EFFECT OF DURATION OF BIOSTIMULATION ON OESTRUS EXPRESSION, RESUMPTION OF OVARIAN ACTIVITY, CONCEPTION RATE AND PERIPHERAL CORTISOL LEVEL IN POSTPARTUM ANOESTRUS NILI-RAVI BUFFALO DURING LOW BREEDING SEASON

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ABSTRACT

The aim of experiment was to evaluate whether biostimulation by mature intact bull to anoestrus buffalo alters breeding performance associated with CIDR based oestrus synchronization protocol during low breeding season. Anoestrus buffalo that had calved 170.0±10.6 (132 to 208) days before the start of the trial, were either exposed to bull (n=30, BE) or not exposed to bull (n=10, BN) for 12 days after CIDR placement (day 0). Animals received $PGF_{2\alpha}$ on day 9, CIDR was removed on day 10 and Timed AI (TAI) was performed with frozen thawed semen at 48 and 60 h after CIDR removal. Buffalo were observed for oestrus detection twice daily for three days after CIDR removal. Blood sampling was done on day 9, 12 and 20 of the experiment to monitor the progesterone and cortisol levels (5 per group). Pregnancy test was done 60 days after insemination. Oestrus expression was noted in 68.97% (BE) and 60% (BN) animals (P>0.05). Progesterone rise above 1 ng/ml on day 20 was recorded in 4/5 (80%) in BE group and 2/5 (40%) in BN group (P>0.05). Conception rate was 37.9% (11/29) and 30%, (3/10) in BE and BN groups respectively (P>0.05). It is concluded that CIDR and fixed time artificial insemination can be used to overcome partially the problem of anoestrus and seasonality in buffalo. However, biostimulation in CIDR treated anoestrus buffalo did not increase the number of ovulating/oestrus buffalo during low breeding season probably due to a shorter exposure period (only 12 days) to bull.

Keywords: *Bubalus bubalis*, buffaloes, biostimulation, oestrous, conception rate, CIDR

INTRODUCTION

Water buffaloes are well-adjusted to harsh environments (Perera, 2011). However, reproductive parameters like delayed puberty, long inter calving interval, poor oestrus expression and seasonality in breeding (Barile, 2005) need to be improved to further enhance the usefulness of this important dairy animal. Artificial insemination (AI) is practiced in more than 90% dairy cattle in developed countries (Machatya *et al*., 2012) but use of AI is limited in buffalo due to poor fertility rates with frozen thawed semen (Anzar *et al*., 2003). Many hormonal protocols have been developed that control the time of ovulation and

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thus avoid the need of oestrus detection in cattle (Lane *et al*., 2008) and buffalo (Neglia *et al*., 2013). Gonadotropin releasing hormone and $PGF_{2\alpha}$ (in the form of Ovsynch and its modifications) and Progestogens like Control Internal Drug Release (CIDR) are extensively used for synchronizing oestrus in cattle (Lane *et al*., 2008). These protocols have also been tested in buffalo (Warriach *et al*., 2008). Progesterone based protocol resulted 80 to 93% oestrus induction in buffalo with a pregnancy rate varying from 20 to 50% (De Rensis and Lopez-Gatius, 2007; Jabeen *et al*., 2015). Seasonality of breeding may also be overcome to some extent in buffalo through the hormonal application (Barile, 2005) as round the year breeding is a desired trait for dairy animals for successful and feasible dairy entity. CIDR has been shown to be more effective than ovsynch to induce heat during low breeding season in buffalo (De Rensis and Lopez-Gatius, 2007).

Management is an important aspect influencing fertility results. Therefore the synchronizing protocols need to be further tested in combination with improved management practices for induction of fertile oestrus especially during low breeding season when most of the buffaloes are in anoestrus condition. One management strategy that has the potential to increase the proportion of postpartum cows that resume cycling is the use of the biostimulatory effect of bulls (Tauck, 2008). Exposure of postpartum beef cows to bulls or their excretory products prior or during the oestrus synchronization regimen improved fixed-timed AI conception (Berardinelli *et al*., 2007). It has been shown that biostimulatory effect of bulls is mediated by pheromones that are mainly present in urine in case of mammals (Izard, 1983). However, the mechanism of activation of hypothalamo-hypophyseal-ovarian (HPO) axis through pheromones is not fully understood. There is indication in mice and rats that adrenal activation may be involved in the response of female to male pheromones (Mora and Sanchez-Criado, 2004). Cortisol rise was also recorded in primiparous beef cows exposed to bull (Tauck *et al*., 2007), however, in a later study it was suggested that cortisol secretion pattern (and not the level) was altered by male stimuli in beef cattle (Tauck, 2008).

The present experiment was designed to investigate the effect of oestrous synchronization using CIDR coupled with biostimulation on oestrus expression and conception rates during low breeding seasons in anoestrus buffalo.

MATERIALS AND METHODS

Animals and management

This experiment was conducted during March-April (low breeding season for buffalo) at Livestock experiment Station Rakh Gulaman, District Bhakkar (Pakistan) with longitude and latitude 31.44^{o}N, 71.51^{o}E. Daily temperature averages during the experiment period was 28 to 36^{o}C (maximum) and 17 to 25^{o}C (minimum). Non pregnant, lactating Nili-Ravi buffalo (n=40) with a normal calving history and in their 2nd to 4th lactation were used for the study. Animals had calved 170.0±10.6 days before the start of the trial. These animals were part of a buffalo herd comprising of 850 animals (adult and young stock). Animals were healthy with body condition score ranging from 2 to 3. Heat detection was done in the adult buffalo twice a day using a penile deviated teaser bull since calving. These forty animals were not detected in heat since calving, so they were considered as anoestrus. The animals were kept under loose housing conditions in clean paddocks.

Animals were brought to sheds only for feeding and milking. The sheds had a concrete floor with asbestos roofing with a capacity of 100 animals each. Animals were stall fed with Egyptian clover (30 kg/animal/day) and wheat straw (15 kg/animal/day) and concentrate (commercially available cattle mixture "Anmol Wanda") at the rate of 2 kg/animal/day. Fresh water was available ad libitum. Animals were sent out daily for grazing natural grasses in the morning at 8 am and brought back at milking time (3 pm). The farm grew Lucerne, oats, canola and Egyptian clover during winter and maize and Mott grass during summer. Anthelmintics were used quarterly for deworming. Vaccination for Foot and Mouth Disease, Black quarter and Hemorrhagic septicemia was done once a year.

Oestrus synchronization and bull exposure (biostimulation)

All the 40 buffaloes received intra-vaginal CIDR (Controlled internal drug release containing 1.38 gram progesterone in molded silicone, DEC Int. NZ Ltd, Hamilton, New Zealand) for oestrus synchronization. Oetrus synchronization protocol was as follows.

DAY 0: CIDR was inserted intra-vaginally in all forty animals.

DAY 9: Administration of 150 µg Cloprostenol (Synthetic analogue of $PGF_{2\alpha}$, Dalmazin, FATRO Italy, 2 ml i/m).

DAY 10: CIDR was removed.

After CIDR placement the animals were assigned to one of the following treatment groups.

Bull exposed (BE) group (N=30)

Buffalo were kept with a buffalo bull round the clock. The bull also acted as a teaser for oestrus detection after CIDR removal while roaming among the buffaloes.

Bull not exposed (BN) group (N=10)

Buffaloes were kept without a buffalo bull. The BN group was kept at a distance of 0.5 km from the BE group in complete isolation from the bull so that no excretory products or bull pheromone might reach this group. The routine teasing for heat detection was stopped for this group one day before the start of the trial.

Effect of bull exposure in heat synchronized buffalo was compared by oestrus expression, progesterone level on day 20 and conception rate after timed AI.

Oestrus expression

Animals were monitored for heat signs twice a day (morning, evening) for half an hour each time, starting 24 h after CIDR removal. This was done by visual observations in BN group and both visually and by a bull in BE group. Visual signs taken into account were, mucous discharge, swollen vulva, bellowing, frequent micturation, restlessness, mounting by other buffalo, mounting by Bull, standing to be mounted.

Animals showing one or more than one of the above behavioural oestrus signs were considered to be in oestrus.

Ovarian activity by progesterone assay

Ovarian activity was monitored by the progesterone assay. For progesterone assay blood was collected from five randomly selected animals per group on following days.

Day 9 of treatment (before $PGF_{2\alpha,}$ injection, when CIDR was yet in vagina).

Day 12 of treatment (before first insemination, 48 h post CIDR removal).

Day 20 of treatment (i.e. 8 days after first

AI)

Blood samples were collected by jugular veni puncture in heparinized tubes and immediately put in the ice box. The samples were centrifuged at 3000 rpm (1006 g) for 15 minutes and plasma was stored at -20^{o}C until analyzed. Progesterone concentration was measured in duplicate using solid phase ^{125}I Radioimmunoassay (Immunotech, Beckman Coulter Company, France) at Nuclear Medicine, Oncology and Radiotherapy Institute Islamabad (NORI). The analytical sensitivity was 0.05 ng/ml. The intra and inter assay coefficient of variation were 10.1 and 9.3% respectively.

Fixed time AI and conception rate

Animals were inseminated using frozen, thawed semen from bulls with proven fertility. Timed insemination was performed at 48 and 60 h after CIDR removal (i.e. Day 12 and 12.5 of treatment). Buffalo were mixed with the main herd after insemination. Conception rate was determined by rectal palpation 60 days after insemination.

Cortisol assay

The blood samples collected for progesterone assay were also used for cortisol analysis. Plasma Cortisol concentrations were measured in duplicate by using ELISA kits (MicroLISA. Amgenix, CA, USA). The Assay was performed according to the manufacturer's instructions. The Intra-Assay and Inter assay Coefficient of Variation was 9.8 and 10.2.

Statistical analysis

Response to oestrus synchronization treatment, oestrus incidence and conception rates were compared by Chi-square statistics. Cortisol levels on day 9, 12 and 20 were compared with students T test. Progesterone and cortisol levels are presented as mean±SEM. Statistical significance was set at P<0.05.

RESULTS

One buffaloes lost CIDR from the BE group so it was excluded from the analysis.

Oestrus expression

One or more than one behavioural oestrus signs were observed in 20/29 (68.97%) buffalo in Bull Exposed and in 6/10 (60%) buffaloes in Bull Not Exposed groups respectively. Although a higher percentage of buffalo showed behavioural oestrus in BE compared to BN group, the difference was non-significant (P>0.05).

Plasma progesterone level

In all (five buffaloes from each group) the sampled buffalo, plasma progesterone was >1 ng/ml at day 9 of treatment when CIDR was in place and it declined to bellow 1 ng/ml at day 12 (i.e. two days after CIDR removal). Progesterone rise above 1ng/ml on day 20 was recorded in 4/5 (80%) in BE group and 2/5 (40%) in BN group. So a higher number of animals were found responsive to CIDR protocol in BE group as compared to BN group. All the animals that showed a rise in P4 level at day 20 of treatment were found pregnant at day 60 post insemination.

Conception rate

Conception rates did not differ significantly between BE (37.9%, 11/29) and BN (30%, 3/10) groups.

Plasma cortisol level

No significant difference (P>0.05) was observed between BE and BN groups in plasma cortisol level on day 9, 12 and 21.

DISCUSSION

Present experiment studied the effect of oestrus synchronization (using CIDR) in buffalo that were either exposed to (biostimulation) or not exposed to bull during the synchronization period on oestrus expression, progesterone profile and conception rate during low breeding season. All the animals included in the study were anoestrus as they had not shown oestrus signs since calving to start of the trial as noted by regular teasing with a bull after calving.

Behavioral oestrus signs noted both in BE (68.97%) and BN (60%) groups during the present study indicated that heat can be induced in anoestrus Nili Ravi buffalo using the CIDR treatment in low breeding season. Plasma progesterone levels measured in a part of the animals also confirmed the finding where all the 10 buffaloes had progesterone level above 1 ng/ml while CIDR was in the vagina, and six out of ten buffaloes showed a progesterone rise above 1 ng/ml at day 8 after insemination indicating ovulation after CIDR removal. It has been noted that progesterone released from CIDR may sensitize the Hypothalamo-Pituitary-Gonadal axis of buffaloes for resumption of ovarian activity (Singh, 2003). In dairy cattle, progesterone is an important regulator of frequency of pulsatile secretion of LH and hence plays an important regulatory role in preovulatory follicular development (Herlihy *et al*., 2012). Induction of ovulatory oestrus might be due to similar endocrine impact in buffalo.

There are observations that during the low breeding season oestrus signs are covert and silent oestrus increases in buffalo (Singh *et al*., 2000), however ≥60% animals showed overt oestrus signs after CIDR treatment in the present study indicating its effectiveness to overcome the problem of silent heat and covert signs in buffalo to some extent. It has been reported that peak P4 and estradiol concentration were lower in anoestrus rural buffalo in summer months than in winter. The expression of oestrus and LH secretion requires an appropriate balance between estradiol and progesterone (Dobson *et al*., 2008). The low progesterone concentration in hot months in comparison to cooler ones is likely to be responsible for the weak oestrus signs during this period. However CIDR delivers high content of P4 and results in overt estrus signs expressed by buffaloes even during low breeding season.

The buffalo used in the present study were daily exposed to bull twice a day since calving for the purpose of estrus detection. Continuous exposure or non exposure to bull over next 12 days of oestrus synchronization and timed AI had no impact on expression of behavioral oestrus in the present study. There is a possibility that non-exposure window of less than two weeks might not be enough to result in a negative impact on oestrus behavior and oestrus incidence in buffalo synchronized for heat. A 45 day exposure to bull for 12 h daily was able to induce difference in beef cows in terms of ovarian activation (Tauck *et al*., 2010b). Positive effects of biostimulation have also been reported in zebu (*Bos indicus)* cows (Rekwot *et al*., 2000a). Biostimulation was also effective to reduce anoestrus periods in buffalo (Gokuldas *et al*., 2010) with exposure time of 50 days. The results of the biostimulatory effect of bulls may depend on the intensity of exposure (frequency of exposure, duration of exposure, and quantity of stimuli) of pheromonal stimuli produced by bulls (Gokuldas *et al*., 2010). A non significant difference observed in BE and BN groups in the present study might be due to insufficient intensity of exposure in terms of duration.

Conception rates did not differ between bull exposed and bull not exposed groups (37.9 and 30%, respectively) in the present study, however, induction of fertile oestrus in a proportion of anoestrus buffalo during low breeding season is a positive point observed here. Use of progestagens along with eCG induced estrus in anestrous buffaloes with pregnancy rates up to 30% in the Mediterranean (Neglia *et al*., 2003) and Egyptian buffalo (Hattab *et al*., 2000). Use of CIDR alone in anestrus Murrah buffalo gave an ovulation rate of 47.4% and a conception rate of 27.3%, while CIDR + eCG gave an ovulation rate of 81% and conception rate of 40.6% (Murugavel *et al*., 2009). So the use of progestagens alone or in combination with eCG (as reported above) or biostimulation during oestrus synchronization (in the present study) is able to induce fertile heat in 30 to 40.6% in anoestrus buffalo. However, there is potential to improve the conception rate as the number of ovulating buffalo or buffaloes in standing heat is almost double than those conceiving. Improved management practices (such as better feeding and cooling) need to be investigated in combination with hormonal therapy. There is indication in temperate dairy cattle that a progestagen device maintaining higher circulating P4 levels might be beneficial in inducing fertile oestrus, during synchronization programs and this might be due to improved oocyte quality (Werven *et al*., 2013). Benefits of intravaginal device with higher progesterone level also need to be investigated in anoestrus buffalo. In Brazil a study on anestrous *Bos indicus* cows achieved pregnancy rates between 40 and 55% by insertion of an intravaginal device containing 1.9 g of progesterone (CIDR) plus 2.0 mg i/m estradiol benzoate (Meneghetti *et al*., 2009). In Pakistan 36.8% conception rate was observed in CIDR treated buffalo during low breeding season (Jabeen *et al*., 2015). While in another study in anoestrous Nili-Ravi buffalo 30% pregnancy rates were achieved, when alone CIDR was used (Naseer *et al*., 2011). Singh, (2003) reported an overall conception rate of 44.4% (16/36) in adult buffalo synchronized with CIDR during summer however animals in this study were naturally served at observed oestrus. Furthermore the anoestrus status of the buffalo in the above study was not accurately determined either by repeated ultrasonography, progesterone assay or regular teasing by a bull. Variable results have been obtained when cows were exposed to males before or during an estrous synchronization treatment. Exposure of postpartum beef cows to bulls or excretory products of bulls before or during estrous synchronization treatment increased fixed-timed AI conception rates (Berardinelli *et al*., 2007).

In contrast, others did not find any positive effect on conception rates with direct contact (Ungerfeld, 2010) or fence-line exposure (Fike *et al*., 1996; Tauck and Berardinelli, 2007) to males before the beginning of anoestrus synchronization treatment in postpartum cows and heifers.

Cortisol levels (one sample per day) at day 9, 12 and 20 during the present experiment did not differ in BE and BN groups indicating no effect of biostimulation on plasma cortisol levels. The present results corroborate with reports in beef cattle (Tauck *et al*., 2010) where exposing cows to bulls for 5 h daily over a 9 days period did not alter mean concentrations of cortisol compared to mean concentrations of cortisol in cows exposed to steers.

However characteristics of temporal patterns of cortisol were altered by increasing LH pulse frequency and decreasing cortisol pulse frequency. Thus amplitude and frequency of cortisol pulses in buffaloes need to be investigated to understand the mechanism of biostimulatory effect.

CONCLUSION

It is concluded that CIDR and fixed time artificial insemination can be used to overcome partially the problem of anoestrus and seasonality in buffalo. However, biostimulation in CIDR treated anoestrus buffalo did not increase the number of ovulating/oestrus buffalo during low breeding season.

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