Timed Artificial Insemination Methods involving Ovulation of Second Follicular Wave *vis-à-vis* Extension in Growth of Dominant Follicle- New Alternatives to Enhance Fertility in Buffalo *(Bubalus bubalis)*

Bilal Ahmad Ganaie[1], Shahbaz Singh Dhindsa[2]*, Dinesh Dadarwal[3] and M. Honparkhe[4]

[1]Animal Reproduction Gynaecology and Obstetrics Division, ICAR-National Dairy Research Institute, Karnal, INDIA
[2]Directorate of Livestock Farms, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, INDIA
[3]Large Animal Clinical Sciences, RM 2434, WCVM, University of Saskatchewan, CANADA
[4]Department of Veterinary Gynaecology and Obstetrics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, INDIA

*Corresponding author: SS Dhindsa; Email: shahbazdhindsa76@yahoo.co.in

ABSTRACT

The study was undertaken to assess the ovarian and fertility responses following ovulation of dominant follicle (DF) from second follicular wave (2nd FW) through application of modified synchronization protocols in 46 cyclic postpartum buffaloes. All buffaloes were administered PGF2α (PGF; Cloprostenol, 500 mcg) on day -2, gonadotrophin releasing hormone (GnRH; Buserelin) on days 0 (10 mcg) and 7 (20 mcg). After that, all buffaloes were randomly divided into 2 groups on the basis of administration of second dose of PGF (500 mcg) on day 14 (Group I, n= 20) and 15 (Group II, n= 26). Third dose of GnRH (10 mcg) was administered on day 16 (Evening) and 17 (Morning) in group I and II, respectively, followed by artificial insemination (AI) at 12-16 h later. The pregnancy diagnosis was performed at day 45 post AI. Progesterone (P_4) concentrations remained higher during 2FW in buffaloes that became pregnant compared to nonpregnant ones. A larger (P<0.01) diameter of the DF was observed on day of third GnRH and AI in group II compared to group I buffaloes. A comparable corpus luteum (CL) diameter was observed on all days of protocols in both groups. Ovulatory response following last GnRH administration and first AI pregnancy rate were 90 vs. 96 % and 35 vs. 46 % in group I vs. II, respectively. In conclusion, ovarian and fertility responses following ovulation of DF from 2nd FW showed an improved trend in buffaloes where growth of DF was extended by delaying the luteolysis.

Keywords: Buffalo, Estrus synchronization, Follicular wave, Pregnancy rate

The buffalo is the backbone of dairy industry in India contributing more than 55 per cent of national total milk production. Reproductive efficiency is the primary factor affecting productivity of buffalo. Low reproductive efficiencies due to problems of subestrus, anestrus, and repeat breeding are considered as major constraints in buffalo farming (Dhindsa *et al.,* 2016). In fact, decline in conception rate and increase in calving interval due to AI at wrong time is a major problem in the progress of buffalo dairy industry. Applying estrus synchronization treatments in buffaloes provide a potential alternative for increasing their productive period as these regimens eliminate the problem of estrus detection. Even though a number of protocols have been implicated, a partial success has been achieved with available estrus synchronization regimens till date in buffaloes (Ghuman *et al.,* 2010).

Moreover, most of the protocols involve timed AI (TAI) following ovulation of the DF from first follicular wave (1st FW). However, the DF of the 1st FW and 2nd FW grows in different P_4 environments. Various studies have demonstrated favourable effect of P_4 environment during DF development on subsequent CL development, CL functionality, estrus behaviour and fertility in bovine (Lonergan, 2011; Wiltbank, 2012; Dadarwal *et al.,*

2013; Bilal *et al.,* 2017). Studies in cattle (Denicol *et al.,* 2012) have shown that fertility decreases when 1st FW DF is allowed to ovulate than the 2nd FW. Thus the present study was conducted to suggest modified estrus synchronization protocols involving ovulation of 2nd FW DF and to ascertain the effect of delaying luteolysis during the treatment on ovarian response and hormonal profile of buffaloes.

MATERIALS AND METHODS

Animals

The present study was carried out on 46 post-partum (> 60 days in milk) cyclic buffaloes maintained at dairy farm, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India. The selected buffaloes had body condition score (BCS) ranging from 2.5 to 3.5 on scale of 1 to 5 basis (Edmondson *et al.,* 1989). The animals were managed in a semi-intensive housing system. Only buffaloes in general good health and free from genital abnormalities / discharges were included in the present study. The cyclic status of all buffaloes was assessed by transrectal ultrasonographic examination of ovarian structures at the start of the experiment.

Experimental design

All buffaloes were pre-synchronized at random stages of the estrous cycle by administering single injection of PGF2α (PGF: Cloprostenol B.P 500 μg, im, Provimi Vetcare) on -2 day. Two days later (day 0), all animals were administered a gonadotrophin releasing hormone analogue (GnRH: Buserelin acetate 10 mcg, im, MSD) to induce ovulation and a new FW. Again on day 7 all animals were given GnRH (20 mcg) to ovulate DF of 1st FW and initiate 2nd FW. Thereafter to study the effect of increased P_4 exposure on the growth of 2nd FW DF, all buffaloes were randomly divided into 2 groups (Group I, n=20; Group II, n=26) on the basis of administration of second dose of PGF (500 μg) that was injected one day late in group II compared to group I buffaloes (15 vs. 14 day). A third dose of GnRH (10 mcg) was administered on day 16 (Evening) and 17 (Morning) in group I and II, respectively, followed by AI at 12-16 h later.

Blood collection and transrectal ultrasonography

All buffaloes of both groups were subjected to blood sampling and transrectal ultrasonographic examination of ovarian structures on respective days of treatments as shown in fig. 1. Blood samples (5-10 ml, jugular venupuncture) were collected into heparinised (1:1000) tubes which were immediately placed in ice box and transferred to the laboratory. Plasma was separated by centrifugation (3000 rpm, 15 minutes) and stored at -20°C until the hormone assay. Transrectal ultrasonographic examination was performed using brightness mode (B mode) ultrasound device, Z 5 diagnostic ultrasound machine (Shenzhen Mindray Biomedical Electronic co. ltd). The animals were restrained properly to avoid distortion in the ultrasound image. During each ultrasonographic examination the number and diameter of DFs and CLs were measured after freezing the best image with minimum distortion and maximum clarity.

Progesterone analysis

Plasma P_4 was estimated by liquid phase Radioimmunoassay (RIA) procedure using progesterone antisera raised in the department of Veterinary Gynaecology and Obstetrics, GADVASU, Ludhiana (Ghuman *et al.,* 2009). The sensitivity of the assay was 0.1ng/ml for P_4. The mean intra- and inter-assay coefficients of variance were 6.0 and 9.3 percent, respectively.

Statistical analysis

The data were presented as mean ± standard error of mean. Chi square test was applied to compare ovulation and pregnancy rates. Plasma P_4, DF and CL values were analysed using "Unpaired T-test" to compare between groups and "One way ANOVA" to evaluate the effect within the group. The statistical significance of tests was determined at P<0.05 and P<0.01 levels.

Ethical approval

This study was approved by the Institutional Animal Ethics Committee, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana.

RESULTS AND DISCUSSION

Exposure to insufficient concentrations of P_4 during the growth of the ovulatory follicle is one of the important factors that affect fertility in high-producing dairy animals (Wiltbank *et al.,* 2012; Bisinotto *et al.,* 2010). Low P_4 concentration during the development of DF reduces the subsequent fertility in bovine (Wiltbank *et al.,* 2012). The DF from 1st FW develops under low circulatory P_4 whereas DF of 2nd FW/ovulatory wave grows under luteal level (> 2 ng/ml) of P_4, prior to luteolysis (Denicol *et al.,* 2012). In present study, P_4 concentrations on days of second PGF, third GnRH and AI were 1.84±0.17, 0.54±0.02 and 0.09±0.05 ng/ml in group I, while the corresponding levels in group II were 1.96±0.18, 0.44±0.02 and 0.16±0.08 ng/ml, respectively. The present results support the hypothesis that higher P_4 levels are required during the growth of ovulatory follicle to enhance pregnancy rate. In group I, P_4 concentrations during 2nd FW on days of PGF, GnRH and AI in pregnant vs. non-pregnant buffaloes were 2.14±0.19 vs. 1.67±0.23, 0.52±0.07 vs. 0.55±0.05 and 0.13±0.02 vs. 0.06±0.02 ng/ml, whereas the corresponding values in group II were 2.17±0.27 vs. 1.78±0.24, 0.51±0.03 vs. 0.43±0.06 and 0.21±0.00 vs. 0.11±0.01 ng/ml, respectively.

The mean DF and CL size on various days of modified synchronization protocols in group I and II is presented in Table 1. The DF on the day of third GnRH and AI was significantly (P<0.01) larger in group II compared to group I buffaloes (13.06±0.25 and 14.47±0.26 vs. 11.53±0.20

and 11.92±0.14 mm, respectively). The size of the CL did not differ significantly (P>0.05) between the groups. This finding supported the earlier report suggesting uncertain correlation between size of the CL determined by conventional ultrasonography and P_4 secretion or subsequent pregnancy (Russo *et al.,* 2010).

In group I and II, 90 and 96 percent buffaloes ovulated following last GnRH injection, respectively. The observed ovulatory responses during second wave in buffaloes were higher than in earlier studies (78.1%: Souza *et al.,* 2008; 83.3%: Hoque *et al.,* 2014). The overall first AI pregnancy rate was 35 and 46 percent in group I and II, respectively. Earlier research has revealed that increasing P_4 during growth of the follicular wave increased fertility more than 10 percent to the subsequent AI in bovines (Bisinotto *et al.,* 2010). During the current study, a higher pregnancy rate by 11 per cent was observed in group II (46%) compared to group I (35 %). The pregnancy rate obtained in the present study is in corroboration with earlier studies comparing conception rate following second wave ovulated AI (Cunha *et al.,* 2008; Hoque *et al.,* 2014). The exact mechanisms that produce increase in fertility are unknown. However, the possible underlying mechanism by which P_4 modulates fertility likely involves a change in the pattern of LH release (Wiltbank *et al.,* 2012). Changes in LH pulse frequency are associated with alterations in the process of follicular maturation and subsequent embryo survival (Cerri *et al.,* 2011). In buffaloes only a few reports are available supporting the above hypothesis

Table 1: Size of dominant follicle and corpus luteum (Mean±SE) on various days of modified synchronization protocols in buffaloes

Treatment group	Days of protocol				
	Day-2 [M]	Day 7[M]	Day 14[M]	Day 16[E]	Day 17[M]
	Mean diameter of dominant follicle (mm)				
Group I (n=20)	7.46±0.18[a]	8.89±0.16[b]	9.12±0.14[b]	11.53±0.20 [c1]	11.92±0.14[c1]
	Mean diameter of corpus luteum (mm)				
	11.56± 0.34[d]	10.40± 0.28[c]	13.10± 0.27[e]	8.68 ± 0.22[b]	6.14± 0.17[a]
	Day -2[M]	Day 7[M]	Day 15[M]	Day 17[M]	Day 17[E]
	Mean diameter of dominant follicle (mm)				
Group II (n=26)	7.72±0.13[a]	8.20±0.16[a]	9.46±0.08[b]	13.06±0.25[c1]	14.47±0.26[d1]
	Mean diameter of corpus luteum (mm)				
	9.49 ± 0.23[c]	10.53± 0.24[d]	13.52± 0.22[e]	8.79 ± 0.16[b]	6.60 ± 0.18[a]

Values bearing different superscripts (a - e) along the row differ significantly (P<0.05)
[1]: Significantly different within the column (P<0.01)
[M] and [E]: Time of treatment/AI; Morning (9.00 am) and Evening (6.00 pm)

(Hoque *et al.,* 2014). Moreover, no literature is available to compare the present results of extending growth of DF under high P_4 environment during 2nd FW on subsequent fertility in buffaloes. Future studies are required in this direction to define the mechanism involved and support the hypothesis.

CONCLUSION

The results of the present study indicate that TAI protocols involving ovulation of the 2nd FW may successfully be incorporated to enhance the reproductive efficiency of buffaloes. The delay in luteolysis during 2nd FW may increase the preovulatory DF size by extending its growth under high P_4 environment that subsequently improves the oocyte quality and pregnancy rate in buffaloes.

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