Philipp J Vet Anim Sci 2019 45(3):178-186

EFFECTS OF DIFFERENT PROSTAGLANDIN ($PGF_{2\alpha}$) ANALOGUES DURING SYNCHRONIZATION OF OVULATION IN DAIRY BUFFALOES (*Bubalus bubalis*)

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ABSTRACT

The study was conducted to determine the effects of prostaglandin analogues in buffaloes subjected to Fixed-Time AI (FTAI) using CIDR-Synch protocol. In Study 1, buffaloes were treated with GnRH and CIDR on day 0 and after the removal of CIDR on day 7, the animals were randomly assigned to different prostaglandin treatments: Dinoprost-Tromethamine (T1), Chloprostenol (T2), and D-Chloprostenol (T3). Blood samples were collected on days 7, 8, and 9 for progesterone assay. In study 2, buffaloes were subjected to the same treatments and FTAI protocol; signs of estrus were observed on the day of AI (day 10) and pregnancy diagnosis was performed on day 30-40. In the subset of animals per treatment, dominant follicle (DF) was measured at the time of AI. Results revealed progesterone concentrations of 1.67, 2.20 and 1.63 ng/ml in T1, T2 and T3, respectively, on day 7 and linear decline were observed thereafter with concentrations of 0.51, 0.59 and 0.70 ng/ml, respectively, on day 9. The average sizes of DF were 11.74, 13.3 and 13.02 mm, respectively. Estrus manifestation, DF diameter, and pregnancy rates were not significantly different among the groups. The work demonstrated the effectiveness of prostaglandin analogues in inducing luteolysis and estrus, and that chloprostenols are effective alternative luteolytic agents for FTAI in buffaloes.

Key words: fixed-time artificial insemination, progesterone, prostaglandin

INTRODUCTION

Artificial insemination (AI) is widely used in many Asian countries and is accepted as a technology that can bring about rapid genetic improvement in cattle and buffaloes. However, optimum conception rates will only be achieved if the quality of semen used is good, the insemination is done at the most appropriate time in relation to the estrus period, and the technicians have adequate training and skills in the procedure. The above factors, together with other socio-economic considerations specific to smallholder production systems and inadequate infrastructure for the efficient delivery of AI services, have often led to poor success rates. In an effort to assist producers in managing reproduction in a more

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effective manner, synchronization protocols have been developed with the use of prostaglandin ($PGF_{2\alpha}$) with the efforts to create timed artificial insemination protocols to assist in estrus detection (Stevenson *et al.*, 1987).

Prostaglandins have a positive and negative effect on reproduction. They play important role in the regulation of the female estrous cycle through the hypothalamic-pituitary-gonadal axis relationship and in the resumption of postpartum ovarian cyclicity. Prostaglandins are used to synchronize estrus, terminate pseudo-pregnancy in mares, induce parturition in sows, and treat retained placenta, luteal cysts, pyometra, and chronic endometritis. However, it will cause abortion when it is injected into pregnant cows, buffaloes and goats due to its luteolytic activity (Weems *et al.*, 2006).

To synchronize the estrous cycle, ovarian activity is manipulated so that the time of ovulation can be predicted. This is achieved by controlling the luteal phase of the cycle through the administration of $PGF_{2\alpha}$ or progesterone (P_4) analogues, or controlling the follicular development and ovulation using different combinations of $PGF_{2\alpha}$. Prostaglandin causes lysis of the corpus luteum (CL) during the responsive phase and a consequent decrease in the levels of P_4 leading to the development of follicles of the next wave (Galina and Orihuela, 2007). The use of gonadotropin releasing hormone (GnRH) and $PGF_{2\alpha}$ has proven to be very successful in synchronizing estrus in cattle and buffaloes (Lamb *et al.*, 2004; Amaya-Montoya *et al.*, 2007).

Understanding and knowing the rate of decrease of progesterone level in animals treated with luteolytic agents under synchronizing ovulation protocol are very important in order to achieve more synchronous estrus, ovulation and proper timing of insemination to increase pregnancy rate and overall AI efficiency in dairy buffaloes.

Therefore the general objective of the study is to determine the effects of different prostaglandin analogues during ovulation synchronization in dairy buffaloes under Controlled Internal Drug Release (CIDR)-based synchronizing ovulation and Fixed-time Artificial Insemination (FTAI) program.

MATERIALS AND METHODS

Open post-partum buffalo cows with approximately ≥ 2 cm size of ovaries and with body condition score (BCS) of not less than three were selected at PCC-CLSU dairy farm and PCC Dairy Cooperatives in Nueva Ecija. Evaluation of BCS was done according to the method described by Alapati *et al.*, (2010). Briefly, a BCS of 1 stand for emaciated animals; a BCS of 2 with a prominent hips, pins tail head and ribs; BCS of 3 with visible ribs and little fat cover and dorsal spine are barely visible; BCS of 4 is for animals that are smooth and well covered, but with no marked fat deposits; and BCS of 5 is with the dorsal spines, ribs, hooks and pins fully covered and unable to be felt even with firm pressure.

Progesterone Concentration of Buffaloes Treated with Different Prostaglandin Analogues

For Study 1, a total of 24 post-partum buffaloes were used to determine the hormonal response to the treatments. The experimental animals were randomly allocated into three (3) treatment groups having eight (8) replications each and were administered with different prostaglandin ($PGF_{2\alpha}$) analogues: T1- 5ml Dinoprost-Tromethamine (Lutalyse, 25 mg; Pharmacia and Upjohn Co., MI, USA), T2- 2ml Chloprostenol (Bioestrovet, 500 mcg;

Vetoquinol-Biowet, Gorzon WLKP, Poland) and T3- 2ml D-Chloprostenol (Gestavet Prost, 150 µg; Laboratorios HIPRA, Girona, Spain).

Experimental animals were subjected to Controlled Internal Drug Release (CIDR, 1.38-g progesterone; (Eazi-Breed CIDR, DEC International, NZ. Ltd.) + Ovsynch (CIDR-Synch) ovulation synchronization protocol (Carvalho *et al.*, 2007) with slight modification. Briefly, on day 0, 2ml gonadotropin releasing hormone (GnRH, Cystorelin®, 100 µg, Merial Ltd., GA, USA) was injected intramuscularly per animal and at the same time, insertion of CIDR into the vagina was done using an applicator. On day 7, the CIDR was removed followed by the injection of different prostaglandin analogues described above. On day 9, 2ml of human chorionic gonadotropin (hCG, Chorulon®, 10,000 IU; Intervet Inc., Summit, NJ 07901, USA) was administered. Timed AI was performed on day 10 and animals were observed for behavioral signs of estrus.

Every collection time, 15-20 ml of blood samples were collected from the jugular vein prior to $PGF_{2\alpha}$ treatment (day 7) and two days after treatment (day 8 and 9) and placed in a vacutainer tube containing heparin. Samples were centrifuged at 15 rpm for 20 min and thereafter, plasma was collected and placed into individual conical tubes (Falcon®, 5 ml Polystyrene Round-Bottomed Tubes), sealed with parafilm and stored at −20°C until used for progesterone (P_4) analysis.

The conventional P_4 extraction protocol established at the Reproductive Biotechnology Laboratory, Philippine Carabao Center was adapted. Briefly, the serum sample (0.5 ml) was pipetted into a glass tube (A), added with diethyl ether and was mixed for 15 minutes. Thereafter, the tubes were submerged into an acetone dry ice bath up to the level of the sample. When distinct solid portion was formed on the bottom of the tube, the remaining ether was allowed to decant in another tube (B). The decanted solutions were left to evaporate while the solid portions were left to thaw. The procedure was repeated twice and once all the liquid has evaporated, the tubes (B) containing progesterone were covered with parafilm and kept at -20˚C until use.

Progesterone Assay

For the progesterterone assay, Twenty (20) µl each of the standards and samples were pipetted into wells and 120 µl of assay buffer was pipetted into the "blank" wells. One hundred microliter (100µl) of anti-body solution was dispensed on all wells except blank ones; followed by 100 µl of HRP labeled hormone for all the wells. The plates were covered with plastic film and wrapped with aluminum foil to avoid exposure to light. The samples were incubated for 16 to 18 hrs at 4°C with gentle mixing.

Thereafter, all the solutions from the wells were discarded and then each plate was washed four times. After this, 150 µl of substrate solution was dispensed into all wells and the plate was incubated at 37°C for 40 min. Thereafter, 50 µl of stop solution was added to all wells and plate was left to stand for 5 minutes. Absorbance (optical density) was read at 450 nm using a microplate reader.

External and internal manifestations of estrus such as the presence or absence of vaginal mucus discharge and tonicity of the uterus were recorded. The tonicity of the uterus was graded following the protocol developed by PCC wherein the soft/flaccid uterus is considered as tone 1; slightly hard as tone 2; and, very hard as tone 3. Essentially, animals are considered in estrus when mucus discharge is present and the uterine tone observed is 2 to 3.

In addition, the size of the dominant follicle mainly at the time of AI was measured

using an ultrasound scanner (HS-1600®, Honda Electronics Co., Ltd. Japan) equipped with a 5.0 MHz probe. The probe was placed over an ovary and scanning was accomplished in several planes to identify the dominant follicle which was described as one which grew to around 10 mm and exceeded the diameter of other follicles. Measurements were taken from still images using a built-in caliper system and were saved to an external memory device.

Pregnancy Rate of Dairy Buffaloes Treated with Different Prostaglandin Analogues under FTAI Program

For Study 2, a total of 150 post-partum buffaloes were selected and randomly allocated into three treatment groups and were subjected to FTAI - CIDR-Synch protocol as described in Study 1. Signs of estrus and tonicity of the uterus were determined at the time of AI as previously described.

All the treated animals (n=150) were artificially inseminated 72 hr after $PGF_{2\alpha}$ injection, or 14-16 h after hCG injection and were repeated eight hours later, using the frozen-thawed buffalo semen from the Semen Processing Laboratory of PCC at CLSU. The semen used was derived from bulls with proven fertility with at least 30% post-thaw motility and with a concentration of approximately 50 million in a 0.5 ml capacity straw. The insemination was done by two selected trained AI technicians.

At day 30 post-FTAI, blood samples were collected from the artificially inseminated animals and pregnancy associated glycoprotein (PAG) concentration was measured from the extracted plasma using an ELISA Kit (Bio Pryn®). Furthermore, all experimental animals were subjected to trans-rectal ultrasonography at day 40 post-AI to detect pregnancy. Cows were considered pregnant based on the presence of CL, uterine fluid and embryo with a heartbeat (Fricke, 2016). Confirmation of pregnancy was done on day 60 post-FTAI by rectal palpation.

Statistical Analysis

Data were subjected to Analysis of Variance (ANOVA) with Least Significant Difference (LSD) as post hoc test. A probability value of $P<0.05$ was considered statistically significant. All analyses were performed using SPSS (Version 16.0).

RESULTS AND DISCUSSION

Reproductive inefficiency and low fertility are the major problems limiting the productivity of the livestock industry. In relation to this, protocols for synchronizing ovulation and FTAI were introduced and applied. These involve the use of gonadotropins and prostaglandin to improve the production of good quality oocytes at the time of ovulation and subsequently provide an optimal environment for embryo development leading to pregnancy. The widely used FTAI protocols are those that involved CIDR insert as source of exogenous progesterone to enable more synchronous growth of follicles and minimize premature ovulation thus achieving more precise timing of AI among the treated animals. The original ovulation synchronization protocol is Ovsynch developed for dairy cows which involves the injection of GnRH 7 days before and 2 days after prostaglandin administration (Pursley *et al.*,1995). Essentially, the injection of GnRH on day 0, induces the release of gonadotropins from anterior pituitary with luteinizing hormone (LH) causing the ovulation of the dominant follicle present in the ovary. The treatment leads to the emergence of a new follicular wave

at day 2-3 and formation of a new dominant follicle at around $PGF_{2\alpha}$ injection on day 7 of the protocol. Prostaglandin induces luteolysis of the CL formed from the previously GnRH-induced dominant follicle ovulation. Concurrent removal of CIDR implant in CIDR-based protocol and $PGF_{2\alpha}$ injection result in a drop of circulating progesterone, allowing E2-induced GnRH-LH release from the anterior pituitary to cause ovulation. Synchronous ovulation is induced by injection of GnRH/hCG on day 9 and timed insemination is fixed at 14-16 hr after GnRH/hCG injection. Ovulation is expected to occur 24-36 hr after GnRH/hCG injection.

Results of progesterone assay (Table 1) revealed a high level of P_4 concentrations: 1.66 ng/ml, 2.20 ng/ml and 1.63 ng/ml for Treatments 1, 2 and 3, respectively, before the injection of prostaglandin on day 7 of the protocol. The amount of P_4 is associated with the presence of functional corpus luteum (CL) that produces progesterone and with the intravaginal P_4 implant which contains exogenous progesterone. The result further implies that the animals for this experiment were in the same stage of the reproductive cycle at the start of prostaglandin treatment. Following $PGF_{2\alpha}$ injection, the results also showed precipitous decrease in progesterone level on day 8 specifically in T1 (Figure 1). The further decline continued until day 9 in three treatments; however, no significant differences in P_4 concentrations were observed among the treatment groups. The continual decline in progesterone concentration is attributed to the luteolytic effect of prostaglandin and its analogues and the removal of CIDR device. In spite of the seemingly similar ability of the analogues to cause lysis of CL, innate differences, however exist among them in terms of potency to induce luteolysis which can be attributed to its active ingredient. The abrupt decline in progesterone from day 7 to day 8 with Dinoprost-Tromethamine in T1 can be mainly attributed to its being a tromethamine salt of the natural $PGF_{2\alpha}$. According to Kindahl *et al.* (1976), Dinoprost-Tromethamine has a short-half-life (7-8 min) as it is rapidly metabolized in a similar manner to endogenous $PGF_{2\alpha}$. Chloprostenol sodium, however, is a synthetic prostaglandin analogue with a benzyl chlorine ring that makes this molecule more resistant to endogenous metabolism compared with Dinoprost-Tromethamine (Bourne *et al.*, 1980). It was therefore reported to have a much longer half-life (approximately 3 hr) compared with Dinoprost-Tromethamine (Reeves, 1978). On the other hand, Chloprostenol sodium was reported to be a more potent synthetic analog of $PGF_{2\alpha}$ since a dose of only 0.5 mg (2 ml) is needed to induce luteolysis while 25mg (5 ml) of Dinoprost-Tromethamine was needed to make this effect (Dukes *et al.*,1974). In addition, the lower dose required for Chloprostenol groups facilitated easier administration of the drug during hormonal treatment.

Moreover, Chloprostenols are characterized having an optic isometry, which means that they are compounds with the same molecular formula but with different structural

Table 1. Progesterone concentrations in CIDR treated dairy buffaloes before and after injection with different prostaglandin analogues.

Treatments	Progesterone Level (ng/ml)		
	Day 7	Day 8	Day 9
T_1	1.66 ± 0.854	0.54 ± 0.38	0.51 ± 0.40
T_2	2.20 ± 0.88	0.93 ± 0.85	0.59 ± 0.23
T_3	1.63 ± 0.52	0.73 ± 0.63	0.70 ± 0.48

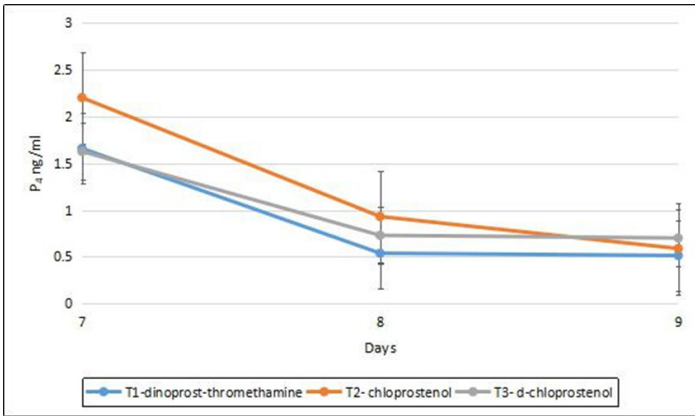

Figure 1. Degree of progesterone concentration decline in CIDR- treated dairy buffaloes before and after injection with different prostaglandin analogues.

forms, and therefore, the D and L isomers of these compounds are with different properties. Isomer D is said to be four times more powerful than the isomer L, and because it has a higher affinity by the receptor, this allows the use of lower doses, achieving higher efficiency and better tolerance. The D-chloprostenol acts as a luteolytic agent, which causes functional and structural regression of the corpus luteum followed by the manifestation of estrus (McCracken *et al.*, 1999). In the current study, however, the same dose of D-Chloprostenol and Chloprostenol sodium was used and yielded the same effects.

Results of $PGF_{2\alpha}$ on follicular size revealed that T1, T2 and T3 yielded average follicular sizes of 11.74, 13.30 and 13.02 mm, respectively. Animals treated with Chloprostenols tended to generate larger follicle sizes than those treated with Dinoprost-Tromethamine; the treatments were found not significantly different (P>0.05) though.

Essentially, progesterone which is produced by CL and other coming from its exogenous counterpart from CIDR implant is known to improve the development of ovulatory follicles following GnRH treatment. A high concentration of P_4 during ovulatory follicle growth is important for oocyte quality which is associated with an increase in fertility in dairy cows (Wiltbank and Pursley, 2014). The decline of circulating P_4 after injection of prostaglandin analogues removes its suppressing action on GnRH-LH release, resulting in final maturation and ovulation of ovulatory follicle. The final diameter of the ovulatory follcile is measured normally at the time of AI and is commonly associated with pregnancy outcome.

There are other factors that determine the success of hormonal treatment in terms of follicular response. The kind of ovarian structure present, whether dominant follicle or CL and the type of hormone used at the start of the protocol influence follicular response and ovarian dynamics in general.

The present study indicated that regardless of $PGF_{2\alpha}$ analogue used in the FTAI protocol, hundred percent (n=150/150) of the treated animals exhibited external and internal signs of estrus based on the criteria established in the study i.e., the presence of vaginal mucus discharge and uterine tone ranging from 2 to 3. Estrus expression near the time of artificial insemination influenced reproductive efficiency and was found correlated with pregnancy success in cattle. This means that estrus has a positive impact on both ovarian

function and uterine environment which affects embryo development and pregnancy maintenance (Pohler *et al.*, 2016). Several studies cited by Brito *et al.*, (2002) suggested that the administration of $PGF_{2\alpha}$ is the most common method to induce estrus in cattle and buffalo because it causes the CL to regress resulting in reduction of blood progesterone concentrations, induction of follicular growth, and ovulation within two to six days after treatment.

In FTAI protocol, the main objective is to improve the productivity of the animal by increasing the pregnancy rate per animal inseminated. The result of the present study in terms of pregnancy is presented in Table 2. Animals were diagnosed pregnant by PAG assay on day 30 and by ultrasonography on day 40 and were confirmed for pregnancy through rectal palpation on day 60. The pregnancy rates of 40% for T2 and 36% for T1 and T3 were found not significantly different among the treatment groups. Previous study on the use of CIDR-Synch FTAI protocol in buffaloes resulted in higher pregnancy rate (56%) compared with the prostaglandin-based estrus synchronization protocol (21%) in water buffaloes (Atabay *et al.*, 2015). The size of the follicle at the end of the treatment is known to influence pregnancy outcome following AI; however, this was not observed in the present study. More number of animals is needed to verify the correlation of follicle size and pregnancy following FTAI. Moreover, other factors exist influencing pregnancy outcomes such as health status and body condition of the animals, the quality of semen used, the time of

In conclusion, the present study demonstrated the effectiveness of the CIDR-Synch-based FTAI protocol using the different prostaglandin analogues mainly for CL lysis. Results showed no significant differences among the treatment groups in terms of their ability to decrease P_4 concentration, follicle diameter at AI, estrus manifestations, and pregnancy rates. The results insemination in relation to the estrus period, and the skill of technicians performing AI, among others. imply that Chloprostenol groups were as effective as Dinoprost-Tromethamine to induce CL lysis and therefore can be used as its alternative in ovulation synchronization protocol. Essentially, the information gained from this study can serve as valuable input in the development of reproductive management program to improve AI efficiency in water buffaloes. Subsequent studies should include measurement of other hormones such as estradiol and luteinizing hormone to fully characterize endocrine responses associated with ovulation synchronization in buffaloes. Generally, result of FTAI is best achieved when it is performed with ultrasonography which allows monitoring of ovarian response primarily follicular growth resulting in a more precise timing of AI among treated animals. Nevertheless, the understanding and technical advancement realized from the attempt to use this reproductive tool provide opportunities to refine and strengthen current efforts to enhance the propagation of water buffaloes in the country.

Table 2. Percent pregnancy of buffaloes treated with different prostaglandin analogues.

TREATMENTS	NO. OF ANIMALS TREATED	NO. OF ANIMALS PREGNANT	PERCENT PREGNANCY
T1	50	18	36
T2	50	20	40
T3	50	18	36

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