Pregnancy Outcomes Following GnRH- or Prostaglandin-Based Timed Artificial Insemination Protocols in Water Buffaloes (*Bubalus bubalis*)

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Abstract: The efficiencies of Timed Artificial Insemination (TAI) protocols in post-partum riverine dairy buffaloes were evaluated in the present research work. In Study 1, GnRH-based ovulation synchronization for Fixed Time Artificial Insemination (FTAI) protocol was evaluated for pregnancy. Buffaloes in Treatment 1 were subjected to the Ovsynch (GnRH-PGF2α-GnRH) protocol. Buffaloes in Treatment 2 were subjected to Controlled Internal Drug Release–Gonadotrophin Releasing Hormone (CIDR-Synch-GnRH) protocol, and buffaloes in Treatment 3 were subjected to CIDR-Synch-human Chorionic Gonadotrophin (CIDR-Synch-hCG) protocol. In Study 2, Prostaglandin-based estrus synchronization protocols were similarly evaluated for pregnancy. Buffaloes in Treatment 1 were treated with Prostaglandin hormone alone; buffaloes in Treatment 2 were subjected to Prostaglandin-GnRH protocol, while buffaloes in Treatment 3 were subjected to Prostaglandin-hCG protocol. Results in Study 1 revealed that supplementation of Ovsynch with CIDR in Treatment 2 and 3 resulted in significantly higher ($P<0.05$) pregnancy rates compared with Ovsynch alone (T1). Meanwhile, the use of hCG as the final ovulatory hormone in FTAI protocol (T3) yielded a significantly higher ($P<0.05$) pregnancy rate than GnRH (T2). In Study 2, results showed that prostaglandin protocols enhanced with GnRH (T2) or with hCG (T3) resulted in significantly higher ($P<0.05$) pregnancy rates (31.88±3.39 and 34.62±1.53), respectively, compared with Prostaglandin alone (T1, 23.91±2.49). However, pregnancy rates in Prostaglandin-based protocols (T2) and (T3) were not significantly different ($P<0.05$). In sum, the present study demonstrated that supplementation with exogenous progesterone (CIDR) improved the efficiency of Ovsynch FTAI protocol while using hCG as the final ovulatory hormone is found to be the best among FTAI protocols. Meanwhile, a Prostaglandin-based protocol enhanced with ovulatory hormones, either GnRH or hCG, on the day of AI improved pregnancy rates in post-partum water buffaloes.

Keywords: GnRH, hCG, progesterone, prostaglandin, timed artificial insemination, water buffaloes.

INTRODUCTION

Artificial insemination (AI) is the very first reproductive biotechnology used in disseminating genetic material from superior sires [1]. Estrus synchronization (ES) has become the tandem technology of AI to maximize its benefit for milk and beef production purposes. Initially, estrus induction and synchronization have been carried out using Prostaglandin (PGF2α) and its analogs, mainly to induce lysis of the corpus luteum (CL) and estrus for pre-determined AI in cattle and buffaloes.

In the Philippines, the use of this tandem technology of ES and AI has been strengthened with the implementation of two-phase UNDP-FAO funded Projects from 1980 to 1990, which have become valuable and widely used reproductive tools in the carabao upgrading program in the country. Complemented by the rigorous initiatives on buffalo semen cryopreservation, thousands of calves are produced annually, contributing to local milk production and the growth of the Philippine dairy industry. However, buffalo reproduction remains a huge challenge due to some inherent reproductive concerns such as poor estrus expression, seasonal infertility, delayed sexual maturity, anestrus, and long inter-calving intervals hampering the full reproductive and production potential of this species [2, 3]. Meanwhile, it was identified that post-partum anestrus in buffaloes is responsible for long calving intervals [4]. Enormous research efforts on AI, reproduction, and associated management systems are continuously pursued to improve fertility in water buffaloes.

Recent advances in AI include the so-called fixed time AI (FTAI), which was brought about by the development of the original ovulation synchronization protocol, Ovsynch, in dairy cattle [5]. This ovulation synchronization protocol involves mainly two times injections of GnRH, seven days before and two days

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after the injection of Prostaglandin in the usual 10-day FTAI program. Synchronization of ovulation followed by fixed-time AI has proven to increase ovulation and pregnancy rates in livestock species. After that, various modifications of the original protocol were generated to achieve better precision and improve the efficiency of FTAI, which benefits the livestock industry [6-9].

Faced with consistent challenges of poor AI efficiency in water buffaloes, FTAI was first attempted in the country in 2014, primarily to address the problem of post-partum anestrus, repeat breeders, and long calving intervals specifically of dairy buffaloes at the Philippine Carabao Center, Buffalo National Gene Pool. FTAI activities in the farm were initiated with trials on Ovsynch protocol coupled with an ovarian examination by transrectal ultrasonography during hormonal injection and the conduct of AI. Thereafter, an intensified reproductive management program was defined, and research initiatives were initially focused on GnRH-based ovulation synchronization and FTAI to improve pregnancy in post-partum dairy buffaloes. Meanwhile, improvement of the Prostaglandin-based estrus synchronization and AI protocol was deemed necessary, being a practical technology for upgrading the native buffaloes in the country. Prostaglandin plus GnRH has proven to be very successful in synchronizing estrus in cattle and buffaloes [10, 11] for a genetic improvement program.

Further research on Timed Artificial Insemination technologies has yet to be pursued to determine the best protocols suitable for a breeding program, breeding season, and reproductive stages, considering the efficiency and economic implications associated with each type of protocol. Specifically, the aim of the present study was to identify an efficient GnRH-based ovulation synchronization protocol and a Prostaglandin-based estrus synchronization protocol to develop a strategic Timed AI and reproductive management program to maximize the productivity of post-partum dairy buffaloes in the country.

MATERIALS AND METHODS

The study was conducted at the Philippine Carabao Center, National Headquarters and Gene Pool, Science City of Munoz, Nueva Ecija and Lomboy Farm, San Jose City, from September 2019 to December 2023. All works and procedures involving the use of animals for scientific research were followed in accordance with the requirements for the protection and welfare of animals of the Philippine Animal Welfare Act of 1998 and were approved for experimentation by the Ethics Committee of the Philippine Carabao Center, Department of Agriculture.

Animal Selection and Determination of Body Condition Score

Riverine buffaloes of at least 60 days post-partum with body condition score (BCS) of not less than three (3) and at least one (1) of the ovaries is equal or greater than two (2) cm in length or width and with dominant follicle size of not less than 7 mm in diameter were selected and used in Study 1. The same criteria were used for Study 2, except that only animals with corpus luteum were selected for the Study.

Evaluation of the Body Condition Score was done according to the method described by Alapati *et al*., 2010 [12]. Briefly, a BCS of 1 stands for emaciated animals; a BCS of 2 indicates a dorsal spine that is pointed to the touch, with the hips, pins, tail head, and ribs being prominent; a BCS of 3 represents those water buffaloes whose ribs are usually visible with little fat cover, and dorsal spine are barely visible; a BCS of 4 is for animals that are smooth and well covered, but with no marked fat deposits; and BCS of 5 is for heavy deposits of fat clearly visible on the tail head and brisket, with the dorsal spines, ribs, hooks, and pins fully covered and unable to be felt even with firm pressure.

Transrectal ultrasonographic examinations and measurements of the ovaries were conducted using an ultrasound scanner (HS-1600, Honda Electronics Co., Ltd., Japan). On Day 0 of the treatment protocol, the sizes of the dominant follicle (DF) present in the left or right ovaries were measured.

Experimental Design

Study 1. Comparative Efficiencies of GnRH-based Ovulation Synchronization Protocols

A total of 404 animals were used in Study 1. In Treatment 1 (n=122), animals were subjected to the Ovsynch protocol for ovulation synchronization followed by FTAI (Figure **1**). Briefly, 1st Gonadotrophin-releasing hormone (GnRH, Fertagyl, 2ml IM) was injected on Day 0, and Prostaglandin ($PGF_2\alpha$, 2ml Veteglan, IM) was injected on Day 7. Injection of the 2nd GnRH was done on Day 9, and the conduct of the 1st and 2nd AI, with 8 hrs intervals, was done on Day 10. Pregnancy diagnosis was conducted by transrectal ultrasonography on Days 35-40 post-AI.

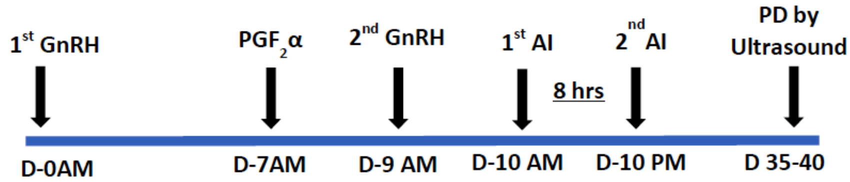

Figure 1: Schematic presentation of Ovsynch protocol. Day 0: Injection of 1st Gonadotropin-releasing hormone (GnRH). Day 7: Injection of PGF2α. Day 9: Injection of 2nd GnRH. Day 10: Conduct of 1st and 2nd AI with 8 hrs intervals. Days 35-40: Conduct pregnancy diagnosis by transrectal ultrasonography.

In Treatment 2 (n=139), the animals were subjected to Controlled Internal Drug Release-Gonadotrophin releasing hormone protocol (CIDR-Synch-GnRH; Figure **2**). In brief**,** CIDR (1.38g of micronized natural progesterone, Pfizer EAZI-Breed) were inserted in the vagina, with simultaneous injection of 1stGnRH (Fertagyl, 2 mL, IM) on Day 0. On Day 7, injection of Prostaglandin (PGF2α, 2ml Veteglan, IM) and removal of CIDR inserts were done. 2nd GnRH was injected after the 1st insemination on the morning of Day 9. FTAI was done twice with an 8-hour interval on Day 10. Pregnancy diagnosis was conducted by transrectal ultrasonography on Days 35-40 post-AI.

In Treatment 3 (n=143), buffaloes were subjected to CIDR-Synch- human chorionic gonadotrophin protocol (CIDR-Synch-hCG; Figure **3**). The procedure was similar to that of T2 from Day 0 to Day 7. On Day 9, however, the animals were injected intramuscularly with human-Chorionic Gonadotrophin (hCG, Chorulon, 10,000 units) instead of GnRH. The 1st insemination was performed on the morning of Day 10, with 2nd AI at 8hrs intervals. Pregnancy diagnosis was conducted by transrectal ultrasonography on Days 35-40 post-AI.

Study 2. Comparative Efficiencies of Prostaglandin-Based Estrus Synchronization Protocols

A total of 440 animals were used in the Study. In Treatment 1 (n=155), the conventional Prostaglandin protocol for estrus synchronization was followed (Figure **4**). Qualified animals were injected intramuscularly with Prostaglandin (PGF2α, 2 ml Veteglan) on Day 0. Artificial insemination was performed twice on Day 3, in the morning and afternoon, 8 hrs apart. Pregnancy diagnosis was conducted by transrectal ultrasonography on Days 35-40 post-AI.

In Treatment 2, buffaloes (n=138) were injected with Prostaglandin (PGF2α, 2ml Veteglan, IM) on Day 0 (Figure **5**). Similarly, twice artificial insemination was conducted on Day 3 but the animals were injected with 2 ml GnRH, as ovulatory hormone, after the first insemination in the morning. Pregnancy diagnosis was

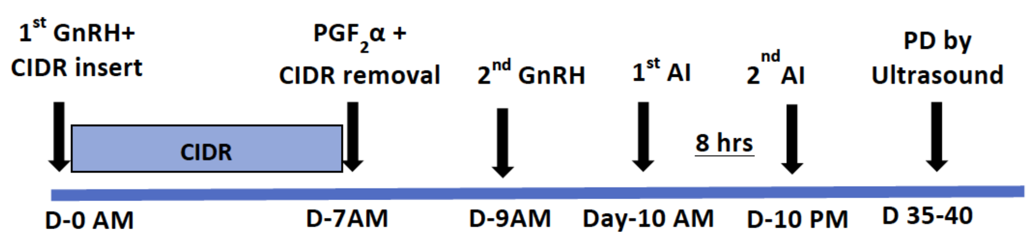

Figure 2: Schematic presentation of CIDR-Synch-GnRH protocol. Day 0: Injection of 1st GnRH and insertion of CIDR. Day 7: Injection of PGF2α and removal of CIDR. Day 9: Injection of 2nd GnRH. Day 10: Conduct of 1st and 2nd AI with 8 hrs intervals. Days 35-40: Conduct pregnancy diagnosis by transrectal ultrasonography.

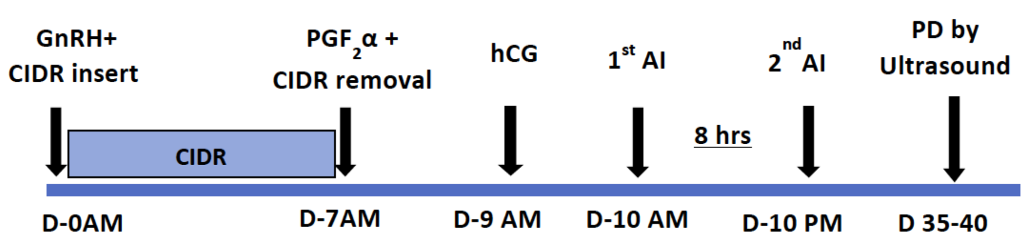

Figure 3: Schematic presentation of CIDR-Synch-hCG protocol. Day 0: Injection of GnRH and insertion of CIDR. Day 7: Injection of PGF2α and removal of CIDR. Day 9: Injection of human-Chorionic Gonadotropin (hCG). Day 10: Conduct of 1st and 2nd AI at 8 hrs intervals. Days 35-40: Conduct pregnancy diagnosis by transrectal ultrasonography.

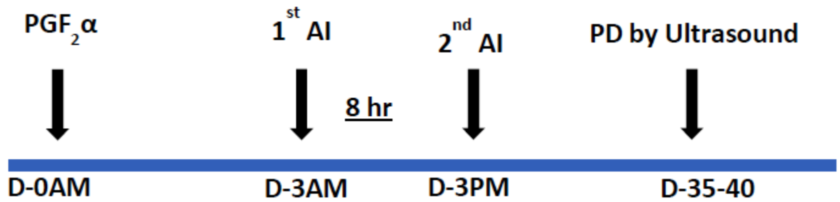

Figure 4: Schematic presentation of Prostaglandin protocol. Day 0: Injection of PGF2α. Day 3: Conduct of 1st and 2nd AI at 8-hour intervals. Days 35-45: Conduct of pregnancy diagnosis by transrectal ultrasonography.

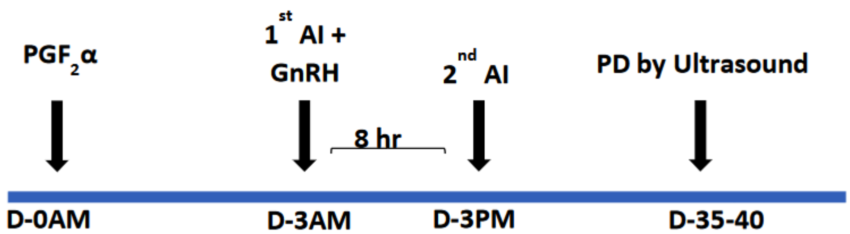

Figure 5: Schematic presentation of Prostaglandin-GnRH protocol. Day 0: Injection of PGF2α. Day 3: Conduct of 1stAI and injection of GnRH and 2nd AI at 8 hrs intervals. Day 35-40 post AI, pregnancy diagnosis was done by transrectal ultrasonography.

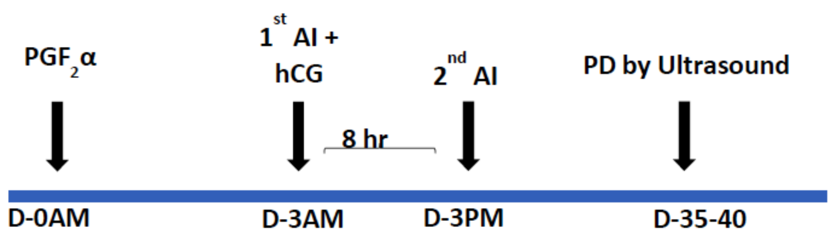

Figure 6: Schematic presentation of Prostaglandin-hCG protocol. Day 0: Injection of PGF2α. Day 3: Injection of hCG and conduct of 1st and 2nd AI with 8 hrs interval. Days 35-45: Conduct of pregnancy diagnosis by transrectal ultrasonography.

conducted by transrectal ultrasonography on Days 35-40 post-AI.

In Treatment 3 (n=147), a similar procedure was performed on Day 0 and Day 3, except that GnRH was replaced by human-Chorionic Gonadotrophin (hCG, Chorulon, 10,000 units).

Artificial Insemination

All the animals were artificially inseminated using frozen-thawed water buffalo semen derived from bulls with proven fertility. Artificial insemination was done by two (2) selected AI technicians from the Philippine Carabao Center. Selections of AI technicians were based on their performances for the last 4 years of insemination. The presence of mucus discharge and tonicity of the uterine horn were determined at the time of artificial insemination.

Transrectal Ultrasonography

The ultrasound examinations were performed using a transrectal ultrasound scanner (Honda, HS-1600V, Japan) equipped with a 7.5 MHz linear array transducer designed for intra-rectal placement [13]. The scanning of uterine horns was performed on their dorsal and lateral surfaces. Pregnancy status was determined following the criteria described by Fricke *et al*., 2016 [14] with some modifications. Briefly, the criteria include the presence or absence of corpus luteum (CL), uterine fluid, and fetus. Cows are considered pregnant when CL is present, along with the presence of both uterine fluid and fetus.

Statistical Analysis

Data gathered in Studies 1 and 2 were presented as means ± SD and were analyzed and compared by Analysis of Variance (ANOVA), followed by Tukeys-

Kramer's HSD as a *post hoc test*. All analyses were performed using JMP Statistical Software (Version 11.1.1 SAS Institute, Inc., Cary, NC, USA). The minimum level of significance was set at P<0.05.

RESULTS

Study 1. Comparative Efficiencies of GnRH-based Ovulation Synchronization Protocols

Efficiencies of different GnRH-based ovulation synchronization and FTAI protocols are presented in Table **1**. The results revealed that the pregnancy rates in treatments with CIDR supplementation (T2 and T3) were significantly higher (P<0.05) from treatment without CIDR supplementation (T1). Moreover, T3 with CIDR supplementation and hCG as ovulatory hormone significantly differed (P<0.05) in pregnancy rate compared with T2, which used GnRH for ovulation. The results imply that the supplementation of Ovsynch protocol with CIDR, an exogenous form of progesterone, improved pregnancy outcomes in post-partum buffaloes. In addition, hCG is a better final ovulatory hormone, yielding higher pregnancy outcomes than GnRH. Overall, the CIDR-Synch-hCG protocol (T3), basically Ovsynch enhanced with CIDR and hCG, is the most efficient among the GnRH-based ovulation and FTAI protocols.

Study 2. Comparative Efficiencies of Prostaglandin-based Estrus Synchronization Protocols

Pregnancy outcomes following Prostaglandin-based estrus synchronization and Artificial Insemination in post-partum dairy buffaloes are presented in Table **2**. The results of the study showed that T2 (Prostaglandin + GnRH) and T3 (Prostaglandin + hCG) are significantly higher (P<0.05) compared with TI (Prostaglandin alone). However, pregnancy rates did not differ (P>0.05) between the use of GnRH (T2) and hCG (T3) in Prostaglandin-based timed insemination protocols. The findings of the present study suggest that the use of Prostaglandin alone will result in lower pregnancy rates, but enhancing the protocol with the ovulatory hormones, either GnRH or hCG, on the day of AI can improve pregnancy in post-partum dairy buffaloes.

DISCUSSION

Timed artificial insemination (TAI) programs provide an organized approach for the induction of estrus and follicle ovulation, which enhances the use of AI and the progress of genetic gain to improve reproductive efficiency in dairy and beef herds [15,16] and buffaloes [17-20]. Timed AI in the present context optimized both GnRH-based ovulation synchronization protocols to manipulate follicle development and ovulation and the Prostaglandin-based estrus synchronization schemes to primarily control the corpus luteum phase of the estrus cycle in post-partum dairy buffaloes.

The present work on the Ovsynch protocol achieved a pregnancy rate of 32.82%, which is within the range reported in cycling lactating buffaloes [17,18]. The result is comparable (33.3%) to spontaneous inseminated buffaloes [19]. In contrast, premature estrus and ovulation, as well as inconsistent and lower

Table 1: Pregnancy Rates Following GnRH-Based Ovulation Synchronization and Fixed Time Artificial Insemination Protocols in Post-Partum Water Buffaloes

Treatments	No. of animals used	No. of animals pregnant	% Pregnancy
T1 (Ovsynch)	122	40	32.82 ± 1.39[c]
T2 (CIDR-Synch-GnRH)	139	54	38.97 ± 1.39[b]
T3(CIDR-Synch-hCG)	143	66	46.04 ± 1.39[a]

Data were presented as Means ± SD, with 4 replications.
[a,b,c]Values with different superscripts within a column are significantly different at P<0.05.

Table 2: Pregnancy Rates Following Prostaglandin-Based Estrus Synchronization and Artificial Insemination Protocols in Post-Partum Water Buffaloes

Treatment	No. of animals used	No. of animals pregnant	% Pregnancy
T1 (Prostaglandin alone)	155	37	23.91 ± 2.49[b]
T2 (Prostaglandin + GnRH)	138	44	31.88± 3.39[a]
T3 (Prostaglandin + hCG)	147	51	34.62 ± 1.53[a]

Data were presented as Means ± SD, with 4 replications.
[a,b]Values with different superscripts within a column are significantly different at P<0.05.

pregnancy outcomes were reported following the Ovysnch protocol, which could probably be due to the variable number of cows in anestrous conditions. Meanwhile, Purohit *et al*. 2019 [20] emphasized that estrus and ovulation can be effectively synchronized in buffaloes using Ovsynch protocols during the breeding season; however, during the non-breeding season, progestogen-based protocols in combination with estradiol, eCG, PG, and GnRH are better options for timing insemination and planning calvings.

On the other hand, supplementation of Ovsynch protocol with CIDR in the initial hormonal treatment resulted in an increased pregnancy rate in post-partum buffaloes, supporting previous beneficial claims of Progesterone supplementation. Accordingly, the exogenous progesterone minimizes the growth and development of the cohort follicles due to its negative effect on the synthesis and production of Follicle Stimulating Hormone until after its withdrawal on day 7, enabling synchronous growth and ovulation [21]. Similarly, insertion of P4-releasing devices for 5–10 days maintains plasma concentrations of P4 for a given period, and as P4 concentrations reach sub-luteal levels during treatment, LH pulse-frequency increases, leading to follicular growth preventing atresia of the dominant follicle [22,23]. The said mechanism enables the growth and maturation of the dominant follicle capable of ovulation, even in anestrous animals, prevents the formation of a short-lived CL, and enables the development and maintenance of pregnancy [24].

The highest pregnancy outcome in the present work was achieved when the second GnRH in the CIDR-Synch-GnRH protocol was replaced by hCG as the final ovulatory hormone. The present finding is consistent with earlier work, which demonstrated that the follicle size and ovulation rate increased in animals injected with hCG compared with animals given GnRH at the end of the hormonal treatment [25]. It was likewise reported that hCG improves the fertility of dairy cows even during summer months and has no effect on pregnancy rates in cool climates. It is considered that season could be an important factor affecting the success of hCG [26]. Further injection of 3,000 IU hCG on Day 5 of the estrous cycle induced alterations in follicle development, enhanced conception rates during the next cycle, and increased embryonic survival, which could be associated with increased progesterone secretion that favors embryonic survival [27, 28]. However, a lower pregnancy rate (34%) was observed in cows that received hCG than those treated with GnRH (49%), and more hCG-treated cows exhibited short estrous cycles following TAI which could be due to luteal deficiency following TAI [29]. It was pointed out that GnRH action is mediated by the hypothalamus-pituitary-gonadal axis, wherein GnRH stimulates the hypophysis to release the endogenous LH. However, if there is a lack of GnRH receptors in the hypothalamus, or if there is inadequate stored LH in the hypophysis, GnRH will not induce LH release, leading to ovulation failure [30]. Overall, incorporating hCG into the CIDR-Synch-hCG protocol in the present study could have enhanced ovulation and fertility, resulting in the highest conception in post-partum buffaloes subjected to the FTAI program.

The above GnRH-based FTAI protocols, however, require a long period (10 days) from the start of hormonal treatment to the conduct of timed AI with implications on labor and cost; thus, shorter estrous synchronization schemes would be desirable. The short Prostaglandin synchronization method relies on a single injection of the hormone and requires only 3-4 days to its completion. It is, however, only effective in cycling animals, which requires evidence of CL at the start of injection for better estrus response. Recent advances in ovarian manipulations, which cover both the induction of estrus and follicle ovulation in FTAI programs, provided the opportunity to use GnRH or hCG to induce estrus and follicle ovulation in Prostaglandin-treated animals. The result of the present study revealed that supplementation of Prostaglandin either with GnRH or hCG at the time of insemination on Day 3 increased pregnancy rate in post-partum dairy buffaloes, which conforms with previous works on buffaloes [31]. Adding GnRH or hCG to Prostaglandin protocol resulted in a more synchronous estrus and ovulation in the herd, ensuring more cows cycling for breeding at the same time, compared with Prostaglandin alone. The injection of GnRH or hCG coinciding with the day of AI is simply dubbed as Enhanced AI under our current reproductive program and operation. In sum, the present study demonstrated that hormonal protocols using combinations of Prostaglandin, CIDR, GnRH, and hCG improved AI efficiency in post-partum dairy buffaloes.

CONCLUSION

Combining CIDR and hCG with Ovsynch in the CIDR-Synch-hCG protocol in the present study efficiently induced both estrus and follicle ovulation, which resulted in improved fertility and the highest pregnancy outcome in post-partum buffaloes. Specifically, in terms of the Philippine Genetic

Improvement Program, the Prostaglandin-GnRH/hCG protocols are recommended for National Carabao upgrading and crossbreeding activities, while the CIDR-Synch-hCG protocol is mainly recommended for purebred buffalo production. Applying these Timed AI protocols in buffalo genetic improvement programs can lead to more efficient reproductive performance and higher profitability from dairy buffalo-raising activities.

AVAILABILITY OF DATA AND MATERIAL

Data and material for this research are available from the main author upon request.

CONFLICT OF INTEREST

We hereby declare that there is no conflict of interest with respect to the publication of this manuscript.

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