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Research Article
Efficacy of Three Synchronization Protocols on the Pregnancy Rate in Lactating Dairy Crossbred Cows after Fixed Time Artificial Insemination

[1]Essam Almadaly, [2]Ibrahim Mansour, [1]Nabil Mansour, [1]Aly Karen, [1]Bahy Serur and [3]Ibrahim M. Abd El-Razek

[1]Department of Theriogenology, Faculty of Veterinary Medicine, Kafrelsheikh University, El-Geish Street, 33516 Kafrelsheikh, Egypt
[2]Sakha Animal Production Station, Agricultural Production Sector, Agricultural Research Center, 33516 Kafrelsheikh, Egypt
[3]Department of Animal Production, Faculty of Agriculture, Kafrelsheikh University, 33516 Kafrelsheikh, Egypt

Abstract
Background and Objective: Ineffective estrus detection is the foremost limiting factor in the fertility of farmed cattle worldwide. Therefore, this study investigates the effect of three synchronization protocols with Fixed Time Artificial Insemination (FTAI) on the pregnancy rate of crossbred dairy cows. **Methodology:** Ninety one cows were assigned to four groups where the Body Condition Score (BCS), age distribution, parity and average daily milk yield were apparently even among groups then each group received 1 of 4 treatments: (1) Ovsynch-CIDR treated cows (n = 24) received 10 µg gonadotropin releasing hormone (GnRH, IM, Buserelin acetate) with insertion of Controlled Internal Drug Release device (CIDR) on day 0 followed by 500 µg prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, IM, cloprostenol sodium) and CIDR removal on day 7. On day 9, cows received the second dose (10 µg) of GnRH followed by FTAI 18 h later, (2) $PGF_{2\alpha}$-synchronized cows (n = 25) received double doses of 500 µg $PGF_{2\alpha}$ analogue (cloprostenol sodium) 12 days apart followed by FTAI 80 h after the second dose of $PGF_{2\alpha}$, (3) Ovsynch treatment (n = 17) with 10 µg GnRH-day 0, 500 µg $PGF_{2\alpha}$-day 7 and 10 µg GnRH-day 9 sequence followed by FTAI 18 h later and (4) Untreated cows (control, n = 25). Blood samples were taken for progesterone (P4) determination on days 0, 7 and on day of AI. **Results:** Our results revealed that pregnancy rate was greater ($p<0.05$) in $PGF_{2\alpha}$ (36%), compared with ovsynch (29.4%), ovsynch-CIDR (20.8%) and control (12%) cows. **Conclusion:** In conclusion, acceptable pregnancy rates can be achieved with FTAI after 80 h of synchronized cows with double doses of $PGF_{2\alpha}$ 12 days apart compared with synchronization of ovulation with (ovsynch-CIDR) or without (ovsynch) P4 supplementation.

Key words: Crossbred cow, ovsynch-CIDR, $PGF_{2\alpha}$, ovsynch, pregnancy rate

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Corresponding Author: Essam Almadaly, Department of Theriogenology, Faculty of Veterinary Medicine, Kafrelsheikh University, El-Geish Street, 33516 Kafrelsheikh, Egypt Tel/Fax: +02-(0)47-323-1311

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

It is well known that estrus synchronization and Artificial Insemination (AI) are the most important and widely applicable assisted reproductive technologies used to improve cattle industry[1] and commonly used in lactating dairy and beef cattle to bring a large group of cows into heat at a preplanned time[2] for efficiency in AI and embryo transfer practice[3]. Moreover, the lack of an efficient and accurate method of estrus detection is the foremost limiting factor in obtaining the optimum breeding outcomes in lactating dairy cattle[4]. The main benefits of estrus synchronization in lactating dairy cattle include reduced time and labour assigned for estrus detection as well as reduced variability in the duration of days open resulted in achievement of the optimum calving intervals within a dairy herd[5]. Nowadays, hormonal treatments have been commonly used to synchronize ovulation in cows, allowing breeding without estrus detection and consequently increasing service rates[6], culling for infertility and shorten days open[7]. Synchronization of ovulation (ovsynch) protocols have been developed to allow insemination of all cows at a Fixed Time AI (FTAI)[8], also helping in fixing the breeding time within a short predefined period and thereby scheduling the calving time at the most favorable season when the newborns can be reared in suitable environment[9].

Synchronization of follicular wave using both gonadotropin releasing hormone (GnRH) and prostaglandin $F_{2\alpha}$ [($PGF_{2\alpha}$), administration of GnRH 7 days before and 2 days after $PGF_{2\alpha}$ and 16 h before timed AI (ovsynch) or administration of GnRH 7 days before $PGF_{2\alpha}$ and at the time of AI (CO-synch)] have been investigated by various study groups[10-12]. Nevertheless, it has been reported that from 5-15% of cyclic cows exhibit estrus at or before $PGF_{2\alpha}$ injection which adversely affect pregnancy outcomes for FTAI in beef cows treated with these protocols[13].

Indeed, using exogenous GnRH, $PGF_{2\alpha}$ and/or P4 to synchronize ovulation in cyclic and non-cyclic cows before FTAI produce acceptable pregnancy rates[11,14]. These GnRH-based synchronization protocols such as ovsynch[15], CO-synch[11,14] and select synch[11] include injection of GnRH on day 0 (approximately 10 days before AI) to initiate a new follicular wave. This is followed by injection of $PGF_{2\alpha}$ on day 7 (approximately 64 h before AI) to lyse the Corpus Luteum (CL) and a second GnRH injection is performed to induce ovulation either at the time of AI (day 10) in the CO-synch protocol or 16 h (day 9) before AI in the ovsynch protocol. Moreover, certain methods of estrus synchronization using P4 supplemented ovsynch protocols succeeded in cattle farms[16]. There are several factors influencing the outcomes of ovsynch protocols such as Body Condition Score (BCS)[17,18], season[19], heat stress[19,20], milk production[18], stage of estrous cycle at the initiation of the protocol[6], parity[18] and breed[21] of cows. Therefore, the present study was conducted to compare between the effect of estrus synchronization using double doses of $PGF_{2\alpha}$ and synchronization of ovulation with (ovsynch-CIDR) or without (ovsynch) supplementation of P4 followed by FTAI on the pregnancy rate of Egyptian crossbred dairy cows.

MATERIALS AND METHODS

Experimental animals: This study was conducted at Sakha Animal Production Station, Agricultural Production Sector, Agricultural Research Center, Ministry of Agriculture, Egypt. The experiments were carried out with a total of 91 crossbred dairy cows (Baladi×Friesian, 3-9 years old). The cows were randomly assigned to four groups. These groups were selected such that the BCS, age distribution and parity were apparently similar among groups then each group received 1 of the 4 treatments. All cows calved more than 60 days and have an average milk yield of nearly 10 kg day^{-1}. Also, BCS of animals was determined using the scale of 1 (emaciated) to 5 (over conditioned) according to Wildman *et al.*[22]. The cows were housed in open-yard and fed Concentrate Feed Mixture (CFM) and roughages according to National Research Council[23] requirements. The ration offered daily for each cow composed of 5 kg ration containing 16% crude protein plus 0.5% of its body weight straw. Also, cows were grazed on green alfalfa in winter and on dry alfalfa in summer. All cows were healthy and free from brucellosis, external and internal parasites and were vaccinated periodically against the common infectious diseases. All animal experiments were performed following approval from the local Animal Ethics Committee of the Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh, Egypt.

Synchronization protocols: All experimental animals were randomly allocated into four groups (3 treatments+1 control) and each group received one of the following treatments.

Ovsynch-CIDR (n = 24): This protocol was applied according to Kasimanickam *et al.*[24]. In this treatment CIDR (Eazi-breed CIDR™; 1.38 g of P4, Pfizer Animal health, New Zealand) was inserted intravaginally by using a sterile applicator on day 0 concomitant with IM injection of 10 µg GnRH analogue (Buserelin acetate, Receptal, MSD, Germany). On day 7, cows received 500 µg $PGF_{2\alpha}$ analogue (IM, Cloprostenol Sodium, Estrumate, Schering-Plough, Germany). A second dose of GnRH analogue (10 µg) was administered on day 9 followed by FTAI 18 h after the second GnRH on day10 as illustrated in (Fig. 1a).

$PGF_{2\alpha}$ (n = 25): Following this method, synchronization was initiated by IM administration of 500 µg $PGF_{2\alpha}$ analogue (Estrumate, Schering-Plough, Germany) on day 0. Each milliliter of Estrumate contained 250 µg Cloprostenol Sodium; the second dose of $PGF_{2\alpha}$ (500 µg) was administered 12 days later[25]. All cows were inseminated 80 h after the second dose of $PGF_{2\alpha}$ (Fig. 1b).

Ovsynch (n = 17): In this study ovsynch protocol was applied according to[26] where, an initial dose (10 µg) of GnRH analogue (Buserelin Acetate, Receptal, MSD, Germany) was injected on day 0 to synchronize ovulation in cows. The $PGF_{2\alpha}$ (500 µg Estrumate) was given IM 7 days later to remove the resulting CL. The second dose of GnRH analogue (10 µg) was given 2 days after $PGF_{2\alpha}$ (day 9) to increase the synchrony of ovulation. All cows of this group were inseminated at 18 h after the second dose of GnRH (Fig. 1c).

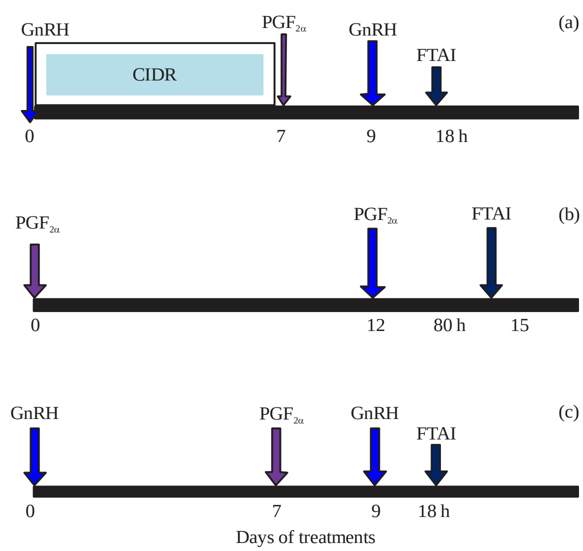

Fig. 1(a-c): Schematic representation of (a) Ovsynch-CIDR, (b) $PGF_{2\alpha}$ and (c) Ovsynch synchronization protocols, (a) Ovulation synchronization in the presence of controlled internal drug release device (CIDR), GnRH: Gonadotropin releasing hormone, FTAI: Fixed time artificial insemination, (b) Estrus synchronization with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and (c) Ovulation synchronization protocol

Control (n = 25): Twenty five cows were set aside as a control group where no hormones were administered to them and finally, all cows were observed for estrus detection and inseminated according to AM/PM system.

Pregnancy diagnosis: Real-time B-mode ultrasonography (Litrascan 1700 Alliance Inc., Amos Lachine, Quabic, Canada) equipped with a 5 MHz linear assay transducer was used to diagnose the pregnant cows at days 28-35 post-insemination[27] and reconfirmed again at days 60-90 by rectal palpation. Finally, FTAI pregnancy rate was calculated by the No. of cows pregnant to FTAI divided by the total No. of cows inseminated.

P4 assay: To evaluate concentrations of serum P4, blood samples were collected via jugular venipuncture and immediately placed on crushed ice. On day 0 and on day of FTAI blood samples were collected from all treated cows (n = 66). Additional blood samples were collected on day 7 from ovsynch-CIDR treated cows only. Blood samples were centrifuged at 1500×g for 15 min at 4°C and the serum was transferred into 1.5 mL micro-centrifuge tubes and frozen at -20°C until assayed. To detect circulating concentrations of P4, samples were assayed using mini-VIDAS (VIDAS TESTS, BIOMÉRIEUX, France) according to the manufacturer's instructions. Assay sensitivity for a 200 µL sample was 0.25 ng mL^{-1} P4. Also, inter and intra-assay variation coefficients were 12 and 7%, respectively. All samples for a single cow were analyzed within the same assay and treatments were run in a random order.

Statistical analysis: All data were analyzed with a statistical software program (GraphPad Prism Version 5.0, GraphPad Software, San Diego, CA, USA). Results are presented as the Mean±SEM. The obtained data were subjected to repeated measures ANOVA or t-test. When difference was significant by ANOVA, individual means were further tested by Tukey's multiple comparison tests[28]. Proportions of cows detected pregnant were compared using the Chi-square test. A $p<0.05$ was considered statistically significant.

RESULTS

BCS, parity and milk yield of experimental animals: Statistical analysis of Means±SEM of BCS and parity of the treated and control cows revealed non-significant ($p\geq 0.05$) difference among treatments as presented in Table 1. On contrary, daily milk yield showed significant

Table 1: BCS, parity, milk yield and pregnancy rate of treated and control cows (Means±SEM)*

Parameters	Ovsynch-CIDR (n = 24)	$PGF_{2\alpha}$ (n = 25)	Ovsynch (n = 17)	Control (n = 25)
BCS (2-3)	2.47 ± 0.07^{a}	2.44 ± 0.08^{a}	2.47 ± 0.07^{a}	2.70 ± 0.12^{a}
Parity (1-7)	3.08 ± 0.38^{a}	2.92 ± 0.33^{a}	3.47 ± 0.42^{a}	3.00 ± 0.54^{a}
Milk yield (kg)	9.01 ± 0.43^{ab}	9.69 ± 0.36^{a}	7.92 ± 0.34^{b}	8.27 ± 0.29^{b}
FTAI pregnancy rate (%)	20.8^{c} (5/24)	36.0^{a} (9/25)	29.4^{b} (5/17)	12.0^{d} (3/25)

Ovsynch-CIDR: Ovulation synchronization in the presence of controlled internal drug release device (CIDR), BCS: Body condition score, $PGF_{2\alpha}$: Prostaglandin $F_{2\alpha}$, Ovsynch: Ovulation synchronization and FTAI: Fixed time artificial insemination, *Values within the same row bearing at least one common superscript were not significantly different at p<0.05

Table 2: Serum P4 concentrations (Means±SEM) in treated cows*

Sampling day	Ovsynch-CIDR (n = 24)	$PGF_{2\alpha}$ (n = 25)	Ovsynch (n = 17)
Day 0	1.11 ± 0.10^{b}	1.59 ± 0.15^{a}	1.19 ± 0.12^{ab}
Day 7	2.02 ± 0.17^{a}	-	-
Day of FTAI	1.56 ± 0.14^{ab}	1.59 ± 0.18^{a}	1.68 ± 0.29^{a}

*P4 concentration expressed as ng mL^{-1}, Values within the same column and row bearing at least one common superscript were not significantly different ($p\geq0.05$)

(p<0.05) difference among treatments where cows in $PGF_{2\alpha}$ protocol showed the highest (9.69±0.36 kg) daily milk yield in comparison with those of ovsynch (7.92±0.34 kg) and control (8.27±0.29 kg) groups but was nearly equal with the daily milk yield of cows in ovsynch-CIDR (9.01±0.43 kg) protocol (Table 1).

Pregnancy rate: Results presented in Table 1 revealed that pregnancy rate analyzed by Chi-square test showed highly significant (p<0.001) difference among synchronization protocols and control where the lowest (12%) pregnancy rate was recorded in the control group but the highest (36%) rate was achieved by using $PGF_{2\alpha}$ synchronization protocol (Table 1). Moreover, P4 supplementation of ovsynch protocol adversely affects the pregnancy rate in the present study where it achieved 20.8% pregnancy outcome versus 29.4% for P4 non-supplemented ovsynch protocol (Table 1).

Serum P4 concentration: Table 2 shows the Means±SEM concentration of serum P4 (ng mL^{-1}) measured in treated cows. The obtained results revealed that on day 0 there was a significant difference in the P4 concentration among treatments where P4 concentration in $PGF_{2\alpha}$ treated cows (1.59±0.15) was significantly (p<0.05) higher than ovsynch-CIDR (1.11±0.10) treated cows but was non-significantly different from ovsynch (1.19±0.12) treated cows. On day 7, P4 concentration in ovsynch-CIDR (2.02±0.17) treated cows was higher (p<0.05) than its basal level (1.11±0.10) on day 0. On day of FTAI, the P4 concentration did not show any significant difference among treatments and also was non-significantly differed from the corresponding P4 concentration on day 0 (Table 2).

DISCUSSION

Even though, to increase the utilization of AI in cattle industry, it is imperative to provide them with an estrus synchronization protocol with minimal time, cost and labour inputs. This can be achieved by minimizing cattle handling and by reducing or eliminating heat detection aid. Monitoring of ovarian follicular dynamics has led to recent protocols such as ovsynch and ovsynch modifications which enhances the usefulness of AI in dairy and beef cattle industry. However, it is also important not to compromise the chance of pregnancy by eliminating heat detection. This might be one of the plausible explanations for the lower FTAI pregnancy rates achieved in the present study for both P4 supplemented and P4 non-supplemented ovsynch protocols in crossbred dairy cows.

The results of the present study indicated that there was a highly significant (p<0.001) difference in terms of the pregnancy outcomes among ovsynch-CIDR, $PGF_{2\alpha}$, ovsynch treatments and control (Table 1). In previous study[29], FTAI pregnancy rate for the ovsynch-CIDR was ranged from 48.8-55.6% and recently, Kasimanickam *et al.*[24] achieved 46.4% FTAI pregnancy rate for ovsynch-CIDR whereas, in the current study, FTAI pregnancy rate for ovsynch-CIDR was 20.8% (Table 1). The most straightforward explanation for this discrepancy might be the different breed, season of year and the dose of GnRH as well as the product of GnRH (Receptal versus Cystorelin). Because induction of an LH surge, potentially in the absence of estrus, with exogenous GnRH can induce ovulation; if a responsive dominant follicle is present on the ovary and the GnRH-induced LH surge is of sufficient magnitude. It has been found that the LH surge is greatly reduced when GnRH is given in the presence of elevated P4 compared with GnRH treatment when circulating P4 is low[30]. Therefore, the first GnRH treatment is likely to require a higher dose than the second GnRH to induce an LH surge of adequate magnitude to induce ovulation. Moreover, Silcox *et al.*[31] demonstrated that 100 µg of GnRH induced either ovulation or luteinization of growing and dominant follicles even during the luteal phase of estrous cycle in heifers. Conversely, in the current study the dose of GnRH (10 µg) was much lower than that (100 µg) of the previous studies either with[24,25,32,33] inclusion or without[34,35] inclusion of estrus detection aid in their GnRH-based protocols.

An early study[36] concluded that GnRH could induce an LH surge in cows during the luteal phase, although the magnitude of the LH surge was greatly reduced compared with cows with follicular cysts. Also, it has been reported that the success of ovsynch protocol is influenced by the number and the length of follicular waves[15] as well as the stage of estrous cycle when the initial dose of GnRH is administered[6,37]. Unfortunately, this point did not consider carefully in the present study which might be the reason of the lower FTAI pregnancy outcomes of both ovsynch-CIDR and ovsynch protocols compared with $PGF_{2\alpha}$. Thus, the first dose of GnRH, when administered at a random stage of the estrous cycle, induces ovulation only in cows with a functional dominant follicle. Moreover, it has been confirmed that ovsynch-synchronized animals may exhibit estrus prior to FTAI associated with ovsynch protocols. For instance, it has been reported that up to 72% of cows show estrus before or immediately after the $PGF_{2\alpha}$ administration[29] and 17% of cows detected in estrus after administration of $PGF_{2\alpha}$ but before the second dose of GnRH in ovsynch-CIDR and CO-synch-CIDR protocols[32]. Interestingly, Dirandeh *et al.*[38] found that using double ovsynch protocol in heat-stressed lactating dairy cows yielded greater ovulation rate to the first GnRH and a greater synchronization.

Although, Perry *et al.*[39] concluded that GnRH-induced ovulation of follicles $\geq$11 mm resulted in decreased pregnancy rates and increased late embryonic/foetal mortality but follicle size had no effect on pregnancy rate when ovulation occurred spontaneously. Based on the review of Wiltbank and Pursley[37] which introduced ovsynch, more as a concept of induced ovulation this may be another possibility for the lower FTAI pregnancy rates in the current study. Hence, all these findings suggest that it is essential to investigate follicular dynamics before initiation of all ovsynch protocols according to Souza *et al.*[40] because the fertility after a FTAI program was related to the stage of the estrous cycle (stage of the first follicular wave). Moreover, recently[41] it has been confirmed that ultrasonographic examination of the uterus and ovaries in cows submitted for service facilitates the exclusion from service of proestrus and diestrus cows and thus reduces the number of pointless services. Additionally, Colazo *et al.*[34] reported that although the ovulatory follicle diameter was not associated with pregnancy per AI at 32 or 60 days but large ovulatory follicle size was identified as a predictor of pregnancy loss in lactating Holstein cows subjected to ovsynch protocols for FTAI. Noteworthy, the combined use of CIDR, ultrasound scanning and GnRH succeeded to identify and resynchronize nonpregnant cows and heifers on day 21 after FTAI and improved pregnancy outcomes for FTAI according to Kelley *et al.*[35]. A previous study[24] revealed that there was a 4.4% increase in FTAI pregnancy rates for CO-synch-CIDR group than ovsynch group. In addition, among the ovsynch-CIDR group cows that were inseminated after detection of estrus, there was a 7% increase in FTAI pregnancy rates for cows that received a second GnRH when compared with cows that did not receive a second GnRH[24]. Likewise, it is possible that the administration of GnRH 48 h after $PGF_{2\alpha}$ to the cows that were in estrus in the ovsynch-CIDR and ovsynch groups might induce ovulation of smaller sized follicles resulted in formation of smaller CLs that secreted less P4 in agreement with Vasconcelos *et al.*[42] resulting in the observed lower pregnancy rate of ovsynch protocols compared with that of estrus synchronization using double doses of $PGF_{2\alpha}$.

Previous studies have discussed the reduction in fertility when the preovulatory follicular wave develops in a low P4 environment[43,44]. Recent studies have shown improvements of approximately 5-9% in pregnancy rate using a P4 vaginal implant during the ovsynch protocol before AI[45,46]. Thus, much of the effect of supplementation with a single intravaginal P4 implant might be due to improvements in synchronization rate, in addition to elevating P4 during preovulatory follicle growth in synchronized cows. Supplementation with a single vaginal P4 implant might not elevate circulating P4 sufficiently to optimize fertility and two P4 implants might be needed in dairy cows that lack a CL at the initiation of ovsynch protocols[45,47]. This might be another explanation for the lower pregnancy (20.8%) outcome of ovsynch-CIDR. Thus, circulating P4 during the ovsynch protocol might, at times, be insufficient for optimal fertility, particularly in dairy cows that are anovular, near estrus, in the early luteal phase or in the late luteal phase. Accordingly, supplementation during ovsynch protocol, with sufficient P4, in these types of dairy cows is likely to improve fertility. The obtained results were inconsistent with the findings of Kojima *et al.*[48] and Bader *et al.*[49] who concluded that using progestins, along with or preceded by GnRH-$PGF_{2\alpha}$ enhanced the response to estrus synchronization in cycling as well as in anestrous cows. Regardless of the individual variations of P4 concentration within and among treated groups FTAI yielded lower conception rate in absence[50,51] or presence[26,52] of estrus detection aid.

A study[25] using both of a charge-coupled device camera and direct visual observation for estrus detection reported that both first service conception rate and cumulative conception rate were significantly higher in the CIDR and ovsynch treated cows compared with $PGF_{2\alpha}$ treated cows, fortunately, this first service conception rate (40%) of $PGF_{2\alpha}$ protocol was comparable to the pregnancy rate (36%) obtained herein using $PGF_{2\alpha}$. Based on these results, it seems likely that inclusion of estrus detection aid in ovsynch protocols followed by either natural breeding or AI will be profitable in cattle industry in agreement with Kasimanickam *et al.*[24] and Lee *et al.*[25]. On contrast, $PGF_{2\alpha}$ synchronization protocol might be efficient with or without estrus detection aid in improvement of cattle reproduction through achievement of greater pregnancy outcome especially during the early postpartum period in crossbred dairy cows.

Postpartum negative energy balance is associated with a delayed resumption of ovarian cyclicity and reduced fertility in beef cows[53]. Obviously, the means of BCS and parity were non-significantly different among the treated and control cows which exclude any significant effect of both BCS and parity on the obtained FTAI pregnancy outcomes. Our finding supports the previous study by Larson *et al.*[29] those reported that BCS did not enhance fertility. In addition, they speculated that the usage of GnRH and CIDR may induce cyclicity and negate the associated negative effect of lower BCS. Collectively, our findings emphasize the fact that ovsynch protocols are beneficial tools to induce animal cyclicity early postpartum rather than to improve the pregnancy outcomes after FTAI in absence of estrus detection aid. Although, several studies have been investigated the impact of ovsynch protocols in dairy and beef farms, there is still substantial need for further studies to improve the synchronization, efficacy, simplicity and practical application of these protocols. Thus, dairy producers could use ovsynch to shorten "days open", not because of better fertility after AI but because of better service rates.

CONCLUSIONS

In conclusion, the results of the present study revealed that, although all synchronization protocols had a positive effect on the reproductive efficiency of crossbred dairy cows but $PGF_{2\alpha}$ achieved greater FTAI pregnancy outcomes compared with P4 supplemented and P4 non-supplemented ovsynch protocols. Moreover, the current study required further investigation using higher doses of GnRH with ultrasound scanning of ovarian follicular dynamics throughout the whole time schedule of GnRH-based protocols particularly with FTAI to achieve greater pregnancy rates especially during the early postpartum period in dairy cows.

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