B-4333
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RESEARCH ARTICLE Indian Journal of Animal Research

Presynch-Heatsynch: A New Approach to Improve Ovarian and Fertility Responses in Water Buffalo (*Bubalus bubalis*)

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10.18805/IJAR.B-4333

ABSTRACT

Background: Standard estrus synchronization regimens resulted in variable outcomes in buffaloes. The present study evaluated ovarian and fertility responses following presynchronization in Heatsynch protocol administered in postpartum buffaloes.

Methods: In group-I (Presynch-Heatsynch group, n = 30), $PGF_2\alpha$ was administered on days -14 and -2. Then, GnRH analogue, $PGF_2\alpha$ (Cloprostenol) and Estradiol benzoate were administered on day 0, 7 and 8, respectively followed by fixed-time artificial insemination (FTAI) 48 hours later. In group-II (Heatsynch group, n = 30), rest protocol was same, except that first two $PGF_2\alpha$ injections were not administered.

Result: The progesterone concentrations differed (P<0.01) between the two groups on days -2, 0 and 7. Post-treatment, progesterone profiles were also higher in pregnant compared to non-pregnant buffaloes in both the groups except on day 10. CL diameter differed (P<0.01) between groups on days -2, 0 and 7. It was larger in pregnant than non-pregnant buffaloes on day 7 in both the groups. Dominant follicle diameter remained larger on days -2, 0 and 8 in group-I than -II. Buffaloes getting pregnant had a larger (P<0.01) dominant follicle size on the day of FTAI in group-I than of group-II. Ovulatory response of 93.33 and 90.00% was observed in group-I and II. The conception rate was higher (66.66 vs. 40.00%; P<0.05) in group-I than Group-II. Presynchronization improved reproductive efficiency in Heatsynch treatment and may aid for better fertility in buffaloes.

Key words: Buffalo, Estrus synchronization, Fertility, Heatsynch, Presynchronization.

INTRODUCTION

Buffalo is the backbone of dairy industry in India that contributes >55% to the total national milk production. Low reproductive efficiency is considered as a major constraint in dairy buffalo farming. Untimely artificial insemination (AI) leading to decline in conception rate and increase in the calving interval is a major problem in progress of the buffalo dairy industry. Application of estrus synchronization treatments in buffalo provides workable strategy to increase their productive period as these regimens eliminate the problem of estrus detection. A number of these protocols employed in buffalo had achieved partial success with variable results (Ghuman *et al*., 2008).

Heatsynch protocol has been developed that makes use of a combination of GnRH-$PGF_2\alpha$-estradiol benzoate injection followed by FTAI (Fernandes *et al*., 2001; Pancarci *et al*., 2002 and Mohan and Prakash *et al.*, 2014). The major advantages of Heatsynch protocol are reduced hormone costs, increased estrus intensity, and easy in scheduling and implementation. Certain limitations of Ovsynch and Heatsynch protocols in buffaloes are reduced pregnancy rates ranging from 20 to 40% were reported in earlier studies (Mohan and Prakash *et al.*, 2014 and Singh *et al.*, 2017). It is well established that ovulation of follicle in response to first GnRH injection of any GnRH-based program is a prerequisite for its success (Thatcher *et al.*, 2002 and Cirit *et al.*, 2007). However, at certain stages of the estrus cycle, first GnRH dose of Ovsynch/Heatsynch treatment failed to ovulate the follicle leading to reduced conception (Geary *et al.*, 2000; Ozturk *et al.*, 2010 and Mirmahmoundi *et al.*, 2014).

Presynchronization of estrus prior to incorporation of actual GnRH based program may increase pregnancy rates in bovines (Moreira *et al.*, 2001; El-Zarkouny *et al.*, 2004 and Navanukraw *et al.*, 2004). The conventional method of presynchronization involves administration of two $PGF_2\alpha$ injections at 11 to 14 days apart, with the last injection given 14 days before initiation of any GnRH based breeding protocol (Moreira *et al.*, 2000). The major draw back of this method is long duration of protocol and subsequently

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How to cite this article: Sandhu, H.S., Dhindsa, S.S., Singh, H., Honparkhe, M., Singh, P. (2021). Presynch-Heatsynch: A New Approach to Improve Ovarian and Fertility Responses in Water Buffalo (*Bubalus bubalis*). Indian Journal of Animal Research. DOI: 10.18805/IJAR.B-4333.

Submitted: 15-10-2020 **Accepted:** 24-05-2021 **Online:** 11-06-2021

prolonged service period and the calving interval. Hence, development of short duration pesynchronization method is warranted. However, presynchronization of estrus with double PGF$_2$α injections before Heatsynch (Presynch-Heatsynch) has neither been reported in buffaloes nor in cattle. The evaluation of the economical alternative protocols to improve the conception in buffaloes and clear doubts associated with fixed time AI (Singh *et al*., 2006) are warranted. Hence, it was hypothesized that the presynchronization would lead to enhanced submission rate with shorter calving intervals. Thus, the present study was taken up to evaluate the ovarian activity and fertility following modified Heatsynch protocol by addition of two PGF$_2$α injections (12 days apart, last PGF$_2$α injection given two days prior to first GnRH) to shorten the duration of the protocol.

MATERIALS AND METHODS

Experimental animal

The experiment was conducted in 2016-17 on post-partum (> 60 days in milk) cyclic buffaloes *(Bubalus bubalis)* maintained at dairy farm of Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India. Buffaloes with history of any pre- and post-partum reproductive and or metabolic issues were excluded. The selected buffaloes had body condition score of 2.5 to 3.5 (Edmondson *et al.*, 1989). Cyclicity of buffaloes was confirmed before enrolling into the experiment by transrectal ultrasonography. The buffaloes were managed in a semi-intensive loose housing system and identified with ear tags.

Experimental design

The sixty buffaloes enrolled were randomly allocated into two groups and were subjected to experimental protocol as depicted in Fig 1 (Group-I, Presynch-Heatsynch group, n = 30; Group-II, Heatsynch group, n = 30). Real time B-mode ultrasonography using 7.5 MHz frequency trans-rectal transducer (Z5, Shenzhen Mindray Biomedical Electronics Co. Ltd., Germany) was carried out to record the number and diameter of dominant follicle (DF) and corpus luteum (CL).

Plasma was harvested by centrifugation (3000 rpm, 15 min) from the blood samples taken by jugular venipucture and stored in aliquots at -20°C till further progesterone (P$_4$) and estradiol (E$_2$) estimation by Enzyme Immunoassay kits (Arbor Assays, Michigan, USA). This study was duly approved by the Institutional Animal Ethics Committee.

Statistical analysis

The Chi-square test was employed to compare ovulation and conception rates. Data pertaining to P$_4$, E$_2$, DF and CL profiles was analyzed using Unpaired t-test to compare between groups and One way ANOVA to evaluate the effect within the group.

RESULTS AND DISCUSSION

The plasma P$_4$ and E$_2$ concentrations, diameter (Mean ± SEM) of CL and DF on different days of intervention; and comparative analysis of these parameters based on subsequent pregnancy status of group-I (Presynch-

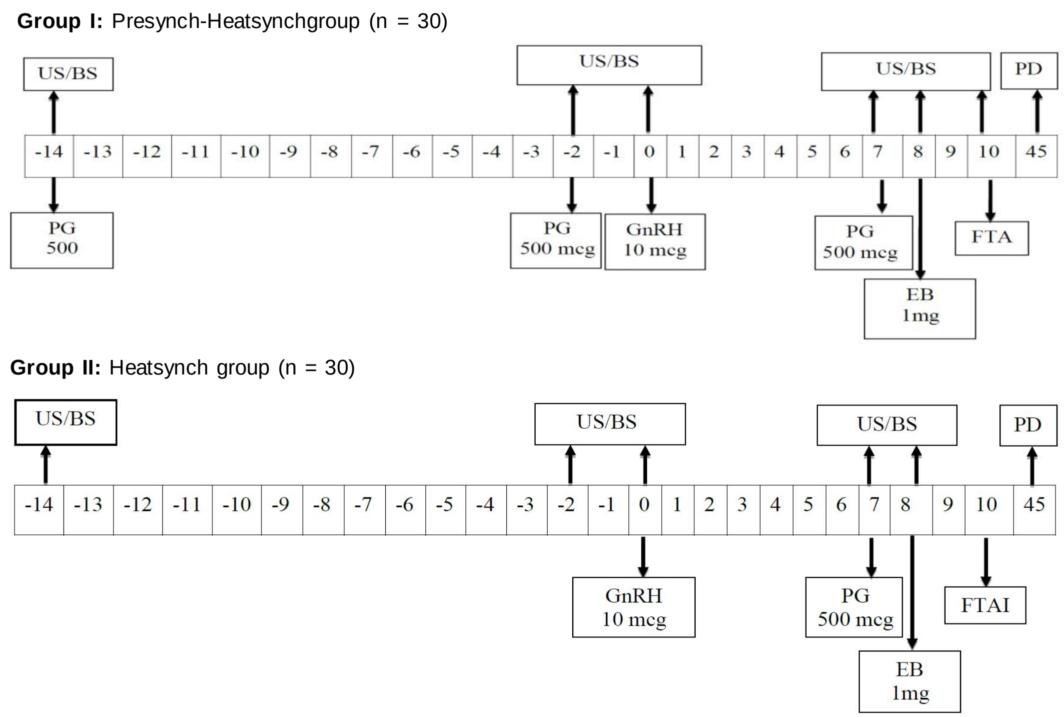

Fig 1: Protocols of estrus synchronization treatments employed in buffaloes.

PG- Prostaglandin F$_{2α}$; GnRH- Gonadotrophin Releasing Hormone; EB-Estradiol Benzoate; BS-Blood Sample; US- Ultrasound Examination; FTAI- Fixed time artificial insemination.

Heatsynch) and group-II (Heatsynch group) have been presented in (Tables 1 and 2), respectively.

Effect on ovarian parameters

Dominant follicle diameter

The diameter of DF on day -2 as well as on day 0 remained larger (P<0.05) in buffaloes of group-I than of group-II (Fig 3). Furthermore, DF diameter on the day of EB injection (P<0.05) and FTAI (P<0.01) in group-I remained higher compared to group-II buffaloes. Within each group, size of DF during second follicular wave (that emerged after induction of ovulation by GnRH injection on day 0) increased (P<0.05) by the time of FTAI after $PGF_2\alpha$ on day 7 in both groups (Group-I: 10.21±0.25 to 15.04±0.35; Group-II: 10.01±0.29 to 12.81±0.31 mm, respectively).

However, in group-I, DF diameter was larger (P<0.05) on the day of first GnRH injection in buffaloes that became pregnant compared to non-pregnant buffaloes *i.e.* 9.54±0.35 versus (vs.) 8.18±0.53 mm, respectively. Likewise, DF diameter on rest of the days (Day 7, 8 and 10) during ovulatory wave remained larger (P<0.01) in buffaloes that became pregnant than non-pregnant (Table 1). Similarly in group-II, DF diameter was larger on the day 0 (P<0.05), days 8 and 10 (P<0.01) in pregnant compared to non-pregnant buffaloes (Table 2).

Corpus luteum diameter

The diameter of CL on commencement of the study (day -14; 9.66±0.6 vs. 8.38±0.54 mm) did not differ (P>0.05) between the groups. However, CL diameter differed (P<0.01) between the two groups on days -2, 0 and 7. Diameter of CL decreased abruptly (P<0.05) after $PGF_2\alpha$ injection on day -2 (Group-I) and day 7 (Group-II) (Fig 2).

In Group-I, CL diameter remained significantly (P<0.01) larger in pregnant buffaloes on all the days of three $PGF_2\alpha$

Table 1: Different parameters in relation to pregnancy in buffaloes subjected to Presynch-Heatsynch protocol.

Fertility status	Day -14	Day -2	Day 0	Day 7	Day 8	Day 10
Diameter of dominant follicle (mm)						
P	7.74 ± 0.21^{a}	7.64 ± 0.19^{a}	$9.54 \pm 0.35^{*b}$	$10.73 \pm 0.26^{**c}$	$13.18 \pm 0.20^{**d}$	$16.13 \pm 0.25^{**e}$
NP	7.28 ± 0.18^{a}	7.05 ± 0.15^{a}	$8.18 \pm 0.53^{*ab}$	$9.17 \pm 0.39^{**b}$	$12.09 \pm 0.28^{**d}$	$12.86 \pm 0.35^{**d}$
Diameter of corpus luteum (mm)						
P	$11.32 \pm 0.24^{**b}$	$11.44 \pm 0.20^{**b}$	3.34 ± 0.11^{a}	$13.96 \pm 0.28^{**c}$	$3.51 \pm 0.09^{**a}$	3.01 ± 0.11^{a}
NP	$6.36 \pm 0.14^{**c}$	$9.00 \pm 0.18^{**d}$	4.17 ± 0.55^{ab}	$9.96 \pm 0.31^{**d}$	$4.50 \pm 0.23^{**b}$	3.23 ± 0.13^{a}
Plasma estradiol concentration (pg/ml)						
P	$12.54 \pm 0.44^{**a}$	11.93 ± 0.64^{a}	24.14 ± 1.158^{b}	21.26 ± 2.70^{b}	$39.45 \pm 2.21^{*c}$	46.46 ± 1.93^{c}
NP	$19.9 \pm 3.31^{**ab}$	14.18 ± 1.01^{a}	24.22 ± 2.74^{bc}	16.78 ± 1.40^{ab}	$30.10 \pm 1.80^{*c}$	45.26 ± 2.64^{d}
Plasma progesterone concentration (ng/ml)						
P	$1.85 \pm 0.11^{**c}$	$2.22 \pm 0.08^{**c}$	0.56 ± 0.05^{a}	$3.18 \pm 0.14^{**d}$	$1.15 \pm 0.08^{**b}$	$0.20 \pm 0.01^{**a}$
NP	$1.05 \pm 0.13^{**bc}$	$0.98 \pm 0.07^{**bc}$	0.40 ± 0.07^{a}	$1.48 \pm 0.20^{**c}$	$0.53 \pm 0.07^{**ab}$	$0.42 \pm 0.05^{**a}$

Values bearing different superscripts along the row differ significantly (P < 0.05).

* and **: Significantly different within the column (P < 0.05) and (P < 0.01), respectively.

P = Pregnant buffaloes (n = 20); NP: Non-pregnant buffaloes (n = 10).

Table 2: Different parameters in relation to pregnancy in buffaloes subjected to Heatsynch treatment.

Fertility status	Day -14	Day -2	Day 0	Day 7	Day 8	Day 10
Diameter of dominant follicle (mm)						
P	$9.48 \pm 0.56^{**b}$	7.00 ± 0.34^{a}	$8.94 \pm 0.47^{*b}$	10.69 ± 0.42^{b}	$12.81 \pm 0.34^{**c}$	$14.36 \pm 0.32^{**c}$
NP	$5.37 \pm 0.27^{**a}$	6.12 ± 0.37^{ab}	$7.41 \pm 0.32^{*b}$	9.55 ± 0.37^{c}	$10.11 \pm 0.52^{**c}$	$11.77 \pm 0.28^{**d}$
Diameter of corpus luteum (mm)						
P	$11.55 \pm 0.50^{**e}$	$7.45 \pm 0.17^{*c}$	$9.90 \pm 0.37^{**d}$	$12.64 \pm 0.41^{**e}$	4.24 ± 0.26^{b}	$2.53 \pm 0.12^{**a}$
NP	$6.27 \pm 0.29^{**b}$	$6.22 \pm 0.23^{*b}$	$5.88 \pm 0.25^{**b}$	$8.51 \pm 0.35^{**c}$	4.74 ± 0.23^{a}	$3.94 \pm 0.21^{**a}$
Plasma estradiol concentration (pg/ml)						
P	18.64 ± 0.50	11.38 ± 0.52	15.5 ± 1.41	17.11 ± 1.33	39.45 ± 2.95	42.68 ± 3.06
NP	8.36 ± 0.30	9.30 ± 0.34	13.67 ± 0.81	15.17 ± 1.25	28.52 ± 2.14	38.06 ± 2.51
Plasma progesterone concentration (ng/ml)						
P	$2.61 \pm 0.14^{**d}$	$0.92 \pm 0.04^{*b}$	$2.98 \pm 0.18^{**d}$	$3.10 \pm 0.18^{**d}$	$1.50 \pm 0.10^{*c}$	$0.19 \pm 0.01^{**a}$
NP	$0.35 \pm 0.04^{**a}$	$0.68 \pm 0.04^{*ab}$	$0.61 \pm 0.07^{**ab}$	$0.91 \pm 0.09^{**b}$	$0.86 \pm 0.15^{*b}$	$0.59 \pm 0.08^{**ab}$

Values bearing different superscripts in a row differ significantly (P<0.05).

* and **: Significantly different within the column (P<0.05) and (P<0.01), respectively.

P = Pregnant buffaloes (n = 12); NP: Non-pregnant buffaloes (n=18).

injections compared to their non-pregnant counterparts (Table 1). In trend to the serum P_4 profiles, CL diameter decreased abruptly (P<0.05) in both pregnant and non-pregnant buffaloes following luteolysis caused by $PGF_2\alpha$ administration on day 7 of the treatment.

The diameter of CL remained comparatively greater in group-II on day -14 (Table 2). The CL size decreased abruptly (P<0.05) following $PGF_2\alpha$ injection on day 7 of the treatment.

Effect on plasma steroid hormone profiles

Estradiol concentrations

The mean plasma E_2 concentrations on first day (day -14) of the treatment were similar in both the groups. However, E_2 concentrations on days -2, 0 and 10 were significantly higher in group-I compared to -II (Fig 3). After $PGF_2\alpha$ administration on day 7 in both groups, E_2 concentration increased (P<0.05) on the day of EB injection, which further increased (P<0.05) on the day of FTAI. The E_2 profiles, in both groups, were comparable between the pregnant and non-pregnant buffaloes; however, the concentrations remained higher (P<0.05) on the day of EB injection in pregnant compared to non-pregnant buffaloes (Table 1 and 2).

Progesterone concentrations

Plasma P_4 concentration remained similar (P>0.05) in both the groups on first day (day -14) of the experiment (Fig 2). However, P_4 concentration between the two groups differed significantly (P<0.01) on days -2, 0 and 7. On the day of AI (day 10) P_4 concentration remained non-significantly higher in group-II. In Group-I, $PGF_2\alpha$ administration on day -14 seems to have caused luteolysis and thus, assisted in ovulation of the DF four-five days later. The second PG shot in this group (on day -2) resulted in the luteolysis of CL formed in response to ovulation of the previous follicular wave.

Unlike group-II buffaloes, luteolysis by 2[nd] PG administration on day -2 was indicated in group-I by significant drop (P<0.05) in plasma P_4 concentration, on day -2 compared to the day 0 (1.80±0.12 vs. 0.50±0.04 ng/ml).

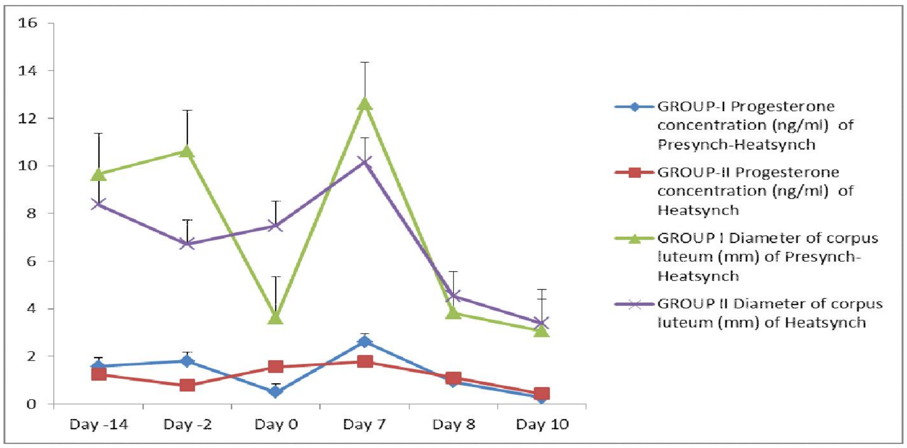

Fig 2: Diameter of corpus luteum (mm) and progesterone concentration (ng/ml) in buffaloes.

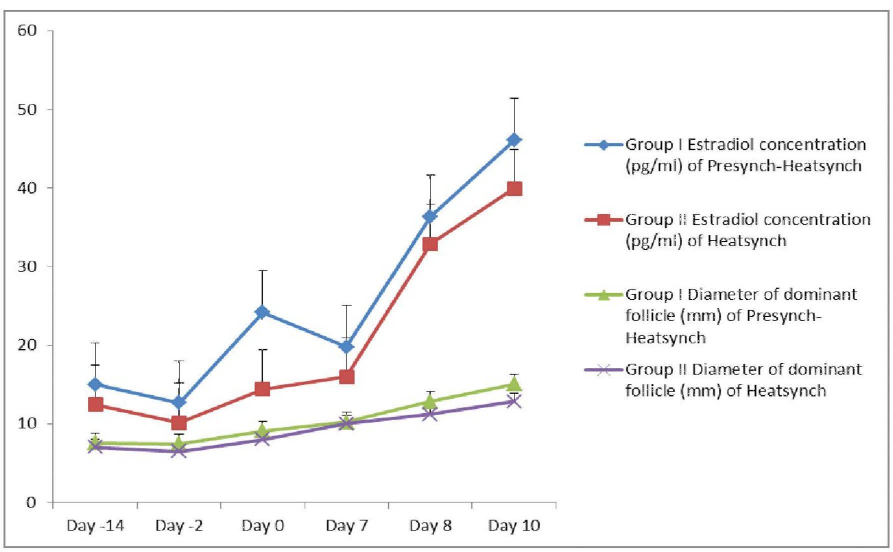

Fig 3: Diameter of dominant follicle (mm) and estradiol concentration (ng/ml) in buffaloes.

In Group-I, P_4 levels remained higher (P<0.01) on days of all three $PGF_{2α}$ injections in buffaloes that became pregnant compared to those who failed to conceive (Table 1). In both pregnant and non-pregnant buffaloes, P_4 levels decreased abruptly (P<0.05) after luteolysis caused by $PGF_2α$ administration on day 7 of the treatment. However, on the day of FTAI, P_4 concentration was higher (P<0.01) in non-pregnant buffaloes than those that became pregnant. Similarly in pregnant buffaloes of Heatsynch group, P_4 levels remained higher on all days of treatment except on the day of FTAI as compared to non-pregnant ones (Table 2). However, on the day of FTAI, P_4 concentration remained higher (P<0.01) in non-pregnant compared to pregnant buffaloes. In pregnant buffaloes, P_4 concentration decreased (P<0.05) following injection of $PGF_2α$ on day 7, unlike in non-pregnant buffaloes. On the day of FTAI, P_4 concentration did not decrease from the day of $PGF_2α$ administration in buffaloes that failed to become pregnant. High P_4 levels on the day of FTAI (Day 10) did not seem to affect ovulatory response as more than 90% animals in both groups ovulated post-AI (Fig 2).

Effect on fertility parameters

In the present study, an ovulatory response of 93.33% (28 out of 30 buffaloes) and 90.00% (27 out of 30 buffaloes) was observed in Group-I and -II, respectively (Fig 2). The conception rate in the present study was significantly higher (P<0.05) with Presynch-Heatsynch than the Heatsynch treatment (66.66 vs. 40.00%, respectively).

Poor reproductive performance, manifested by long calving intervals, could result in huge economic losses due to low milk yield, high replacement costs and culling rates in buffaloes. Estrus synchronization was considered as an effective tool to enhance submission and pregnancy rates in buffaloes. It eliminated or atleast reduced the problem of estrus detection which was considered as the major limitation in reproductive management of buffaloes. Ovsynch protocol was developed to allow timed artificial insemination (TAI) without the need for detection of estrus (Pursley *et al.* 1995). Later, Ovsynch protocol was also applied to buffaloes (Ghuman *et al.* 2008). However, during the last few years another promising estrus synchronization protocol called Heatsynch was developed, that incorporated combination of GnRH-$PGF_2α$-EB injections followed by FTAI. The EB was a less expensive hormone in place of second GnRH injection of Ovsynch protocol. The major advantages of Heatsynch protocol were reduced hormone costs, increased estrus intensity, ease in scheduling and implementation, since all injections and AI are at 24 and 48 h intervals (Mohan and Prakash *et al.* 2014).

Presynchronization of estrus prior to incorporation of Ovsynch program was reported to increase pregnancy rates by 10-12% in bovines (Moreira *et al.* 2001; Navanukraw *et al.* 2004). Moreover, presynchronization could be incorporated during the voluntary waiting period, thereby eliminating the delay in the first postpartum service. Therefore, incorporation of presynchronization prior to Heatsynch might prove beneficial in buffaloes. The conventional method of presynchronization involved administration of two $PGF_2α$ injections 11 to 14 days apart, with the last injection given 14 days before initiation of any GnRH based breeding protocol (Moreira *et al.* 2000). However, in the current study, a modified presynchronization was adopted before initiation of Heatsynch (Presynch-Heatsynch) protocol that involved administration of two $PGF_2α$ injections at 12 days apart, last $PGF_2α$ injection given 2 days prior to the first GnRH of Heatsynch protocol was investigated in 60 lactating buffaloes with the aim of developing a shortened protocol.

In the present study, favourable effect of additional presynchronization was indicated by larger diameter of DF on day -2 as well as on day 0. Administration of $PGF_2α$ before first GnRH injection in any of the GnRH based protocol enhanced the pituitary release of LH in response to GnRH (Mirmahmoudi *et al.*, 2014). It had been established for GnRH based protocols that animals ovulating after first GnRH injection are more likely to have functional DF capable of ovulation after final GnRH injection (Vasconcelos *et al.*, 1999). Larger diameter of DF during ovulatory wave in pregnant than in non-pregnant buffaloes of the present study indicated that in pregnant buffaloes, first GnRH possibly ovulated the existing follicle of larger size resulting in functional DF capable of ovulation after injection of EB on day 8 of the protocol. Hence, the better ovulation induction after GnRH administration could be one of the most important reasons for the greater fertility in pregnant animals of both the groups. These observations were in corroboration with the earlier report of peak E_2 concentration (of 35.8 pg/ml) one day prior to estrus in bovines (Bachalaus *et al.*, 1979). In buffaloes, E_2 concentration at the time of estrus increases upto 45-50 pg/ml (Batra and Pandey, 1983). The E_2 concentrations were positively related to the size of follicle and as bigger sized follicles secreted more E_2 compared to smaller ones (Palta *et al.*, 1998). This relationship was also consistent in the present study.

The sufficient P_4 concentrations are pre-requisite for proper growth of the ovulatory follicle (Bisinotto *et al.*, 2010 and Wiltbank *et al.* 2011). It was believed that effect of P_4 on fertility involved a change in the pattern of LH release. The changes in LH pulse frequency were linked with altered follicular maturation and subsequent embryo survival (Cerri *et al.*, 2011). Several earlier reports emphasized the importance of high concentration of P_4 during the follicular growth of pre-ovulatory DF for successful establishment of pregnancy (Meisterling and Dailey, 1987 and Bilal *et al.* 2016). Previous studies have indicated that the P_4 concentrations during the development of DF influenced the fertility since DFs of first and second follicular wave grow under different P_4 environments (Denicol *et al.*, 2012). The first wave DF grows under sub-luteal phase P_4 level (<1.5 ng/ml) for a few days followed by luteal phase level, whereas DF of ovulatory wave grows under luteal phase level (>2 ng/ml) of P_4, prior to luteolysis (Sartori *et al.*, 2004 and Denicol *et al.*, 2012).

The ovulation following PG administration occurred at 60-156 h in bovines (Brito *et al.*, 2002). Thus, administration of the GnRH on day 0 could have facilitated the ovulation of the follicular wave DF that was initiated after ovulation at around day 10. Administration of the third PG on day 7 resulted in luteolysis, whereas injection of EB on day 8 resulted in LH peak required for subsequent ovulation of the ovulatory follicle with better expression of estrus.

Administration of GnRH on day 0 in group-I animals was able to induce ovulation of DF and formation of CL as indicated by higher (P<0.05) P_4 concentration on day 7 (2.61 ± 0.18 ng/ml) than on day 0 and 8 (0.50 ± 0.04 and 0.94 ± 0.80 ng/ml, respectively).

In group-II of present study, P_4 concentrations on day 0 and 7 were similar due to the fact that no $PGF_2\alpha$ was administered prior to first GnRH injection. However, after luteolysis, the P_4 concentration decreased abruptly (P<0.05) after day 8. Similarly in group-I, P_4 levels reduced (P<0.05) after day 7 till day of FTAI. The above findings indicated that pre-synchronization before start of Heatsynch protocol resulted in low P_4 environment before first GnRH injection (day 0) leading to ovulation in most of the buffaloes post GnRH injection. This might have further led to optimum DF size at days of EB injection and FTAI for ovulation and pregnancy to occur. The significantly higher P_4 levels in buffaloes that became pregnant compared to those that failed to become pregnant in the present study, clearly indicated that ovulatory follicle developed well under higher P_4 environment in pregnant buffaloes of both groups. This finding corroborated with the earlier reports of Cerri *et al.* (2011). Increased P_4 level throughout ovulatory follicle development was related with increased intra follicular levels of IGF-1 and improved pregnancy rates (Wiltbank *et al.*, 2011). High P_4 during growth of ovulatory follicle may increase pregnancy rates by atleast 10 percent in bovines after FTAI (Bisinotto *et al.*, 2010). Another important finding of the present study was presence of supra-basal levels of P_4 at the time of FTAI in buffaloes that failed to conceive in both groups. High P_4 concentration near AI might reduce pregnancy rate by altering gamete transport through compromised oviductal or uterine contractility, thereby, reducing the fertilization rates (Hunter, 2005). The conception rate obtained in the present study was higher than those reported by Mohan *et al.* (2009) and Mirmahmoudi *et al.* (2014) in buffaloes subjected to Heatsynch protocol (32.5 and 33.33%, respectively). Further, conception rates obtained in Presynch-Heatsynch group (66.66%) was higher than reported in earlier studies comparing conception rate following AI on second wave ovulation in buffaloes (Hoque *et al.*, 2014) and dairy cows (Sartori *et al.*, 2009) (44.4 and 48.0%, respectively).

CONCLUSION

In conclusion, the present results indicated that additional presynchronization in Heatsynch protocol might successfully enhance the reproductive efficiency of buffaloes. Moreover, the modified protocol is comparatively of shorter duration (25 vs. approximately 37 days) than the conventional method and might aid to reduced service period and subsequent calving interval in buffaloes.

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