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Timed artificial insemination protocols in dairy cattle: Functioning, shortcomings, and improvements

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Abstract: Oestrus detection is currently at the top of the factors limiting the success of artificial insemination in dairy herds. This limitation becomes more pronounced as milk production increases because of higher metabolic clearance rates of steroid hormones leading to low circulating oestradiol concentration near oestrus. The standard OvSynch has overcome this problem by enabling fixed-time artificial insemination, for it synchronises follicular wave emergence, luteolysis, and ovulation. However, it did not improve fertility compared to cycles with spontaneous ovulation, which motivated the development of "fertility programs". These later are nothing but improved OvSynch protocol. This article explains what went wrong with the original OvSynch protocol, discusses the improvements made by "fertility programs", and highlights the advantages and limits of the newly tested molecules.

Keywords: OvSynch protocol; fertility program; dairy herd; synchronised ovulation

With an increase in feed intake and a genetic selection oriented toward higher milk production, the intensification of dairy cattle breeding has disrupted the reproductive physiology of dairy cattle (Harrison et al. 1990; Lopez et al. 2004; Wiltbank et al. 2006). This resulted in weaker oestrous behaviour (Lopez et al. 2004), adversely impacting female fertility, especially in large herds, where oestrus is difficult to observe (Holman et al. 2011). In response to this problem, oestrus synchronisation protocols allowing blind artificial insemination have been proposed and constantly improved.

The OvSynch protocol was first developed by Pursely et al. (1995) to facilitate insemination without needing oestrus detection and reduce infertility caused by dominant follicle (DF) persistence. Although the OvSynch program did not improve fertility (Pursley et al. 1997; Rabiee et al. 2005), it allowed for inseminations to be scheduled without oestrus detection, thus breaking free from the traditional voluntary waiting period (Pursley et al. 1997).

The main weaknesses of the OvSynch protocol include a lack of synchronisation of follicular waves and incomplete lysis of the corpus luteum (CL) after the first gonadotropin-releasing hormone (GnRH) and prostaglandin F (PGF) treatments, respectively (Martins and Pursley 2016). Subsequent modifications to the OvSynch protocol, referred to as "fertility programs", addressed these issues.

The most successful strategies to improve fertility include presynchronisation to increase the ovulation rate to the first GnRH, and complete CL regression before the final GnRH (Carvalho et al. 2018). The presynchronisation strategy using synthetic progesterone (P4), PGF, or the PGF-GnRH combination enabled the OvSynch protocol to be initiated earlier in dioestrus, improving the response of the DF to the first GnRH treatment (Moreira et al. 2001;

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Bello et al. 2006; Souza et al. 2008). Another adjustment to the OvSynch protocol was to apply a final GnRH 56 h after PGF (instead of 48 h) and 16 h before insemination (instead of 24 h) (Brusveen et al. 2008). In order to reduce animal handling, decrease the duration of preovulatory follicle dominance and increase the pro-oestrus length, the interval between the first GnRH and PGF was reduced (5 days instead of 7), and insemination was performed concomitantly with the final GnRH 72 h after PGF. This protocol, known as 5-day cosynch-72, required a second PGF to overcome the refractory state of the young CL on day 5 (Santos et al. 2010).

The most beneficial modification to the 7-day OvSynch protocol was the administration of a second PGF 24 h after the one administered on day 7 (Carvalho et al. 2015a; Wiltbank et al. 2015; Barletta et al. 2018) improving luteal regression. Finally, P4 supplementation between the first GnRH and PGF improved the pregnancy risk and reduced pregnancy loss in cows regardless of the presence or absence of CL at the start of the protocol (Bisinotto et al. 2015).

This review focuses on the weaknesses of the OvSynch protocol that led to the development of "fertility programs" and the technical solutions these programs provide to improve fertility in cattle bred using fixed-time artificial insemination (FTAI) protocols. The physiological mechanisms of these programs will be addressed more than any other aspect.

DIFFERENT APPROACHES TO THE PROBLEM OF OESTRUS SYNCHRONISATION

There are different ways to approach the oestrus synchronisation problem. (1) Controlling the luteal phase by shortening the lifespan of the CL through the use of luteolytic agents or simulating the CL function by using P4 analogues, (2) controlling both follicular and luteal growth dynamics by combining GnRH and prostaglandin. This approach allows programmed inseminations without oestrus detection. When GnRH is used, the follicular phase is modified by inducing the ovulation of a DF, leading to the emergence of a new follicular wave (Pursley et al. 1995). The same result can be obtained by using oestradiol (E2). The initiation of a new wave of follicles occurs in this case after atresia of the DF (Bo et al. 1995; Martinez et al. 2005). More recently, control of the follicular phase has been achieved through the use of aromatase inhibitors (Letrozole), thus extending the lifespan of the DF and delaying the ovulation timing (Yapura et al. 2012; Zwiefelhofer et al. 2020a).

Control of the luteal phase

Progesterone-based protocols. Protocols that use progesterone or synthetic analogues (progestins) aim to extend P4 levels, suppressing the LH peak and preventing ovulation. To achieve this, intravaginal P4 impregnation provided by an intravaginal device (PRID – progesterone releasing intravaginal device or CIDR – controlled internal drug release) must last at least as long as a natural luteal phase because a natural CL might outlive short-term P4 treatment. However, extended P4 treatment can cause overdominance of the preovulatory follicle, leading to an oocyte of reduced fertility (Sirois and Fortune 1990; Adams et al. 1992). The time to ovulation after the removal of the progesterone-releasing device is variable. Due to this lack of precision, it is necessary to perform single insemination at observed oestrus or two inseminations, one at 56 h and the other at 72 h after device withdrawal (Kasimanickam et al. 2015).

Prostaglandin-based protocols. Protocols that use prostaglandin act by inducing premature luteolysis, which results in oestrus at different intervals depending on the growth stage of the follicle at the time of treatment. Due to this variability, it is recommended to inseminate when oestrus is observed, even though blind insemination can be done 80 h after the second dose of PGF, as suggested by Stevenson et al. (1999). Luteolytic treatment works only if prostaglandin is injected between six and 17 days of the cycle. Indeed, during the first 5–6 days of the luteal phase (met-oestrus), the CL is unresponsive to exogenous prostaglandin (Tsai and Wiltbank 1998; Miyamoto et al. 2005), which can cause single prostaglandin injections to fail. Therefore, two injections are necessary, administered 11–14 days apart. The interval between the injections is shorter for heifers (11 days) than for cows (14 days) because of the heifer's ability to recover a prostaglandin-sensitive CL more quickly (Wiltbank 1997).

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The interval to oestrus is longer when using PGF than when using P4 insert. This difference can be attributed to the time required for the regression of the CL to bring the P4 level back to basal, a condition that is rapidly reached after the removal of the progesterone-releasing device (Smith et al. 1979; Brusveen et al. 2009).

PGF treatment has proven to be the simplest and least expensive (especially if many cows are fertilised after the first injection), but no true anoestrus or prepubertal female will respond to luteolytic prostaglandin treatment (Stevenson and Pursley 1994)

Simultaneous control of the luteal and follicular phase

Oestradiol/progesterone-based protocol. Oestradiol is used in ovulation synchronisation for its ability to cause regression of the follicles, synchronising follicular wave emergence regardless of the stage of follicular development at the time of treatment (Bo et al. 1994). In an E2/progestin-based protocol, the progestin blocks E2-induced LH release (Bo et al. 1994), preventing ovulation from occurring during the treatment, while E2 injection suppresses FSH secretion (Bo et al. 1994; Martinez et al. 2003). The rise of FSH in parallel with the decrease in the concentration of exogenous oestradiol, causes the emergence of a new wave of follicles. However, the interval to wave emergence is still variable and seems to be related to both oestradiol preparation (Colazo et al. 2005) and dose (Burke et al. 2003). Treatment with 5 mg oestradiol valerate resulted in a longer and more variable interval to follicular wave emergence than treatment with 5 mg oestradiol-17b (Colazo et al. 2005). Also, the time to wave emergence is shorter in cows with high milk production than in cows with low milk production (3.5 days vs 4.6 days) (Souza et al. 2009), probably due to higher oestradiol metabolic clearance rates associated with high-production milk (Wiltbank et al. 2006). Finally, E2/progestin-based protocol is less efficient in lactating dairy cows than in beef cattle (Souza et al. 2009)

OvSynch program

"The OvSynch program was designed to synchronise the growth of follicular waves and ovulation of dominant follicles, allowing for timed insemination without the need for oestrus detection. To achieve this goal, the PGF injection in the OvSynch protocol is flanked by two GnRHs, one seven days before and the other 56 h after, followed by AI between 16 to 24 h afterwards (Brusveen et al. 2008).

The OvSynch protocol consists of controlling (1) the beginning of the new follicular wave by one first injection of GnRH (GnRH-I), (2) the lifespan of the spontaneous CL and the GnRH-induced one by injecting a PGF, and (3) the ovulation time of the GnRH-induced DF by a second GnRH injection (GnRH-II).

When coinciding with a DF, GnRH-I causes its ovulation with the formation of a secondary CL, and a new follicular wave will emerge 1–2 days later (Pursley et al. 1995). The GnRH-induced follicle wave will give rise to a DF 7 days later, i.e., at the time of the PGF injection. This dose of PGF leads to lysis of the primary and secondary CL, allowing the DF to continue its maturation. Two days later, GnRH-II is injected to reinforce the preovulatory LH peak so that ovulations in the treated females are spread out over a shorter period, allowing the use of single FTAI, i.e., 16 to 24 h after the injection of GnRH-II, despite the fact that the oestrus behaviour is decoupled from ovulation.

The synchronisation rate obtained by the OvSynch protocol is acceptable in dairy cows. However, 40% may not respond to the treatment (Martins and Pursley 2016). Unsynchronised cows are:

Those that receive GnRH-I during the early stage of the cycle (1–4 days) in the presence of freshly emerged follicles. Such cows cannot develop and subsequently ovulate DF in response to GnRH injection (Bello et al. 2006). In this case, the spontaneous DF would be 10–11 days old at the time of the PGF injection and would already be undergoing atresia (Vasconcelos et al. 2003), allowing the start of a new follicular wave, which will result in a selected follicle that would be too immature to respond to a precocious GnRH-II (same scenario as with GnRH-I) (Figure 1).

Those with spontaneous luteolysis induced by endogenous PGF between GnRH-I and PGF injection. Such cows will be detected in oestrus one day before or one day after PGF administration. In this case, ovulation occurs well before the FTAI, so a low proportion of females will conceive the timed insemination (Figure 2).

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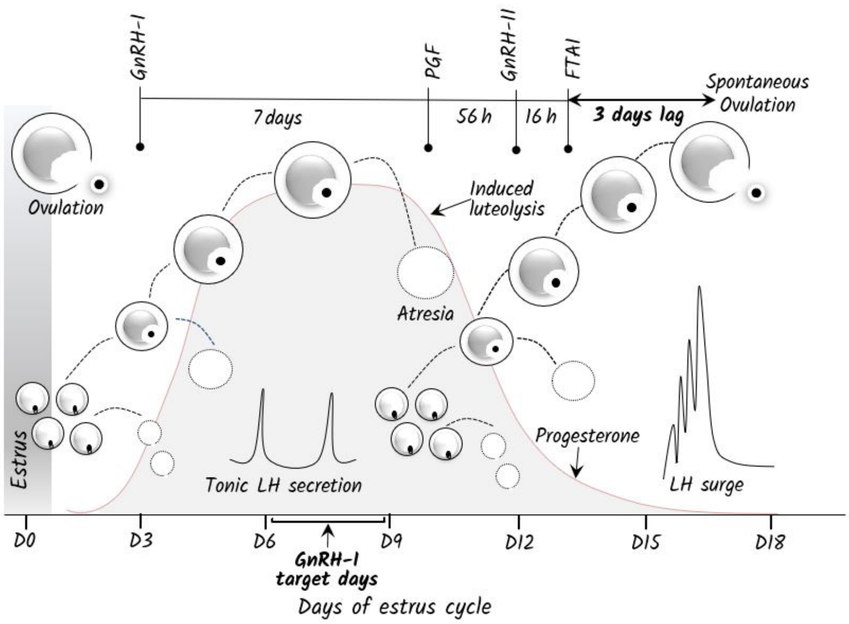

Figure 1. Untimely fixed-time artificial insemination (FTAI), occurring when the OvSynch protocol starts at the early stages of the oestrous cycle

GnRH = gonadotropin-releasing hormone; LH = luteinizing hormone; PGF = prostaglandin F

GnRH-I and GnRH-II fail to synchronise follicular waves and ovulations, respectively, because coinciding with dominant follicles that are too immature. The optimal period to start the protocol is mentioned (days 6–9)

IMPROVEMENTS OF THE OVSYNCH PROTOCOL: FERTILITY PROGRAMS

The success of the OvSynch program in terms of synchronisation and conception rate requires (1) coinciding the first GnRH with a sensitive DF, (2) coinciding the final GnRH with an appropriately sized and aged follicle, (3) the development of the GnRH-induced follicle during an adequate P4 environment (Giordano et al. 2013), (4) the complete regression of the spontaneous and/or induced CL in response to PGF (Carvalho et al. 2015b)

The original OvSynch program cannot always meet all of these conditions. This problem was the origin of the modified OvSynch programs, also called "fertility programs" (Table 1).

Coinciding the first GnRH with a sensitive dominant follicle

A randomly selected cow has an average 50% chance of having a DF (Bello et al. 2006; Colazo et al. 2009). Failure of the OvSynch protocol is most often related to the absence of a sensitive DF at the time of the first injection of GnRH (Vasconcelos et al. 1999) because, in this case, ovulation is delayed with respect to the blind AI.

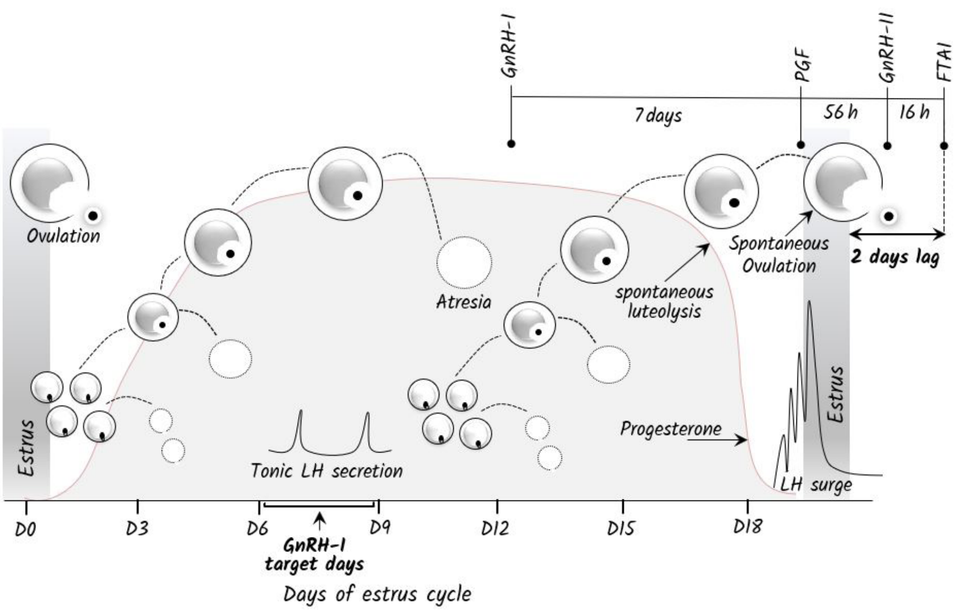

Figure 2. Delayed fixed-time artificial insemination (FTAI), occurring when spontaneous luteolysis takes place between GnRH-I and PGF injections

GnRH = gonadotropin-releasing hormone; LH = luteinizing hormone; PGF = prostaglandin F

The optimal period to start the protocol is mentioned (days 6–9)

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Table 1. The solutions provided by fertility programs to fill the main shortcomings of the OvSynch protocol

OvSynch's issues	Fertility programs	
	alternative solutions	benefits
Absence of a sensitive dominant follicle at the time of GnRH-I	Presynchronisation to initiate the ovsynch protocol at 6–9 days	• Optimises timing of FTAI • Prevents the ovulation of older follicles ➔ improves embryo quality
Poor quality embryo derived from a GnRH-I-induced dominant follicle	P4 implement between GnRH-I and PGF	• Optimises P4 concentration during the growth period of the dominant follicle targeted by the final GnRH • Prevents early ovulation ➔ increases oocyte maturation ➔ improves embryo quality
Incomplete induced luteolysis and overdominance of the preovulatory follicle targeted by GnRH-II	2 PGFs 24 h apart (day 5 and day 6)	• Decreases the P4 level at the final GnRH-induced ovulation ➔ improves oviductal and uterine motility ➔ optimises spermatozoa's transport • Gives an appropriately sized and aged follicle of reduced lifespan

FTAI = fixed-time artificial insemination; GnRH = gonadotropin-releasing hormone; PGF = prostaglandin F

While it is possible for cows to become pregnant without responding to GnRH-I treatment, ovulation during the first GnRH injection can increase the likelihood of a positive response to subsequent treatments in the remainder of the OvSynch protocol (Vasconcelos et al. 1999). This can help ensure the presence of an appropriately sized follicle at the final GnRH and the production of embryos of better quality (Cerri et al. 2009).

To maximise the proportion of females at a more optimal stage of the oestrous cycle at OvSynch start, treated cows must be presynchronised (Vasconcelos et al. 1999) (Figure 3). This optimal cycle stage corresponds to the beginning of the dioestrus phase (D6 to 9) (Bello et al. 2006). To ensure the presence of a DF at the time of GnRH-I administration and if a sensitive CL is present, an injection of PGF 12 days before will induce luteolysis to allow ovulation 3–5 days later. This would put the cow at days 5–7 of the cycle at the time of the GnRH-I injection, i.e., at the beginning of the dioestrus phase, the ideal time to start the OvSynch program (Vasconcelos et al. 1999; Moreira et al. 2000).

Because presynchronisation with a single PGF might fail in cows with insensitive CL, it was recommended to presynchronise with two injections of PGF (PreSynch) 14 days apart with initiation of the timed AI protocol 12 or 14 days later (Moreira et al. 2001). This program referred to as PreSynch-OvSynch protocol, significantly increased the artificial insemination pregnancy rate (P/AI) in cycling cows (43% for presynchronised cows vs 29% for cows submitted to a standard OvSynch) (Moreira et al. 2001). Shortening the interval between the PreSynch and the OvSynch from 14 to 11 days increased P/AI by seven percentage points (Galvao et al. 2007). However, further shortening this interval could reduce fertility (Colazo et al. 2013a).

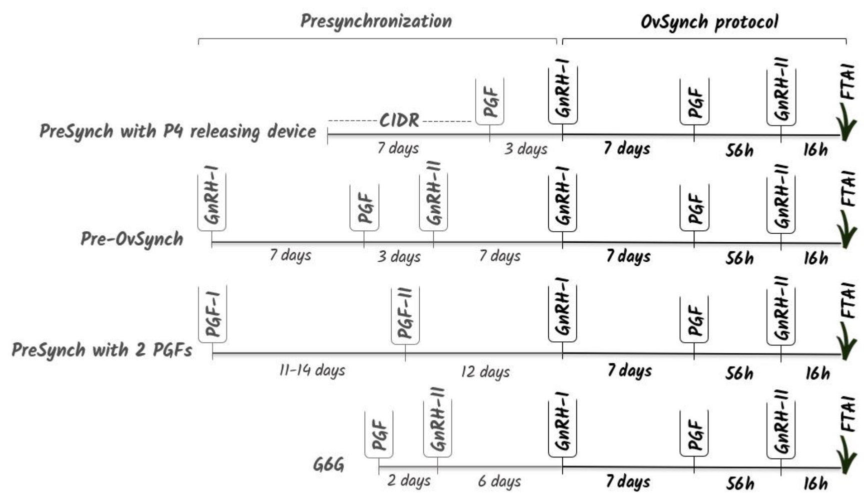

Figure 3. Presychronization protocols that allow the OvSych to start to coincide with the target days (day 6–9)

CIDR = controlled internal drug release; FTAI = fixed-time artificial insemination; G6G = presynchronization with prostaglandin F and gonadotropin-releasing hormone, 6 and 8 days before starting the OvSynch protocol, respectively; GnRH = gonadotropin-releasing hormone; PGF = prostaglandin F

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Overall, the PreSynch strategy with two PGFs improved P/AI compared with OvSynch at random stages of the oestrous cycle (55% vs 46.8%) (Gumen et al. 2012). However, according to Borchardt et al. (2016), negating the presynchronisation effect by inseminating cows observed in oestrus after the second PGF of a PreSynch protocol leads to a significant decrease in P/AI. This decrease could be as much as 14 percentage points (30.5% vs 44.3%), as suggested by a meta-analysis conducted by Strickland et al. (2010).

Other more convoluted programs include Double OvSynch (pre-OvSynch and breeding-OvSynch seven days apart) and G6G protocols (PGF followed by GnRH, 8 and 6 days before the OvSynch program, respectively) (Martins and Pursley 2016). During the post-partum period, presynchronisation with a pre-OvSynch seems to be more efficient than presynchronisation with PGF (Souza et al. 2008; Dirandeh et al. 2015), probably because inactive ovaries do not respond to the luteolytic hormone after parturition (Kinder et al. 1996), while the two GnRH doses of the pre-OvSynch accelerate the recovery of ovarian activity.

Note that the beneficial fertility effect of the Double OvSynch protocol is less pronounced in multiparous compared to primiparous females (Herlihy et al. 2012; Astiz and Fargas 2013). This is likely due to the positive correlation between parity and incomplete luteal regression (Wiltbank et al. 2015). Souza et al. (2008) suggested that the efficiency of the Double OvSynch in primiparous is attributable to its ability to treat anovulation, which is commonly observed in primiparous at the end of the voluntary waiting period (Santos et al. 2009).

Compared to the Double OvSynch, presynchronisation with G6G yields higher P/AI in multiparous cows (Astiz and Fargas 2013). Extending the interval between PGF and GnRH from 2 to 4 days in the G6G protocol enhances the ovulatory response to the first GnRH of OvSynch, increases circulating P4 during OvSynch, and improves P/AI. This modification appears to promote further follicle growth, thereby increasing the likelihood of ovulation at the GnRH of presynchronisation (Heidari et al. 2017).

Presynchronisation can also be achieved through the use of intravaginal inserts containing progesterone (PRID or CIDR). Cows receive a progesterone insert for seven days, and the PGF is then injected at the time of removal of the vaginal device, three days before starting the OvSynch program (Rutigliano et al. 2008).

Silva et al. (2018) recently developed a P4-Synch protocol involving the insertion of an intravaginal P4 device 10 days prior to the start of the OvSynch protocol. The device was withdrawn on the day of PGF administration (D7 of OvSynch protocol) to achieve a 17-day period of P4 impregnation. The aim was to enhance the persistence of a DF and increase the response to the first GnRH of the OvSynch protocol. In a study of 440 dairy cows randomly assigned to P4-Synch or Double OvSynch protocols, there were no significant differences in ovulation rates to GnRH-I (81.2% and 86.3% for P4-Synch and Double OvSynch, respectively) or to the final GnRH (90.9% vs 86.0%). No significant differences were found in pregnancy loss (10.2% vs 3.5%) or pregnancy rates at day 60 (34.8% vs 38.7%). The authors concluded that simpler, shorter, less expensive, and less manipulative animal protocols could be an effective alternative to the use of Double OvSynch to achieve similar outcomes.

Coinciding the final GnRH with an appropriately sized and aged follicle

Unmanipulated oestrus can be either 2- or 3-follicular waves. The emergence of the ultimate wave giving rise to the ovulatory follicle is later in 3-wave cycles than in 2-wave cycles (day 16 vs day 11) (Ginther et al. 1989). This shortens the lifespan of the ovulatory follicle in the 3-wave cycles (three days difference) (Sirois and Fortune 1988; Bleach et al. 2004), which are also considered the most fertile (Sartori et al. 2004; Wolfenson et al. 2004). According to Bleach et al. (2004), when inseminated on spontaneous oestrus, cows that failed to become pregnant had a mean interval from follicular wave emergence to ovulation one day longer than those that were able to conceive. This overdominance-associated reduced fertility seems to be related to poor oocyte quality and impaired embryonic development (Ahmad et al. 1995; Cerri et al. 2009).

Because reducing the period of follicular dominance improves fertility (Bleach et al. 2004; Santos et al. 2010), it was recommended to advance the PGF injection to day 5 in the OvSynch program (Bridges et al. 2008). An earlier dose of PGF (day 5) followed by a second dose 24 h later (day 6) ensures not only the regression of the spontaneous and the newly

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formed CL but also the development of an appropriately sized and aged follicle of reduced lifespan and duration of physiologic dominance (Santos et al. 2010). It should be noted that beef cows enrolled in the 5-day FTAI protocol showed improved pregnancy rates only when they were inseminated at the same time as receiving the last GnRH, 72 h after luteal regression (Bridges et al. 2008). This protocol, referred to as 5-day CoSynch72, prolongs the pro-oestrus stage, maintaining high levels of oestradiol for a longer period of time, and allows for higher oestradiol concentrations by triggering luteal regression earlier relative to follicular wave emergence. Indeed, shortening the interval between the first GnRH and the PGF by 48 h allows for obtaining younger follicles (3–4 days), which have a greater potential to secrete oestradiol than older ones (Bridges et al. 2008). An increase in oestradiol concentration at the second GnRH-induced ovulation was found to improve fertility in cows receiving an FTAI protocol (Perry et al. 2005; Lopes et al. 2007).

By guaranteeing the presence of a responsive DF at the OvSynch start (day 6–9 of the cycle), Presynchronisation also shortens the period of follicular dominance and prevents the ovulation of older follicles. Cerri et al. (2009) observed that embryos recovered from cows receiving the first GnRH on day 6 of the cycle were larger and more viable than those collected from cows starting the OvSynch on day 3 of the cycle.

It should be noted that induction of ovulation from very small follicles after a short period of dominance also reduces the fertility of cows (Mussard et al. 2007; Atkins et al. 2013). On 622 lactating dairy cows subjected to a classic OvSynch program, Souza et al. (2007) reported significantly higher fertility for cows that ovulated medium-sized follicles (15–19 mm) compared with cows that ovulated smaller (< 14 mm) or larger follicles (> 20 mm), with pregnancy rates of 47.4%, 36.2%, and 38.2% respectively.

Development of the GnRH-induced follicle during an adequate progesterone environment

An analysis of a large dataset of P4 profiles during an OvSynch protocol revealed that 26% of cows with lower P/AI exhibited suboptimal P4 concentration at the first GnRH (Carvalho et al. 2018). Furthermore, cycling cows with high P4 concentration at the start of the OvSynch protocol had a significantly greater P/AI than cycling cows with low P4 concentration or anovular cows (43% vs 31.3% vs 29.7%, respectively) (Bisinotto et al. 2010).

The reduced fertility of cows with P4 < 1.0 ng/ml at GnRH-I has been attributed to an increased LH pulse frequency leading to overstimulation of the oocyte (Wiltbank et al. 2006; Rivera et al. 2011), which would generate a poor-quality embryo. Therefore, it is important to optimise P4 concentration during the growth period of the DF targeted by the final GnRH of the OvSynch protocol (Colazo et al. 2013b).

The increase in circulating P4 concentration can be achieved through intravaginal P4 inserts or by the first GnRH-induced accessory CL. Cows with two CL exhibited greater circulating P4 levels at PGF treatment in comparison to cows with a single CL (3.6 ng/ml vs 2.5 ng/ml) (Wiltbank and Pursley 2014).

One meta-analysis realised by Bisinotto et al. (2015) indicated that an OvSynch modified by an intravaginal P4 insert, given during the seven days between the first dose of GnRH and the PGF treatment, tends to reduce the risk of pregnancy loss and significantly increases the pregnancy rate (10% greater in supplemented cows compared to controls), regardless whether a spontaneous CL was present during treatment (Figure 4). Nevertheless, cows that received the OvSynch program without P4 supplementation, but displayed a CL, had a better pregnancy rate than those that received intravaginal P4 insert but did not have a luteal structure during the seven days between GnRH-I and PGF. This is probably due to a higher

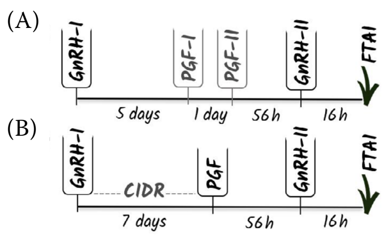

Figure 4. Modified OvSynch protocols that allow complete luteal regression (A) and optimise the P4 level during the selected follicle growth (B)

CIDR = controlled internal drug release; FTAI = fixed-time artificial insemination; GnRH = gonadotropin-releasing hormone; PGF = prostaglandin F

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P4 concentration released by the natural CL than the intravaginal P4 insert.

When cows without CL were given 2 CIDR inserts concurrently at the onset of the 5-day OvSynch protocol, the resulting pregnancy rate was similar to that of cows bearing a CL during a dioestrus (49.9% and 46.8%, respectively) (Bisinotto et al. 2013).

Contrary to expectations, a conventional OvSynch protocol fails to enhance fertility in anovular cows, not due to a lack of GnRH-I-induced ovulation, but rather due to decreased synchronisation (Herlihy et al. 2013). Generally, anovulatory cases respond well to GnRH treatment (Gumen et al. 2003). The decreased synchronisation in anovular cows can be attributed to the development of an anovulatory follicle in a low P4 environment. This explains why P4 supplementation during an OvSynch protocol can enhance fertility in anovular cows. This is particularly important, given that up to 43% of dairy cows are anovular at the end of the voluntary waiting period (Rhodes et al. 2003).

The intimate mechanism by which P4 supplementation improves the pregnancy rate remains to be elucidated. This supplementation probably acts by preventing early ovulation (Colazo et al. 2013b), and/or increasing oocyte maturation (Wiltbank et al. 2011).

P4 supplementation during the OvSynch protocol doesn't seem to improve outcomes for cows inseminated in oestrus before the scheduled day of timed AI (Bisinotto et al. 2015). This supports the notion that the primary benefit of using intravaginal P4 insert during FTAI protocols is ovulation synchrony (Colazo et al. 2013b). Regarding the reduced risk of pregnancy loss, P4 supplementation not only enhances the quality of the early embryo but also promotes uterine vascularisation and glandular function, which have a positive impact on placentation and late embryo development (Shaham-Albalancy et al. 1997).

Complete regression of the spontaneous and/or induced corpus luteum in response to prostaglandin

Lactating dairy cows have approximately a 20% risk of incomplete luteolysis following a single dose of PGF (Carvalho et al. 2015a; Wiltbank et al. 2015). Because of the remaining functional luteal tissue, P4 concentration can be maintained above 0.5 ng/ml at the time of induced ovulation by the final GnRH, and synchronised cows, in this case, will have only a 5% chance of becoming pregnant (Martins et al. 2011; Carvalho et al. 2018). The P4 level at the last GnRH seems crucial for the fertility of cows undergoing an OvSynch protocol. Pregnancy per artificial insemination in cows with P4 < 0.4 ng/ml is significantly better compared to cows with P4 > 0.4 ng/ml (41% vs 14%) (Carvalho et al. 2015b). Furthermore, an increased concentration of circulating P4 as a consequence of incomplete luteolysis may also reduce oviductal and uterine motility, thereby affecting the transport of spermatozoa (Bennett et al. 1988). The decreased endometrial thickness is also a consequence of elevated P4 at the second GnRH, leading to a decrease in fertility (Souza et al. 2011).

To ensure the complete luteolysis and thus optimise the P4 level at the final GnRH-induced ovulation, two successive PGFs at day 5 and day 6 were therefore recommended instead of a single one at day 5 in the 5-day OvSynch protocol (Santos et al. 2010) (Figure 4). This second PGF is aimed at lysing a newly formed CL that is less than five days old at the time of the first dose of PGF, to which it is, therefore, refractory. The second PGF during the 5-day FTAI protocol becomes mandatory in presynchronised cows due to the heightened risk of first GnRH-induced ovulation and hence the occurrence of newly formed CL (Galvao et al. 2007). Let's point out, however, that according to Howard and Britt (1990), accessory CL induced during mid-dioestrus would be able to respond to an exogenous PGF at earlier stages of luteal development than the typical CL that develops during met-oestrus. According to Santos et al. (2010), a single dose of PGF during a 5-day OvSynch protocol was found to be more effective in lysing accessory CL developed under high circulating P4 concentrations rather than low circulating concentrations (59% vs 29%). The second PGF was also preconised at day 8 in a 7-day OvSynch protocol (Borchardt et al. 2018; Tippenhauer et al. 2021). Spacing the two PGFs 24 h apart seems crucial since a double dose of PGF at a single time was insufficient for complete luteolysis (Barletta et al. 2018). However, using cloprostenol at 250% of the normal dose in a 6-day OvSynch produced a similar luteolytic effect as two standard doses given 24 h apart on day 5 and day 6 (Valldecabres-Torres et al. 2013).

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When two PGFs are administered 24 h apart, the 5-day and the 7-day protocols result in similar P/AI. So, the improved fertility provided by the 5-day protocol compared to the standard OvSynch would be attributed to the complete CL lysis resulting from the supplementary luteolytic effect of the second PGF treatment rather than to the reduced period of follicle dominance resulting from the shorter interval between GnRH-I and PGF (Santos et al. 2016). Moreover, a second PGF added to a Double-OvSynch increased P/AI by about 5% in cows receiving a 7-day protocol (Giordano et al. 2013).

LETROZOLE-BASED PROTOCOL

Letrozole is an innovative non-steroidal treatment which offers a safe alternative to estrogen treatment, another method of synchronising wave emergence. While oestradiol regulates FSH secretion through negative feedback (Bo et al. 1995), letrozole is a third-generation aromatase inhibitor capable of specifically and reversibly inactivating the aromatase enzyme, resulting in a dose-dependent decrease of circulating oestradiol concentrations. Contrary to what has been observed in women (Mitwally and Casper 2001; Casper 2007), in the bovine model, letrozole does not appear to cause an FSH surge (which would be a logical consequence of the suppression of the oestradiol inhibitory effect on FSH) and thus hasten the emergence of a new follicular wave (Yapura et al. 2012). Rather, letrozole treatment postpones the emergence of the next follicular wave by causing overdominance of the extant DF, which would be due to an increased pituitary LH secretion (Zwiefelhofer et al. 2020a). Interestingly, this synchronisation effect of letrozole on the follicular waves is systematic and unrelated to the development stage of the follicular wave at the time of treatment (Yapura et al. 2012).

According to Zwiefelhofer et al. (2020b), an intravaginal letrozole-releasing device kept for five days significantly decreased the variation in the onset of oestrus in donor cows despite having undergone a super stimulation treatment [ovulation is usually earlier in donors with a greater number of CL at the time of oocyte/embryo collection (Bo et al. 2006)]. Yapura et al. (2016) also reported that a letrozole-impregnated intravaginal device for four days and a PGF dose at device removal, followed by a GnRH 24 h later, improved the synchrony of ovulation, compared with a PGF-GnRH-based protocol (89% vs 76%).

Although undeniably safer and suitable for FTAI, letrozole-based protocols should be as effective, if not more effective, than oestradiol-based protocols in fertility. Unfortunately, all recent studies that reported good ovulation synchrony also relayed an unacceptably low P/AI when letrozole-based protocols were essayed. According to Zwiefelhofer et al. (2022), a 4-day letrozole treatment followed by GnRH given concomitantly with FTAI 48 h after PGF resulted in a significantly lower P/AI in cows, compared with a 7-day-E2P4 treatment (15% vs 63%). By modifying letrozole-based protocols, Zwiefelhofer et al. (2022) hoped to improve the low pregnancy rate recorded in previous studies. Regrettably, extending the PGF to FTAI interval by an additional 24 h has led to a similar low pregnancy rate reported by Yapura (2013). Similarly, substituting a wax-based dip-coated letrozole device, used by Yang et al. (2021), for a T-shaped silicone letrozole-releasing device has not improved the P/AI.

The factors behind the low pregnancy rates achieved by letrozole-based protocols remain to be experimentally determined. Purely hypothetically, one can speculate that a reduction in peri-ovulatory E2 concentrations as a consequence of the suppressive effect of letrozole on androgen aromatisation could compromise sperm or oocyte transport in the female genital tract (Orihuela and Croxatto 2001). It is unknown whether a three or 4-day letrozole treatment adversely affects oocyte maturation. It is, however, well known that the overdominance of the ovulatory follicle, as seen during letrozole-based protocols, reduces embryo quality (Cerri et al. 2009), thereby decreasing fertility.

CONCLUSION

The initial OvSynch program has overcome the problem of heat detection but failed to improve conception rates in timed-inseminated cows in comparison with those inseminated in oestrus. This shortcoming was the reason for the presynchronisation and modified-OvSynch protocols, often referred to as "fertility programs".

Presynchronisation ensures a sensitive preovulatory follicle is present during the first GnRH injection. Its ovulation triggers the emergence of a new wave of follicles and generates an acces-

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sory CL. The new follicular wave will result in a DF of appropriate age and size at the final GnRH, and the accessory CL will participate in increasing the concentration of P4, thus preventing the LH overstimulation of the oocyte/cumulus complex. On the other hand, modifying the original OvSynch by adding PGF guarantees the complete luteal regression, reinforcing the LH surge and allowing the ovulation to coincide with the predetermined artificial insemination. As for the recent letrozole-based protocol, the reasons for the low fertility they are associated with need to be elucidated.

Conflict of interest

The author declares no conflict of interest.

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