Optimizing Fixed-Time Artificial Insemination Efficiency through Co-Synch Techniques in Water Buffaloes (*Bubalus bubalis)*

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Abstract: The main aim of the study was to optimize the potential of CIDR-CoSynch-hCG protocols developed in water buffaloes. Multiparous animals (163) were assigned to three hormonal treatments. CIDR-Synch-hCG (T1/Control), wherein CIDR and GnRH were given (day 0), prostaglandin (day 7), hCG (day 9), and artificial insemination (AI) was performed twice on day 10. CIDR-CoSynch-hCG-day-9 (T2): Same hormones were given on days 0 and 7, but injection of hCG and AI were concomitantly performed on day 9. CIDR-CoSynch-hCG-day-10 (T3): the same hormones were given on days 0 and 7, but hCG and AI were injected concomitantly on day 10. The size of the pre-ovulatory follicle (POF) at the time of AI was likewise determined to evaluate its influence on pregnancy. The effect of the number of inseminations on pregnancy was determined. Results revealed that pregnancy rates of T1 (43.70%), T2 (43.27%), and T3 (46.07%) were not significantly different (P>0.05). The study demonstrated that simultaneous injection of hCG and AI, either on Day 9 or Day 10 of the CIDR-CoSynch-hCG protocol, is as effective as doing them separately (T1/Control). In addition, large-size (≥12 mm) POF at the time of AI increased pregnancy outcomes (65.52%) compared to medium (41.46%) and small (26.05%) follicles. Meanwhile, twice insemination (51.10%) resulted in significantly higher (P=0.0004) pregnancy rates than single insemination (21.64%). This work identifies key factors and innovative strategies to enhance FTAI implementation in water buffaloes.

Keywords: CIDR-Synch-hCG, CIDR-CoSynch-hCG, pre-ovulatory follicle, artificial insemination, water buffaloes.

INTRODUCTION

Fixed-time artificial insemination (FTAI) following synchronization of ovulation by the OvSynch procedure has been adapted for use in buffalo species [1]. This protocol was originally developed to address non-cyclicity in dairy cattle [2]. The main objective of the original OvSynch procedure was to allow all cows to be inseminated at a similar time post-partum and to improve the herd's pregnancy rate [2, 3]. It also aimed to reduce the number of undetected estrus cows, which could be potentially inseminated and made pregnant [4]. However, one problem with FTAI technology is the strict protocol requiring insemination 14-16 hours after the 2nd gonadotropin-releasing hormone (GnRH) or human chorionic gonadotropin (hCG) injection; otherwise, the timing of artificial insemination (AI) is compromised, leading to reduced pregnancy rates. Numerous modifications to the OvSynch protocol have been implemented since its inception to enhance AI effectiveness. These include, among others, Double Synch, Heat Synch, Pre-Synch-OvSynch, and Select-Synch [5].

In the Philippines, FTAI was initiated in water buffaloes in an attempt to address the problematic animals or repeat breeders at the Philippine Carabao Center (PCC), National Genepool, in December 2014, which started with the validation of the widely used and existing protocol OvSynch and its further improvement involving a controlled internal drug release (CIDR) device, thus the CIDR-Synch protocol. This is the same as OvSynch except for an exogenous progesterone supplementation on Day 0 of the protocol [5]. The above work yielded conception rates of 31.63% for OvSynch and 43.06% for CIDR-Synch, and substituting GnRH with hCG on Day 9 in the CIDR-Synch protocol resulted in the highest pregnancy rate of 58.04% [6]. As a result, the CIDR-Synch-hCG has been used as the working FTAI protocol in activities in buffaloes in many regions of the country.

However, one major drawback of the FTAI protocol is the long duration of the program (11 days), which requires almost 5 times as much time working with animals (at Day 0, 7, 9, 10 am, and 10 pm). The

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sequential activities have important implications for labor costs, travel costs, and the frequency of animal handling. Thus, it is worthwhile to explore ways to reduce or adjust the number of days of animal handling and hormonal treatment for economic and practical considerations. In cattle, shortening the program by 1 day by simultaneously injecting the ovulatory hormone (GnRH) on day 9 with the first AI, called the CoSynch procedure [7], is widely used. In the current study, a similar attempt is being made to develop more practical CIDR-CoSynch-hCG protocols that require less animal handling and are effective in water buffaloes. Injection of hCG and AI was performed simultaneously on day 9 or day 10, thereby emphasizing the size of POF at the time of AI, which can influence pregnancy outcome. The present study likewise examined the optimal number of inseminations to be performed under the CIDR-CoSynch-hCG procedures. It is worthwhile to optimize the efficiency of the CIDR-CoSynch-hCG protocol to make the AI time more flexible, with fewer animal handling events during the program, without compromising efficiency.

MATERIALS AND METHODS

All activities and procedures involving the use of animals for scientific research complied with the requirements for the protection and welfare of animals as outlined in the Philippine Animal Welfare Act of 1998. This compliance was approved by the Ethics Committee for experimentation at the Philippine Carabao Center (PCC) National Headquarters and Genepool, Department of Agriculture.

Animal Selection

The study used dairy Murrah buffaloes maintained at the PCC National Genepool. Animals were raised under confinement and provided with a daily ration of concentrates, fresh forage, and rice straw, with continuous access to potable water. Only adult, multiparous animals (aged approximately 5-9 years) with a body condition score (BCS) greater than 3.0 [8] were subjected to hormonal treatments. Transrectal ultrasound (HS-1600, Honda Electronics Co., Ltd., Japan) was used to check the reproductive status of the cows at the start of the treatment (day 0). Pregnant cows and those with small ovaries (<10 mm) were excluded from the experiment. The study was conducted during the wet season (June to November) to minimize the confounding effects of heat stress on fertility.

Experimental Design

Study 1: Comparative Efficiencies of Different CIDR-CoSynch-hCG Protocols in Water Buffaloes

A total of one hundred sixty-three (163) multiparous animals were randomly assigned using a simple random sampling (lottery method) to three (3) hormonal treatments. The control group (T1) followed a standard CIDR-Synch-hCG protocol (Figure **1**), in which animals received a 2 ml intramuscular (IM) injection of gonadotropin-releasing hormone (GnRH, Cystorelin, 50 µg/ml) simultaneous with intravaginal insertion of a controlled-internal drug release (CIDR, Eazi-Breed CIDR, 1.38 g progesterone) on day 0 (between 0700

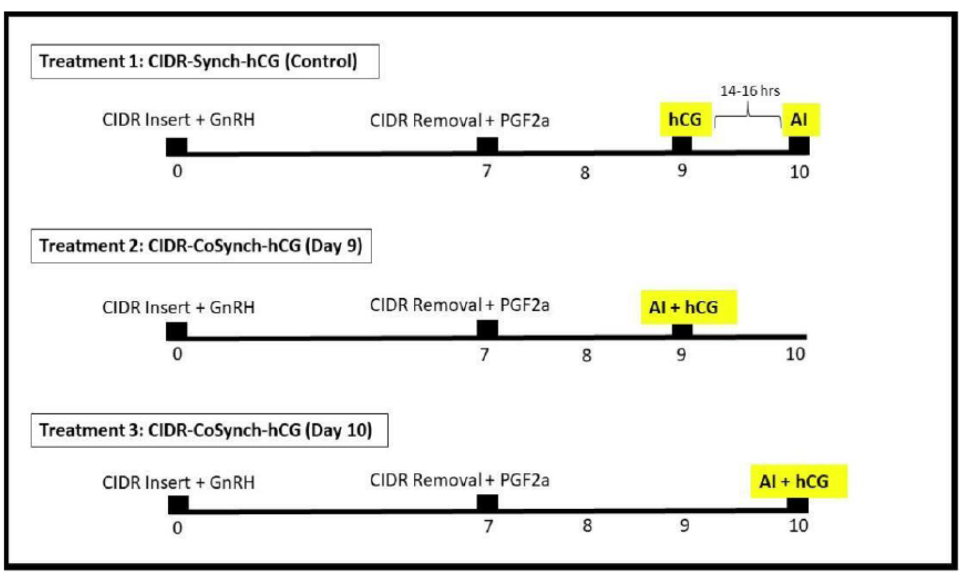

Figure 1: Schematic diagram for CIDR-Synch-hCG (T1; control), CIDR-CoSynch-hCG-Day 9 (T2), and CIDR-CoSynch-hCG-Day 10 (T3) FTAI protocols.

and 0900 h). The CIDR was removed, and cows received an IM injection of 5 ml prostaglandin (PGF2α, Lutalyse, 5 mg/ml) on day 7 (between 0700 and 0900 h). A 2 ml IM injection of human chorionic gonadotropin (hCG, Chorulon, 1,000 I.U./ml) was given on day 9 (between 16:00 and 17:00 h) as an ovulatory hormone, and artificial insemination (AI) was conducted twice on day 10 (14-16 hours and 22-24 hours post-hCG injection). For the CIDR-CoSynch-hCG treatment groups, the same CIDR-Synch-hCG protocol was followed, except that hCG was given concomitantly with the first AI on day 9 (Treatment 2) or with the first AI on day 10 (Treatment 3); AI was also conducted twice (with an 8-hour interval). A schematic diagram of all three protocols is provided in Figure **1**. The experiment was conducted in three replicates.

Follicular growth was monitored in all animals subjected to the CIDR-CoSynch-hCG treatments. The size of the pre-ovulatory follicle (POF) at the time of AI was recorded to evaluate its influence on pregnancy outcome. The POF size was categorized into three groups (small: <10 mm; medium: 10.0-11.9 mm; large: ≥12 mm) to determine the subsequent pregnancy rates.

Study 2: The Effect of the Number of Inseminations on AI Efficiency in Water Buffaloes under the CIDR-CoSynch-hCG program

The pregnancy rates of animals under CIDR-CoSynch-hCG protocols with either single or twice AI (with an 8-hour interval) were determined to evaluate the effectiveness of these protocols in terms of conception rates and overall reproductive performance.

Monitoring, Artificial Insemination, and Pregnancy Diagnosis

Transrectal ultrasound (HS-1600, Honda Electronics Co., Ltd., Japan) was used to monitor follicular development and measure follicle diameter during treatment and the first AI. It is also used to diagnose early pregnancy at days 30-40 post-AI and to confirm it at day 60 post-AI by rectal palpation. The agency's Genetic Improvement Program Coordinator assigned one skilled technician to perform artificial insemination using frozen-thawed buffalo semen from a sire with proven fertility for breeding purposes.

Statistical Analysis

All data were analyzed using JMP Statistical software (version 17.1.1). For Study 1, pregnancy rates among the three treatment groups were compared using a two-way ANOVA with treatment and follicle category as fixed factors. For Study 2, a two-way ANOVA was used to test the effects of treatment, number of inseminations (single vs. double), and their interaction on pregnancy rate. Exact P-values from the ANOVA effect tests are reported. When significant main effects were found ($P<0.05$), post-hoc comparisons were performed using Tukey's HSD test. The minimum level of significance was set at $P<0.05$.

RESULTS

Study 1: Comparative Efficiencies of Different CIDR-CoSynch-hCG Protocols in Water Buffaloes

The present study revealed no significant interaction between treatment group and follicle category on pregnancy rates $F(4,100)=0.09$, $P=0.99$; Supplemental Table **1**). Pregnancy rates attained by the three treatments were not significantly different ($P>0.05$; Table **1**). Hence, AI can be performed simultaneously with hCG injections either on day 9 or 10 without significantly affecting pregnancy rates.

Follicle size was measured at the time of AI on day 9 or 10, and its relationship to subsequent pregnancy rates was analyzed. Data were pooled and classified based on follicle size categories (Table **2**). Pregnancy rates varied across follicle categories: Category III (≥12 mm) had the highest rate (65.52%), followed by Category II (41.46%) and Category I (26.05%). As shown in Supplemental Table **1**, the effect of follicle

Table 1: Pregnancy Rates in Water Buffaloes following Different CIDR-Based FTAI Protocols

Treatment group	No. of animals treated	Pregnancy (30-40 days post-AI)	
		Number	%
T1: CIDR-Synch-hCG (Control)	57	27	43.70 ± 0.08[a]
T2: CIDR-CoSynch-hCG (day 9)	55	24	43.27 ± 0.08[a]
T3: CIDR-CoSynch-hCG (day 10)	51	25	46.07 ± 0.08[a]

[a]Values are means ± SE of 3 replications. Within columns, values with the same superscripts are not significantly different (P>0.05).

Table 2: Pregnancy Rates Based on Follicle Group at the Time of First AI under CIDR-CoSynch-hCG-day 9 & 10

Follicle group	No. of animals treated	Pregnancy (30-40 days post AI)	
		Number	%
Category I (<10mm)	32	8	26.05 ± 0.08[b]
Category II (10.0-11.9mm)	45	20	41.46 ± 0.08[ab]
Category III (≥12mm)	29	21	65.52 ± 0.08[a]

[a,b]Values are means ± SE of 3 replications. Statistical analysis of follicle category effect is presented in Supplemental Table **1**.

Table 3: Pregnancy Rates Based on the Number of Inseminations in Water Buffaloes

No. of insemination	No. of animals treated	Pregnancy (30-40 days post AI)	
		Number	%
Single AI	60	13	21.64 ± 0.06[b]
Double AI	82	42	51.10 ± 0.05[a]

[a,b]Values are means ± SE of 3 replications. Within columns, values with different superscripts are significantly different (P = 0.0004).

category on pregnancy rate was significant in the overall model ($F(2, 103) = 4.02$, $P = 0.021$).

Study 2: The Effect of the Number of Inseminations on AI Efficiency in Water Buffaloes under the CIDR-CoSynch-hCG program

There was no significant interaction between treatment group and number of inseminations on pregnancy rate ($F(2,136)=0.0124$, $P=0.99$; Supplemental Table **2**). While pregnancy rates did not differ significantly among the treatment groups for this subset of animals ($F(2,139)=0.0843$, $P=0.92$; Supplemental Table **2**), the number of inseminations (single vs. double) had a significant main effect($F(1,140)=13.23$, $P=0.0004$; Table **3**). The highest pregnancy rate (51.10%)was achieved with double AI, regardless of the treatment group. This result indicates that double AI is advisable to increase pregnancy rates when using the CIDR-CoSynch-hCG program.

DISCUSSION

The present work showed that the CIDR-CoSynch-hCG and CIDR-Synch-hCG protocols achieve equivalent pregnancy rates. This matches a recent study in Murrah buffaloes, which found a 48.6% conception rate with a CoSynch+CIDR protocol [9], similar to the conception rates in our T2 and T3 groups. Some studies in cows and buffaloes have reported pregnancy rates of 40–55% under a modified CoSynch protocol [10]. However, earlier studies on CoSynch protocols across different cattle breeds showed wide variation, with pregnancy rates ranging from 59–67% [7, 11]. Notably, most CoSynch studies reporting higher pregnancy rates (58.3% to 77.3%) were conducted in heifers [12]. Overall, the current research supports earlier findings that CoSynch methods are simpler, less labor-intensive, require less animal handling, and allow artificial insemination (AI) without a strict time window, without affecting pregnancy outcomes. Also, this study confirms that administering hCG with AI in the CoSynch protocol improves ovulation of pre-ovulatory follicles (POF), helping to better time insemination [13].

On day 10 of the CoSynch protocol, POF greater than 12 mm was associated with higher pregnancy rates. A key new feature of this protocol is the use of hCG to trigger final ovulation and egg maturation. hCG works well and directly affects the ovaries to cause ovulation. A recent study noted that both hCG and GnRH are used to synchronize ovulation in GnRH-based protocols because they have similar effects on ovarian function [14]. Still, hCG is considered a stronger ovulatory agent because it acts independently of the pituitary gland, binding directly to LH receptors on the follicle to trigger ovulation [15]. In contrast, the ovulatory response to GnRH depends partly on pituitary LH release, which is affected by progesterone levels in the blood [16].

Also, the follicle size at insemination strongly affects pregnancy success in water buffaloes [17]. This study similarly found that higher pregnancy rates occurred when POF were ≥12 mm (Category III) at insemination and ovulation in both CIDR-CoSynch-hCG groups [17]. On the other hand, inseminating animals with POF under 10 mm led to lower pregnancy rates. Overall, the group with the largest POF (≥12 mm) on insemination day had very high pregnancy rates, and delaying hCG injection until day 10 increased the number of animals with large POF. Our results agree with another study that found higher pregnancy rates associated with larger POFs (12–16 mm), which are mature enough to form a corpus luteum that can produce optimal progesterone levels to support pregnancy in buffalo cows [17]. Supporting this, a recent study found that cows with follicles measuring 13.5–17.5 mm were more likely to become pregnant than those with smaller follicles [18]. In contrast, follicles that ovulated at smaller sizes were linked to lower pregnancy rates and later embryonic or fetal death [19].

The results of this study should be interpreted with certain limitations in mind. First, the research was conducted at a single location (PCC National Genepool), which may limit the generalizability of the findings to other management systems or environments. Second, only multiparous animals were used; thus, the response of nulliparous heifers to the CIDR-CoSynch-hCG protocol remains unknown. Third, the study was conducted primarily during the wet season, and fertility responses may differ during the dry or hot seasons, when heat stress could confound results. Future research should validate these protocols across different seasons and locations and in heifers.

CONCLUSION

The present study demonstrated the successful implementation of the CIDR-CoSynch-hCG fixed-time artificial insemination (FTAI) protocol by administering the hCG injection and the first artificial insemination (AI) concurrently on either Day 9 or Day 10, rather than on separate days (hCG on Day 9, AI on Day 10). The two treatment groups achieved pregnancy rates comparable to those of the control group, indicating flexibility and reduced labor and procedural demands. Furthermore, pre-ovulatory follicles (POF) measuring ≥10 mm in diameter at the time of AI were associated with improved pregnancy outcomes, while follicles≥12 mm in diameter yielded the highest conception rates, underscoring the significant influence of follicle size on reproductive success. Meanwhile, the practice of double insemination yielded superior pregnancy rates compared with single insemination, suggesting that the ovulatory period in an induced estrus cycle may be longer in water buffaloes, with some POFs experiencing delayed ovulation that necessitates subsequent insemination. A single AI may be particularly useful for inseminating with genetically valuable or sex-sorted semen, an application that warrants further investigation.

In conclusion, the present study demonstrated both efficient and practical CIDR-CoSynch-hCG FTAI and follicle-size-based protocols that have the potential to enhance fertility and reproductive performance in water buffaloes and other livestock species.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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SUPPLEMENTAL TABLES

The supplemental tables can be downloaded from the journal website along with the article.

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