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EFFICACY OF OVULATION SYNCHRONIZATION REGIMENS ON PREGNANCY RATE TO TIMED ARTIFICIAL INSEMINATION IN ACYCLIC POST-PARTUM NILI-RAVI BUFFALOES

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ABSTRACT

The present study compared the two ovulation synchronization treatments combined with a progesterone release device in buffaloes. Ninety acyclic buffaloes were divided into three experimental groups: G I (n=30), G II (n=30), and G III (n=30). The blood samples were collected before the start of the trial to determine the reproductive status (anestrous and sub-estrous). In G I and G II, CIDR was inserted for seven days and treated with PGF2α on the 6th day of protocol. In the protocol, the first estradiol benzoate (EB) and GnRH doses were given on day 0 and 2nd EB and GnRH doses on day 8 to G I and G II groups, respectively. Whereas, G III served as a control group. The G I and G II groups were observed for heat signs, but fixed time twice AI was done at 48 or 60 hours of CIDR removal, whereas G III was inseminated at detected estrus. The blood samples were also collected prior to the execution of the trial from experimental buffaloes for observing the cyclicity status through serum progesterone level. The results of cyclicity showed 78% anestrous and 22% sub-estrous status in included buffaloes. The effects of synchronization protocols revealed that estrus response was significantly greater in G I and G II groups than in the G III group. The ovulation occurrence, ovulatory follicle szie and pregnancy outcomes were also significantly higher in G I and G II compared to G III. Conclusively, including EB or GnRH at the CIDR insertion and removal times effectively enhances estrus, ovulation and pregnancy in sub-estrous and anestrous buffaloes.

Keywords: acyclic buffaloes, estrus, pregnancy, synchronization protocols

INTRODUCTION

The efficiency of female buffaloes is mainly determined by their reproductive performances (Barile, 2005), hampered by delayed maturity, long calving intervals, weak estrus expression and poor function of ovaries during the summer months (Singh, 2003; Das and Khan, 2010). Studies have shown that AI in buffaloes is less successful due to silent heat (30 to 40%), variability in estrus duration (4 to 64 hrs) and hard in predicting the exact time of ovulation (Perera, 2011). In Buffalo, the less breeding output is because of the seasonal breeding pattern, due to which most calving occurs during July and September, and less calving occurs from February to June (Singh *et al.,* 2000). During the summer, buffalo milk yield is low, which ultimately increases the demand for milk (Warriach *et al.,* 2008).

Due to the environmental conditions of south Asian countries, buffaloes showed ovarian dysfunction and reduced functioning of the pituitary, followed by reduced levels of FSH, LH and plasma progesterone concentration (Das and Khan, 2010). Last few decades, many efforts have been made to improve buffaloes' reproductive efficiency by manipulating ovarian activity (Warriach *et al.,* 2008; Yousuf *et al.,* 2015; Naseer *et al.,* 2011; Zaabel *et al.,* 2009). Different protocols have been developed to synchronize estrus response and ovulation and increase the pregnancy rate (De Rensis and Lopez-Gatius, 2007; Kumar *et al.,* 2014; Jabeen *et al.,* 2015). The efficacy of CIDR-based protocol in buffaloes is well-reputed, but the aging of ova following progesterone treatment lowers the fertility rate (Murugavel *et al.,* 2009). The present study proposed the follicular wave emergence and subsequent ovulation in a CIDR-based protocol by injecting EB or GnRH at the start and removing CIDR time. The effects of two different CIDR-based synchronization protocols on estrus expression, ovulation and pregnancy rates in

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anestrous and sub-estrous Nili Ravi buffaloes were determined.

MATERIALS AND METHODS

Experimental animals and site

This study was conducted at Military Dairy Farms of District Okara in Central Punjab, Pakistan. The experiment was carried out in the summer season during the year 2019. The research trial was performed on Nili-Ravi buffaloes (*Bubalus bubalis*). Ninety (n=90) acyclic multiparous Nili-Ravi buffaloes with normal calving history (~150 days postpartum) without any previous reproductive tract complications were selected. The selected buffaloes were further observed through ultrasound for reproductive tract soundness prior to treatments. All these buffaloes were kept under the same housing and feeding condition. The buffaloes with more than 2.5 body condition score points were included in this study. The body condition was assessed by 5-point BCS, where one denotes thin, and five indicates fat (Furgosen *et al.,* 1994).

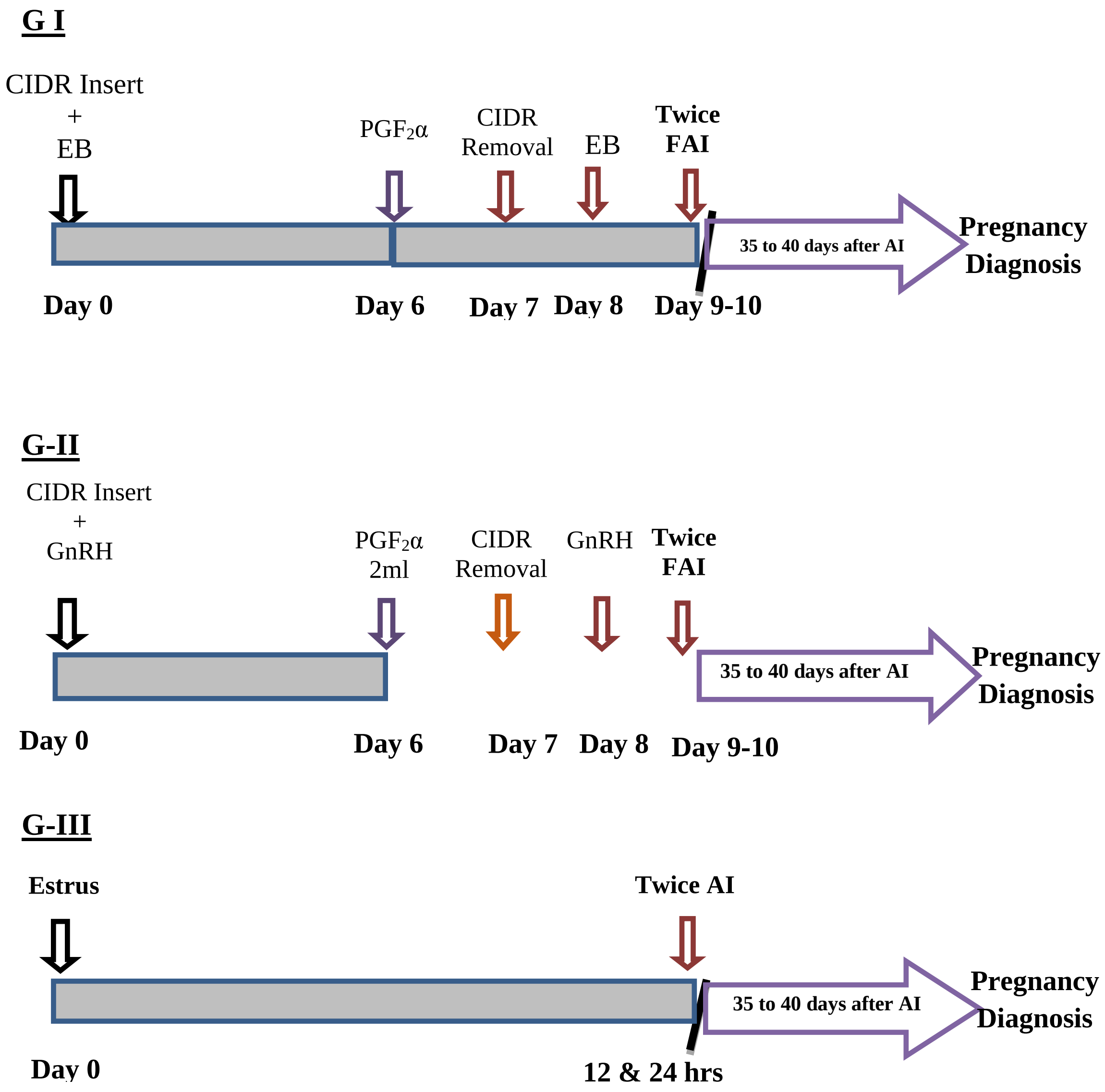

Figure 1. Experimental design of two different synchronization programs applied in acyclic Nili-Ravi buffaloes. G I (EB-CIDR-PG-EB), G II (GnRH-CIDR-PG-GnRH) and G III (Control) treatment groups of Nili-Ravi buffaloes.

Synchronization protocols

The selected buffaloes were divided into three experimental groups: G I (n=30), G II (n=30) and G III (n=30). On day 0, a slow progesterone-releasing device (Eazi-BreedTM; CIDR® Cattle Insert; 1.38 grams Progesterone; Zoetis. USA) was inserted intravaginally for seven days and were injected $PGF_2\alpha$ (Dalmazine®, Fatro, d-cloprostenol 0.150 mg; Italy; 2 ml; i.m.) on day 6 to buffaloes of G I and G II. In G I, an injection of EB (Estradiol Benzoate®, 2mg) was given for follicular wave emergence on day 0 of protocol, and the second injection of EB (2mg) dose for induction of ovulation on day 8. In G II, the first injection of GnRH (Delmeralin®, Lecirelin acetate; Fatro, Italy; 2 ml; i.m.) was given on day 0 of protocol for new follicular wave emergence, followed by a second dose of GnRH on day 8 for ovulation of follicle from a newly emerged wave. The buffaloes in G III were not treated with any hormonal protocol and served as a control group. The buffaloes were monitored for the one-month duration for the display of natural estrus (Figure 1).

Estrus Response, AI, ovulation and pregnancy diagnosis

Estrus expression in buffaloes of all three groups was assessed by visual observation. It was confirmed by swelling of the vulva, vaginal mucous discharge, micturition, bellowing, restlessness, and occurrence of tone in the uterine horns and Graffian follicle on either ovary. The ovulation rate was regularly monitored in estrus buffaloes with the help of the B-Mode Ultrasound Console (Ultrasound HS-1500 Vet/ 7.5 MHz/ Honda, Japan) until ovulation. The ovulation was evidenced by the disappearance of a large dominant follicle during the last ultrasound scanning (Sa Filho *et al.,* 2010).

Twice fixed-time AI was performed to buffaloes of G I and G II groups at 48 to 60 hrs after CIDR removal using frozen-thawed semen. At the same time, the buffaloes in G III group were inseminated at detected estrus after 12 and 24-hour intervals concerning the commencement of standing heat. Pregnancy diagnosis was done 35 to 40 days after the last AI using transrectal ultrasonography. The appearance of amniotic fluid confirmed pregnancy status, amniotic membrane, fetal heartbeat declared positive, and absence of any amniotic fluid or amniotic membrane in uterine horns declared negative.

Hormonal analyses

Blood samples were taken from the experimental buffaloes at the onset of the treatment. The samples were centrifuged within two hours after collection, and harvested serum samples were stored at -4°C until assayed. Serum progesterone levels were checked via solid phase competitive ELISA (Bio Check, Inc. USA). The sensitivity of the progesterone ELISA assay was 0.3 ng/ml, and intra- and inter-assay variation ranged between 10.8 and 12%, respectively. Animals with < 1ng/ml serum progesterone level in samples (Low progesterone level) were classified as anestrous (P4 G-I, n= 70) with no CL on either ovary. Likewise, the samples with concentrations > 1 ng/ml (High progesterone level) of plasma progesterone is considered sub-estrous (P4 G-II, n= 20) with having the CL or active luteal body on the ovary (Naseer *et al.,* 2013).

Statistical analyses

All data were analyzed with a statistical software program (SPSS, Version 19.0 for Windows; SAS Institute, Cary, North Carolina, USA) among G I, G II and G III. The follicular size, growth rate and ovulatory follicle sizes were compared between the groups using a *t*-test. A probability level of ($P < 0.05$) was considered significant among the treated groups. Also, the estrus response, pregnancy and ovulation rates were appraised using a chi-square test.

RESULTS

Cyclicity status of animals

The results of cyclicity determine that 78 % of animals were in an anestrous state and had < 1 ng/ml serum progesterone before the trial, and 22 % of animals were suffering from sub-estrous conditions with > 1 ng/ml serum progesterone level.

Estrus expression, ovulation and pregnancy rate concerning cyclicity of buffaloes.

The comparison of estrus, ovulation and pregnancy rate between anestrous and sub-estrous buffaloes has been presented in Table 1. No significant difference was noted in displaying the estrus between sub-estrous and anestrous buffaloes. Similarly, an equal proportion of buffaloes ovulated between sub-estrous and anestrous animals. Pregnancy rates were numerically lowered in anestrous buffaloes when compared with sub-estrous buffaloes.

Table 1. The comparison of estrus, ovulation and pregnancy rate between anestrous and sub-estrous buffaloes

Characteristics	Anestrous	Sub-estrous	Chi square tests of independence
Estrus response			
Yes (58)	42	16	X^2 (1)=2.72 *p*=0.09 *n*=90
No (32)	28	4	
Total	70	20	
Ovulation rate			
Yes (49)	35	14	X^2 (1)=2.51 *p*= 0.11 *n*=90
No (41)	35	6	
Total	70	20	
Pregnancy rates			
Yes (43)	32	11	X^2 (1)=0.538 *p*=0.46 *n*=90
No (47)	38	9	
Total	70	20	

Estrus expression, ovulation and pregnancy rate in buffaloes following application of synchronization protocols

The comparison of estrus response, ovulation occurrence and pregnancy rates between G-I, G-II and G-III of Nili- Ravi buffaloes are presented in Table 2. The results revealed that estrus response in G-I (80%) and G-II (86.66%) was significantly higher ($p<0.05$) than in G-III (26%) buffaloes. The ovulation occurrence and pregnancy outcomes were also improved significantly ($p<0.05$) in G-I (66.6 and 60%) and G-II (73.3 and 63%) compared to G-III (23.3 and 20%) group. The pre-ovulatory follicle size was also different ($p<0.05$) in G I and G II groups than in the G III group (Figure 2).

Table 2. Comparison of estrus response, ovulation and pregnancy rates in synchronized Nili- Ravi buffaloes

Variables	G-1	G-II	G-III	Chi square tests of independence
Estrus expression				
Yes (58)	24	26	8	X^2 (1)=28.32 *p*=0.001 *n*=90
No (32)	6	4	22	
Total	30	30	30	
Ovulation rate				
Yes(49)	20	22	7	X^2 (1)=17.83 *p*=0.001 *n*=90
No (41)	10	8	23	
Total	30	30	30	
Pregnancy rate				
Yes (43)	18	19	6	X^2 (1)=13.98 *p*=0.001 *n*=90
No (47)	12	11	24	
Total	30	30	30	

DISCUSSION

The present study demonstrated that applying CIDR synchronizes the estrus response and ovulation with optimal pregnancy in sub- and anestrous Nili- Ravi buffaloes. The initial treatments with GnRH had influenced the follicle development, and subsequent GnRH successfully synchronized the estrus and ovulation. Similar results were obtained when EB was injected at the start and removal of CIDR in treated buffaloes.

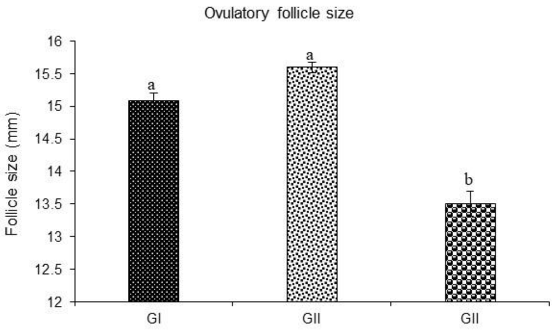

Figure 2. Ovulatory follicle size in buffaloes that ovulated in different groups. Means ± SE followed by different letters within the same row are different ($p<0.05$)

The postpartum anestrous generally coincides with dysfunctions of the hypothalamic GnRH; pituitary FSH and LH secretions are the main contributing factors in cattle and buffaloes (Naseer *et al.,* 2013). This problem has been abolished by the application of hypothalamic GnRH and synthetic progestogen (Progesterone, MGA, Crestar, PRID, and CIDR), which cause the release of pituitary Gonadotropin (FSH and LH) secretion directly or indirectly (Arshad *et al.,* 2017).

Postpartum is one of the major factors that intensify infertility in water buffaloes. Usually, anestrous is classified based on ovarian status, as non-cyclic refers to true anestrous, while cyclic, termed sub-estrous with overt estrous signs, is missing. The present study showed that 78% of buffaloes were in a true anestrous state, whereas 22% suffered from sub-estrous conditions. These findings agree with previous reports on buffaloes (Zabeel *et al.,* 2009; Naseer *et al.,* 2013). In most cases, anestrous is seen longer in buffaloes due to low hormonal concentration, mineral feeding and high environmental stress conditions. The occurrence of anestrous varies under different management conditions, and its rate is more intense in the arid zone than in irrigated areas of Pakistan. Suitable feeding, ample water supply for drinking, wallowing, and calf weaning have been proposed remedies for treating anestrous in buffaloes. But the most reliable treatment of anestrous in buffaloes is to be considered the application of suitable protocols (Bodla *et al.,* 2017).

There are numerous progesterone-based protocols have been applied to resolve this long-lasting issue. But the CIDR was started during the last decade of the 20th century and used widely for estrus synchronization and increased conception rates, especially for treating postpartum anestrous cows (Macmillan and Peterson, 1993). Buffalo is an animal of developing countries that usually suffers from the problem of postpartum anestrous. Among the different protocols, CIDR successfully treats postpartum anestrous buffaloes for better synchronization of estrus and ovulation with optimal pregnancy rates (Naseer *et al.,* 2011; Jabeen *et al.,* 2015; Yousaf *et al.,* 2015). The present study documents that are applying CIDR protocol in farm conditions of irrigated areas results in favorable estrus response with a pregnancy rate in buffaloes. Buffaloes in these areas, like arid zones, also suffer from anestrous conditions. Numerous protocols like PGF2a (Chohan, 1998), GnRH (Shah et al., 1989), PMSG (Chohan *et al.,* 1996) and CIDR (Naseer *et al.,* 2011) have been applied for the treatment of anestrous buffaloes in Pakistan. These protocols also yielded favorable responses in estrus, but the pregnancy rates were variable. But the progesterone-based protocols were always considered the best remedy for postpartum anestrous in cattle and buffaloes (Monteiro *et al.,* 2018; Khan *et al.,* 2018). The physiologically external source of progesterone maintains the circulatory concentration, which in turn exerts negative feedback on the anterior pituitary and hypothalamus for storing FSH, LH and GnRH. After the stoppage of the external source of progesterone (after 7 or 10 days), the circulatory concentration of progesterone suddenly drops, stimulating the release of stored GnRH. This release of GnRH is followed by the secretion of FSH and LH from the anterior pituitary gland, which favors the growth of follicles and maturation (Beal *et al.,* 1988). CIDR device is placed intravaginally that maintains the progesterone level in general circulation through its absorption from the vaginal walls. Its continuous release maintains the progesterone concentration alike to the luteal phase of the estrous cycle and, upon removal, induces the estrus in a large group in a small duration. So this protocol is nowadays popular worldwide for treating postpartum anestrous in buffaloes and cattle.

The ovulation happens under the cascade of events involving the hypothalamo-pituitary gonadal axis, which leads to releasing of ova from ripened follicles. In the present study, the buffaloes in the CIDR group, the ovulation rate was about 85%, and this finding is fairly similar to previous reports where animals were displayed natural (Warriach *et al.,* 2008) or synchronized estrus (Neglia *et al.,* 2003; Naseer *et al.,* 2011, 2013; Yousaf *et al.,* 2015). The pregnancy is the sequel of successful ovulation, subsequent development of the fertilized ovum, zygote division and successful attachment of the embryo with the uterine wall. The ruptured follicle is transformed into a luteal body CL that secretes the progesterone to maintain the mentioned events during embryogenesis. The pregnancy rate in CIDR-treated animals was optimal in the field condition, similar to the previous work of Zaabel *et al.* (2010) and Naseer *et al.* (2011). It is now desirable that the combination of GnRH or hCG with CIDR provides an opportunity for better estrus expression by priming the brain with progesterone and timed ovulation for a better pregnancy rate in anestrous buffaloes (Ali *et al.,* 2012; Rezk *et al.,* 2016; Rathore *et al.,* 2017; Samir *et al.,* 2019). The current data showed a similar influence of EB on estrus expression and ovulation rate. EB successfully sensitizes the hypothalamus for exhibiting improved estrus signs with tight synchrony ovulation. The initial or final treatment with EB showed similar results as previous studies (Naseer *et al.,* 2011; Yousaf *et al.,* 2015; Carvalho *et al.,* 2017). The current results are encouraging and advantageous because applying the CIDR and EB can exaggerate estrous signs intensity without compromising the pregnancy rates in sub- and anestrous buffaloes.

CONCLUSION

In conclusion, the applied synchronization protocols benefit the sub-estrous and anestrous buffaloes by inducing fertile estrus behavior and ovulation with promising pregnancy results. Including EB or GnRH at CIDR insertion or removal times is useful for promoting follicular wave emergence and subsequent ovulation for applying fixed-time AI.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR’S CONTRIBUTION

S. Bashir: Conduct the research and write up.

M. T. Tunio: Supervision, experimental design and analysis.

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