Conceived	5	1.33±0.64	3.23±0.41	0.18±0.02	4.21±0.67
	Non-concd	5	1.52±0.71	2.45±0.91	0.57±0.42	3.04±0.79
	Overall	**10**	**1.43±0.45^{p}**	**2.84±0.49^{qb}**	**0.37±0.21^{p}**	**3.82±0.52^{q}**
Ovsynch	Conceived	6	0.82±0.41	2.75±0.47^{y}	0.38±0.06	5.10±0.49^{y}
	Non-concd	4	1.32±0.70	0.95±0.56^{x}	0.55±0.12	2.24±1.04^{x}
	Overall	**10**	**1.02±0.36^{pq}**	**2.03±0.45^{qab}**	**0.44±0.06^{p}**	**3.95±0.66^{r}**
Ovsynch plus	Conceived	6	1.77±0.71	1.62±0.38	0.16±0.35	3.16±0.69
	Non-concd	4	0.73±0.36	1.48±0.15	0.50±0.33	2.82±0.34
	Overall	**10**	**1.35±0.46^{q}**	**1.56±0.22^{qa}**	**0.29±0.13^{p}**	**3.03±0.42^{r}**

D-0 = Starting the treatment. Means bearing uncommon superscripts within the column differ significantly (P<0.05) between protocols (a,b) or status (x,y), and those within the row (p,q,r) differ between days.

The rise in mean plasma P4 levels noted in cyclic buffaloes under all four protocols on day 7/9 over initial values might be due to luteinization of some of the growing/dominant follicles and/or ovulation of dominant

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follicle and formation of CL, under the influence of first GnRH injection. The sudden drop in plasma P4 on the day of FTAI in all the groups could be attributed to luteolytic effect of $PGF_2\alpha$ injection given 2 days before, on the luteinized follicles and/or CLs, with emergence of next wave of follicles. Moreover, second GnRH injection or the exogenous estradiol benzoate used in Estradoublesynch protocol, probably helped by positive feedback effect on pituitary and hypothalamus in triggering the ovulatory LH surge and thereby better synchronized ovulation in treated animals (Mirmahmoudi *et al.*, 2014; Prajapati *et al.*, 2018[b]). The mean plasma P4 concentrations in conceived and non-conceived animals on day 0, day 7/9 (before $PGF_2\alpha$ injection) or on day of FTAI did not differ significant ($p>0.05$) in any of the protocols, except in Ovsynch on the day of $PGF_2\alpha$ injection, where it was significantly ($P<0.05$) higher in conceived buffaloes (2.75± 0.47 vs. 0.95±0.56 ng/ml), and this could be the reason for better synchrony and fertility in this group. The non-conceived animals (n=4) of Ovsynch group did not respond to first GnRH treatment and hence there was significantly low plasma P4 on day 7 in absence of luteal activity. However, on day 12 post-AI, the conceived buffaloes in all four protocols had higher mean plasma P4 concentrations as compared to non-conceived ones with significant ($p<0.05$) difference in Doublesynch (4.97±0.31 vs. 2.93±0.41 ng/ml) and Ovsynch (5.10±0.49 vs. 2.24±1.04) protocols only (Table 1). In earlier study, the plasma P4 levels above 3.0 ng/ml were reported between days 12 and 22 post-breeding in conceived buffaloes (Sarvaiya *et al.*, 1991; Butani *et al.*, 2016).

The present overall trend and concentrations of plasma P4 recorded on different days of treatment and day 12 post-AI and higher values in conceived than non-conceived animals on day of $PGF_2\alpha$ injection and on day 12 post-AI closely concurred with many previous reports in cyclic/acyclic buffaloes following various estrus synchronization protocols (Mirmahmoudi and Prakash, 2012; Savalia *et al.*, 2014; Dhami *et al.*, 2015; Parmar *et al.*, 2015; Patel *et al.*, 2018 and Prajapati *et al.*, 2018[a]), proving that P4 dominated animals respond better to $PGF_2\alpha$ injection and also exhibit well synchronized ovulatory estrus with better conception rate compared to those having poor luteal activity.

Plasma Total Protein

The plasma protein levels neither varied significantly between days in any of the protocol/group nor between the protocols. The conceived buffaloes had significantly ($p<0.05$) lower plasma total protein concentrations as compared to non-conceived ones in Ovsynch group on day 0 (5.59±0.13 vs. 6.21±0.16 g/dl), but no such differences were noted in other protocols. The inconsistent trend and the overall mean plasma total protein values observed in different protocols under study were in close agreement with the earlier observations made by different researchers (Savalia *et al.*, 2014; Parmar *et al.*, 2016; Patel *et al.*, 2018; Prajapati *et al.*, 2018[b]) in repeat breeder bovines.

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Table 2: Plasma total protein concentrations (g/dl) in repeat breeding buffaloes during different days of various estrus synchronization protocols and on day 12 post-AI

Estrus Synchronization Protocol	Status	No.	Plasma protein on days from treatment/AI			
			D-0	Day of $PGF_2\alpha$ Inj.	Day FTAI	D-12 post-AI
Doublesynch	Conceived	4	6.10±0.32	6.16±0.31	6.45±0.21	6.51±0.25
	Non-concd	6	6.26±0.28	6.23±0.27	6.40±0.23	6.42±0.23
	Overall	**10**	**6.19±0.20**	**6.20±0.19**	**6.42±0.15**	**6.45±0.16**
Estra-doublesynch	Conceived	5	6.14±0.12	6.30±0.17	6.29±0.11	6.32±0.25
	Non-concd	5	5.93±0.06	6.06±0.18	6.25±0.21	6.22±0.17
	Overall	**10**	**6.03±0.07**	**6.18±0.12**	**6.27±0.11**	**6.27±0.14**
Ovsynch	Conceived	6	5.59 ± 0.13^{x}	5.82±0.24	5.71±0.20	6.08±0.26
	Non-concd	4	6.21 ± 0.16^{y}	6.11±0.18	6.36±0.30	6.45±0.09
	Overall	**10**	**5.90±0.13**	**5.94±0.16**	**5.97±0.19**	**6.23±0.17**
Ovsynch plus	Conceived	6	6.02±0.24	5.99±0.21	6.35±0.14	5.89±0.22
	Non-concd	4	5.95±0.18	5.99±0.22	6.04±0.31	6.46±0.47
	Overall	**10**	**5.87±0.17**	**5.99±0.14**	**6.22±0.15**	**6.12±0.23**

D-0 = Starting the treatment. Means of protocol bearing uncommon superscripts within the column for Conceived & Non-conceived (x,y) subgroups differ significantly (P < 0.05).

These results proved that the plasma total protein content is not influenced significantly by the hormonal treatments used in dairy animals, and that higher plasma total protein levels are indicative of better nutritional status/nitrogen balance, which favours the sound reproductive performance in animals. Kavani *et al.* (1987) and Butani *et al.* (2011, 2016) observed significantly ($p<0.01$) lower concentration of total serum protein in infertile as compared to normal cycling/conceiving animals, which could have resulted from inadequate energy supply and output of protein hormones from gonads and pituitary gland.

Plasma Total Cholesterol

The total cholesterol concentration of cyclic (RB) buffaloes did not differ significantly between sampling days in any of the protocols/groups, but variation between the protocols was significant on all days, with higher values in Doublesynch and Estradoublesynch than in Ovsynch and Ovsynch Plus protocols. This is quite obvious as the animals treated were all cyclic RBs with different nutritional and reproductive status. Moreover, the conceived buffaloes had lower mean plasma total cholesterol concentrations as compared to non-conceived ones in Estradoublesynch group at all intervals with significant ($p<0.05$) differences on day 0 and day 12 post-AI, but no such differences were found in other three protocols (Table 3). Similar observations were made by Savalia *et al.* (2014) in buffaloes. As compared to present findings, relatively higher plasma total cholesterol levels were documented by Butani *et al.* (2011), Savalia *et al.* (2014), Parmar *et al.* (2016) and Patel *et al.* (2018) in cyclic and acyclic buffaloes. No more published report could be found in the literature on plasma total cholesterol in cyclic (RB) buffaloes following synchronization protocols followed in our study to discuss the variability of results.

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Table 3: Plasma total cholesterol concentrations (mg/dl) in repeat breeding buffaloes during different days of various estrus synchronization protocol and on day 12 post-AI

Estrus Synchronization Protocol	Status	No.	Plasma cholesterol on days from treatment/AI			
			D-0	Day of $PGF_2\alpha$ Inj.	Day FTAI	D-12 post-AI
Doublesynch	Conceived	4	70.71±4.99	68.62±3.79	76.30±5.89	78.45±6.13
	Non-concd	6	70.59±5.26	71.76±6.02	68.81±5.45	77.24±4.21
	Overall	**10**	**70.64±3.54^{a}**	**70.51±3.77^{ab}**	**71.80±4.00**	**77.72±3.31^{ab}**
Estra-doublesynch	Conceived	5	59.07±2.32^{x}	60.24±4.25	68.30±3.92	66.25±3.47^{x}
	Non-concd	5	69.33±3.09^{y}	71.46±5.54	74.08±5.89	79.24±3.94^{y}
	Overall	**10**	**64.20±2.50^{a}**	**65.85±3.78^{a}**	**71.19±3.47**	**72.74±3.28^{a}**
Ovsynch	Conceived	6	84.96±4.27	77.96±4.75	81.11±5.69	77.33±7.4
	Non-concd	4	80.84±5.33	78.12±3.46	81.83±5.75	84.30±1.73
	Overall	**10**	**83.31±3.21^{b}**	**78.02±3.02^{bc}**	**81.40±3.90**	**80.12±4.47^{ab}**
Ovsynch plus	Conceived	6	81.38±2.15	84.19±4.35	80.21±5.60	85.15±5.72
	Non-concd	4	86.19±2.91	83.77±6.63	83.87±4.65	87.95±4.49
	Overall	**10**	**83.30±1.81^{b}**	**84.02±3.49^{c}**	**81.68±3.70**	**86.27±3.71^{b}**

D-0 = Starting the treatment. Means of protocol bearing uncommon superscripts within the column (a,b) and Conceived &Non-conceived (x,y) subgroups differ significantly ($p<0.05$).

Relatively poor conception rates obtained in animals under Doublesynch and Estradoublesynch protocols could be due to their lower plasma total cholesterol concentration, the precursor of steroid hormones required for synthesis of estrogen and progesterone compared to other two protocols. However, this view needs to be authenticated through further studies involving different parity of animals. Further, the pre-synchronization treatments given in all RB animals was presumed to improve the nutritional status and control the invisible genital infection, if any, which might help to achieve better estrus response/synchrony and conception (Dhami *et al.*, 2009, 2015).

Conclusion

From the results it was concluded that all four hormonal protocols modulated plasma P4 profile and improved conception rates in cyclic buffaloes without altering plasma protein and cholesterol profile. The maximal benefit was with Ovsynch and Ovsynch Plus protocols, hence the same may be adopted by the practicing field Veterinarians to induce ovulatory estrus and reduce calving intervals in repeat breeder buffaloes.

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