Evolution in fixed-time: from synchronization of ovulation to improved fertility

Mario Binelli[1], Roberto Sartori[2], José Luiz Moraes Vasconcelos[3], Pedro Leopoldo Jerônimo Monteiro Jr.[2], Marcos Henrique Colombo Pereira[3] and Roney S. Ramos[1]

[1]Department of Animal Reproduction, School of Veterinary Medicine and Animal Science, University of São Paulo (USP), Pirassununga, SP 13635-900, Brazil; [2]Department of Animal Science, ESALQ, University of São Paulo (USP), Piracicaba, SP, Brazil 13418-900; [3]Department of Animal Production, School of Veterinary Medicine and Animal Science, São Paulo State University (UNESP), Botucatu, SP 18168-000, Brazil.

Summary

Synchronization of ovulation and subsequent timed artificial insemination became a popular practice in modern, large scale, dairy and beef commercial operations. Popularity was because it became a serious option to eliminate a laborious and low efficiency activity in the industry: checking cows for heat. Synchronization protocols involve the sequential administration of reproductive hormones to manipulate the estrous cycle to provide a fertile oocyte for insemination at a predictable moment. In the last 20 years, great effort was put forth in protocol development and adjustment; current rates of synchronous ovulations are 80-90%. Moreover, fertility to current protocols is similar to that obtained in cows inseminated after heat detection. The present wave of protocol development proposes to face the challenge of further improving pregnancy success of current protocols. The guiding principle is that, due to the intense metabolism associated with copious milk production, steroid hormone concentrations are insufficient for adequate reproductive function. Thus, new protocols have incorporated strategies to adjust the endocrine milieu and consequently support specific portions of the synchronization process. This paper discusses the positive fertility impact of protocol design that incorporated the following concepts: controlling P4 concentrations during follicle growth to increase oocyte quality; reducing circulating P4 prior to AI to increase proportion of cows pregnant; providing estrogen priming during proestrus to benefit conception and reduce embryo mortality; and optimizing progesterone priming during early diestrus to enhance conceptus survival. It is expected that adoption of these concepts will lead to a continuous increase in fertility of lactating dairy cows.

Corresponding author E-mail: binelli@usp.br

Introduction

The scientific community owes a lot of respect to the Estrus Synchronization Pioneers. They provided the entire biological basis so that in the 1990's, simple and successful protocols for the synchronization of ovulation and fixed-time artificial insemination (FTAI) were devised and applied massively to bovine production commercial operations around the world (review: Wiltbank & Pursley 2014). Protocols entail strategic administration of reproductive hormones in a sequential manner with a single objective: the ovulation of a follicle carrying a healthy oocyte at a moment compatible with fertilization by AI. Thus, the focus is to program ovarian events to occur in a timely fashion to ultimately provide an oocyte for fertilization and subsequently optimal embryo development leading to a pregnancy. Ovarian events that need effective programming and respective hormones (or pharmaceutical analogs) commonly used for programming are: recruiting a follicular wave [GnRH, estrogens (estradiol 17β and its esters; E2)/progestagens including progesterone (P4)], temporary blocking of ovulations (P4), efficient regression of corpora lutea (CL) to decrease P4 concentrations prior to ovulation [prostaglandin F2α (PGF)], and ovulation [GnRH, E2]. There are two basic categories of protocols. The OvSynch-type protocols, utilize GnRH to synchronize the follicular wave by ovulation of a dominant follicle at the start of the protocol, and to synchronize ovulation at the end of the protocol allowing FTAI. The E2/P4-based protocols start using E2 products in the presence of P4 to induce atresia of follicles and synchronize emergence of a new follicular wave. At the end of E2/P4-based protocol, another E2 treatment in the absence of P4 is used to synchronize ovulation and allow FTAI. Choice of protocols and drugs depend on technical and economic decisions taken by farm personnel and, more importantly, according to regulatory constraints on drug usage, specific to different countries.

Fertility success to protocols is measured in terms of proportion of cows pregnant or pregnancy rate (PR). Mathematical formulation for PR is equal to proportion of cows serviced x proportion of cows pregnant that were serviced. Proportion of cows serviced = the number of cows receiving AI ÷ number of cows eligible to receive AI, and proportion of cows pregnant = the number of cows pregnant ÷ number of cows receiving AI (P/AI). Researchers rationalized that maximization of PR of the herd would result from maximizing both proportion of cows serviced and P/AI. Before FTAI, limitations to proportion of cows serviced were a high proportion of anovular cows, which did not show heat, and inefficient detection of cows in heat. Thus, seminal contributions of FTAI to the animal industry were to both induce cyclicity in anovular cows and eliminate the need for detection of estrus, because all cows eligible to AI were in fact inseminated.

Next, it became important to understand the influence of FTAI protocols on P/AI. Santos *et al*. (2009) compared P/AI between cows inseminated to detected heat and treated to receive FTAI. P/AI were similar between groups. More recently, Nascimento *et al*. (2013a) compiled experimental results involving 48 herds and 83,771 cows. The P/AI of groups of animals treated with hormonal protocols and submitted to FTAI were compared to control groups, in which cows were AI at detected estrus. Again, overall first service P/AI was 35.4% for the detection of estrus group and 36.1% for the FTAI group. Interpretation is that classical protocols achieve P/AI comparable to the cow's natural fertility potential. Next, question became why fertility is low and can it be increased. We propose that the next step in protocol development is to devise treatments aiming to increase P/AI of FTAI cows relative to cows bred to a natural heat. Current protocols must do more than mimic endocrine events to provide an adequately timed oocyte for AI. Modern protocols must incorporate the understanding that the high yield dairy cow has an altered reproductive hormone milieu that affects fertility directly and that is a target for therapeutic interventions. Here, we will discuss concepts that have been applied to novel

protocols for the lactating dairy cow. Specifically, the following topics will be discussed: (1) controlling P4 concentrations during follicle growth to increase oocyte quality and to effectively reduce circulating P4 associated with CL regression prior to AI; (2) providing estrogen priming during proestrus to benefit conception and reduce embryo mortality; and (3) optimizing progesterone priming during early diestrus to enhance conceptus survival.

The abnormal reproductive endocrine profile of the modern dairy cow is associated with reduced fertility

The design of fixed-time protocols to synchronize ovulation requires an understanding and characterization of the reproductive physiology of the modern dairy cow, and how management and nutrition affect it. There is a high positive correlation between dry matter feed intake and milk production. The high feed intake can alter ovarian physiology and hormone concentrations. High-producing cows in lactation have larger size follicles; however, E2 concentrations in blood are lower compared to heifers and non-lactating cows (De La Sota *et al*. 1993, Sartori *et al*. 2002a, Wolfenson *et al*. 2004). It is well known that the size of the ovulatory follicle has a high positive correlation with size of CL (Vasconcelos *et al*. 2001, Sartori *et al*. 2002a) and consequently this may be reflected in circulating P4 concentrations (Moreira *et al*. 2000). However, despite lactating dairy cows having larger CL, their circulating P4 concentrations are also lower than those of heifers and non-lactating cows (Sartori *et al*. 2002a, Sartori *et al*. 2004, Wolfenson *et al*. 2004). Lower circulating P4 has also been related to the increased incidence of follicle co-dominance and double ovulation observed in lactating cows, but not in heifers. Increasing blood P4 by manipulating the estrous cycle with exogenous hormonal treatments has reduced the incidence of multiple ovulations in dairy cows (Wiltbank *et al*. 2012).

The lower circulating steroid concentrations in cattle under high nutritional planes may be due to two causes: 1) there is lower steroidogenesis in follicles or CL of animals fed with high energy diets than those in restricted feeding. However, there is no study that was able to adequately measure production of ovarian steroids in cows submitted to different nutrition regimes; 2) cows ingesting large quantities of feed have higher liver blood flow (Sangsritavong *et al*. 2002, Vasconcelos *et al*. 2003). Consequently, steroid hormones are catabolized at greater rates, resulting in decreased circulating concentrations. Indeed, lactating dairy cows, in relation to non-lactating cows, have a twofold greater hepatic blood flow and, consequently, catabolize much more P4 and E2 (Sangsritavong *et al*. 2002).

Blood E2 concentration has a high positive correlation (0.57) with the proportion of cows presenting estrous behavior and intensity of estrus. Moreover, there is a high negative correlation between milk production and duration of estrus (-0.45; Lopez *et al*. 2004). Thus, lactating dairy cows have shorter duration of estrus than heifers (7.3 *vs*. 10.7 h; $P < 0.05$; Nebel *et al*. 1997), which compromises reproductive management strategies in dairy herds.

In relation to fertility, a longer time between luteolysis and ovulation, or a longer period of follicle dominance may affect embryo quality. Cerri *et al*. (2009) reported that almost 80% of embryos were grades 1 or 2 (i.e., considered viable; Robertson & Nelson 1998) in cows with a 5.5 to 6.0 d period of follicle dominance, whereas only 45% of embryos were considered viable in cows with a period of dominance lasting 8.5 to 11.5 d. Moreover, there is a negative correlation ($r = -0.22$) between the period of follicle dominance and conception rate (Bleach *et al*. 2004). In our studies comparing embryo quality from high producing lactating Holstein versus pubertal heifers or nonlactating cows, a lower embryo viability was detected in lactating cows (Sartori *et al*. 2002b). Among those embryos recovered 5 d after AI from single ovulation

lactating cows, only 48% were considered viable, whereas nulliparous heifers and nonlactating dairy cows yielded 72% (summer) and 82% (winter) of viable embryos, respectively. Moreover, during summer, lactating dairy cows had compromised fertilization compared to heifers (55% *vs* 100%, respectively).

In general, lower blood P4 concentration in cows caused by high feed intake compromises fertility. Cows with lower blood P4 concentrations during the pre-ovulatory follicle growth before AI had reduced fertility (Folman *et al*. 1973, Fonseca *et al*. 1983, Bisinotto *et al*. 2010a,) and supplementation of P4 prior to AI increased conception rate (Folman *et al*. 1990, Wehrman *et al*. 1993, Bisinotto *et al*. 2013). Low serum P4 concentration allows increased pulse frequency of LH (Roberson *et al*. 1989, Adams *et al*. 1992), causing premature maturation of the oocyte (Revah & Butler 1996) with a resulting decrease in oocyte quality at the time of ovulation, and consequently, low embryo quality (Ahmad *et al*. 1995). Reduced P4 concentration after AI also is associated with reduced fertility (Lukaszewska & Hansel 1980, Ahmad *et al*. 1995, Larson *et al*. 1997). Consistently, treatments to reduce P4 during early diestrus impaired embryo development (Lonergan 2011).

Likewise, alteration in circulating E2 concentrations may affect fertilization or transport of an embryo/ovum. Moreover, estrogen-dependent secretory proteins are postulated to be an essential part of a supportive environment for sperm capacitation, fertilization and early embryonic development. An altered oviductal microenvironment in cows bearing a persistent dominant follicle has been observed (Binelli *et al*. 1999). It was postulated that this inappropriate microenvironment contributes to the decreased fertility observed in cows with persistent follicles. Furthermore, excessive exposure to E2 was suggested to be deleterious to the oocyte and early embryo (Inskeep 2004).

Collectively, evidence is accumulating from different research groups and experimental models to suggest that the high-yield, intensively managed, modern dairy cow has abnormally low concentrations of E2 and P4 at different reproductive windows, and this condition negatively impacts fertility. As described in the next sections, positive fertility responses have resulted from using FTAI protocols as tools to supplement the limiting concentrations of steroid hormones at specific moments of the reproductive cycle.

Controlling P4 concentrations during follicle growth to increase oocyte quality and to increase efficiency of CL regression prior to AI

One important premise of FTAI is that protocols can be initiated in cows at random stages of the estrous cycle. However, early in protocol development it was clear that fertility to the protocols varied according to changes in concentrations of P4 through the protocol (Moreira *et al*. 2000). Bisinotto *et al*. (2010a) indicated that fertility was greater when follicle development occurred under greater P4 concentrations, such as those present in the second wave of follicle development versus the first wave. Explanations for these findings are multifactorial and may involve effects of supplemental P4 on quality of the oocyte and resulting embryo, as well as function of the reproductive tract. For example, Rivera *et al*. (2011) measured a greater proportion of excellent, good and fair quality embryos in superstimulated cows treated with supplemental P4 during the superstimulation protocol. Additionally, Cerri *et al*. (2011) showed altered dynamics of endometrial steroid receptors and magnitude of oxytocin-stimulated PGF release when cows had programmed ovulatory follicle development under contrasting concentrations of P4. It was suggested that changes associated with follicle growth under low P4 concentrations could disturb negatively the processes of pregnancy recognition and thereby fertility.

Another critical event involves induction of complete luteolysis. It is now well accepted that slight elevations in P4 concentrations near the time of AI, due to lack of complete CL regression, result in major reductions in fertility of dairy cattle (Souza *et al*. 2007, Brusveen *et al*. 2009, Martins *et al*. 2011b). E2/P4-based protocols are used widely in countries that do not have governmental regulatory restrictions on the use of these pharmaceutical drugs, such as Brazil, Argentina, Australia and Japan. Such protocols consist of injecting E2 concomitant with the insertion of a source of releasable progestin to induce follicle turnover, then removal of the progestin source after 7 to 9 days concurrently with induction of luteolysis (i.e., injecting PGF), and finally induction of ovulation. In E2/P4-based protocols, one possible strategy for more efficacious CL regression is to advance the PGF injection in relation to progestin withdrawal. There are at least two advantageous outcomes from this practice. One is to allow more time for endogenous P4 to decrease and second is to allow for a more uniform decline in P4 among cows. Pereira *et al*. (2013a) evaluated whether there would be an effect on fertility to FTAI or timed embryo transfer (TET) by changing the timing of treatment with PGF (injected PGF on d 7 *vs*. d 8) during an 8d E2/P4-based program for synchronized ovulation. A higher fertility in the d 7 PGF treated group was observed at d 28 and d 60 diagnoses of pregnancy for both primiparous and multiparous cows. The magnitude of the response to earlier treatment with PGF was quite impressive with over a 50% improvement in day 30 P/AI (i.e., 32.9 *vs*. 20.6%). In contrast, the effects of timing of PGF treatment were more subtle in the cows that received TET with only a 13% improvement in pregnancies/timed embryo transfer (P/TET) between cows that received PGF on d 7 *vs*. d 8 (i.e., 47.0 *vs*. 40.7%). Thus, it appears that there is a dramatic effect of advancing the PGF injection that is likely mediated through a series of events such as gamete maturation and transport, fertilization, early embryo development, and changes in the uterine or hormonal environment that manifests itself after embryo transfer on d 7.

One critical point is how low P4 concentration must be at the moment of AI not to be deleterious to pregnancy. Pereira *et al*. (2013b) used ROC curves and selected two important cut-offs of ≤ 0.09 ng/mL at FTAI and ≤ 0.21 ng/mL for TET (i.e., at 7 d before TET). The fertility results gave strong support for these values as definitions for luteolysis. Cows bred by FTAI had much greater fertility if they had P4 concentrations ≤ 0.09 ng/mL at the time of FTAI (34.1%) *vs* cows with greater concentrations (20.2%). Interestingly, the 0.09 ng/mL did not have an effect on fertility in TET, whereas there was a 50.5% decrease in fertility in TET cows with P4 > 0.21 ng/mL on day 10 of the protocol (7 d before TET). Brusveen *et al*. (2009), Santos *et al*. (2010), Martins *et al*. (2011a and 2011b) and Giordano *et al*. (2012) suggested that the highest P4 at AI not to adversely affect pregnancy outcome was 0.4, 0.5 and 0.24 ng/mL, respectively. These concentrations were superior to the 0,09 (ng/mL) established by Pereira *et al*. (2013b). Variations on these values are probably due to differences in protocols and experimental designs, but it is clear that low P4 values at insemination benefit fertility of FTAI programs.

Explanations for the benefit of minimal concentrations of P4 at AI include the greater rates of ovulation of cows presenting less than 0.1 ng/ml P4 at AI (79.8 *vs*. 60.6%; Monteiro Jr *et al*. 2012). Accordingly, P/AI were 49.0 *vs*. 27.3% for cows presenting P4 concentrations less than 0.1 ng/mL versus between 0.1 and 0.3 ng/mL, respectively. Another mechanistic observation includes the effect of P4 on oviduct function. The oviduct epithelium undergoes dramatic morphological and functional changes throughout the estrous cycle (Abe & Oikawa 1993). Changes include variations in the expression of oviductal mRNA coding for steroid receptors (PGR and ESR1) and epithelial markers (i.e., MUC16, OVGP1, HSP90B1) and in the sperm-binding capacity of the epithelium in culture (Chen *et al*. 2013) . At simulated estrus, in vitro, more sperm were bound compared to simulated diestrus. Thus, residual P4 resulting from incomplete luteolysis at AI could cause a decreased sperm binding and fertilizing capacity, contributing to decreased fertility.

Estrogen priming during proestrus benefits P/AI and enhance conceptus survival

Programming of a coordinated sequence of ovarian events is necessary for successful synchronization of fertile ovulations. Ovarian events involve release of steroid hormones for sufficient duration and magnitude. The intense steroid catabolism associated with high milk synthesis reduces steroid circulating concentrations, and this is probably a contributory condition associated with the modern dairy cow sub-fertility syndrome. Portions of the estrous cycle that are critically affected by this phenomenon are proestrus and estrus. Indeed, high producing dairy cows display estrous behavior in much less frequency and intensity than lower producers (Lopez *et al*. 2004). Estrous behaviour results from hypothalamic priming by E2 released by the dominant follicle in the absence of P4 (i.e., after luteolysis). Thus, diminished estrous behaviour likely results from lower circulating concentrations of E2 at expected proestrus-estrus. Most importantly, the presence of estrous behaviour is associated positively with pregnancy success in both beef (Perry *et al*. 2005, Perry *et al*. 2007, Sá Filho *et al*. 2010, Sá Filho *et al*. 2011) and dairy cows (Galvão *et al*. 2004, Souza *et al*. 2007, Hillegass *et al*. 2008, Pereira *et al*. 2014). Moreover, greater endometrial thickness, due to higher circulating E2 near FTAI, also was associated with improved fertility in high-producing dairy cows (Souza *et al*. 2011). The collective beneficial effects to fertility of sufficiently elevated E2 during the proestrus period stimulated the adoption of strategies to achieve this outcome within FTAI programs. Strategies have included the addition of E2 to the end of the Ovsynch protocol (Souza *et al*. 2007), stimulation of ovulation using E2 injections (Pancarci *et al*. 2002, Cerri *et al*. 2004, Stevenson *et al*. 2004), and increasing the interval between the luteolytic injection (PGF) and the time of expected ovulation (Pereira *et al*. 2013b). A longer proestrus period allows a longer period for the pre-ovulatory follicle to grow and allowing it to achieve full estrogenic capacity. Indeed, in the experiment by Pereira and collaborators (2013b), increased fertility and decreased pregnancy losses between days 30 and 60 were associated probably not only with the more complete luteolysis reported for cows receiving advanced PGF injections but also due to a longer proestrus period. A summary of results from various studies with *Bos taurus* beef cattle completed in the laboratory of Michael Day (Bridges *et al*. 2010) indicated that fertility increased consistently as time from PGF until GnRH increased. Similarly, in suckled *Bos indicus* beef cattle with a CL at 4 d before FTAI, Meneghetti *et al*. (2009) reported an increase in P/AI (50.3 *vs*. 36.1%) when PGF was injected 4 d before FTAI rather than 2 d before FTAI, which was the time of CIDR removal and ECP treatment in this protocol. In nonlactating *Bos indicus* beef cattle (Peres *et al*. 2009) the treatment with PGF 4 d before FTAI resulted in lower P4 concentrations on d of CIDR removal, a larger follicle at FTAI, increased percentage of cows that ovulated to the protocol (85.4% *vs*. 77.0%), and increased P/AI (52.0 *vs*. 36.4% for all cows; 60.9 *vs*. 47.2% for cows that ovulated) compared to cows treated with PGF 2 d before FTAI. In grazing dairy cows that were presynchronized with two PGF treatments (Presynch), reducing the interval from first GnRH until PGF from 7d to 5d increased P/AI in a 5d Cosynch FTAI protocol (Bisinotto *et al*. 2010b, Ribeiro *et al*. 2012). Pereira *et al*. (2014) treated lactating Holstein cows with E2/P4-based protocols with different durations (8 *vs*. 9 d). Protocols were identical except that interval between the luteolytic PGF injection and the ovulatory ECP injection was 1 *vs*. 2 d, respectively. Although P/AI was similar between groups, pregnancy losses between 32 and 60 d of pregnancy were decreased ($P = 0.03$) in the 9 d (7.8%) compared to the 8 d (15.2%) protocol.

Pereira *et al*. (2013a) tested the concept of using E2 to induce ovulation in FTAI protocols on dairy cows subjected to summer heat stress. They compared an Ovsynch-type protocol (the 5d Cosynch; Bisinotto *et al*. 2010b, Santos *et al*. 2010) and an E2/P4-based protocol, designed to have similar ages of the ovulatory follicle in synchronized cows. There was increased P/AI

with the E2/P4 protocol (25.6%) compared with the 5d Cosynch protocol (17.7%) and primary reason for the reduced P/AI in the 5d Cosynch protocol was the high pregnancy loss between days 32 and 60 in this group (21.7%) compared with cows in the E2/P4 protocol (6.7%). Finally, although authors did not measure circulating E2 in this trial, the physiological effects of elevated E2 are clearly manifested in the greater expression of estrus in the cows in the E2/P4 protocol (62.8%) compared with the 5d Cosynch protocol (43.4%).

Similar to previous studies, Pereira *et al*. (2013a) observed that follicle size had a positive effect on P/AI in both protocols using either the 32 d or 60 d pregnancy diagnosis. A particularly intriguing finding was that smaller follicles were associated with a greatly increased pregnancy loss from 32 to 60 d for cows in the 5d Cosynch protocol, but not for cows receiving the E2/P4 protocol. Thus, increased circulating E2 near AI may produce changes that reduce pregnancy loss from 32 to 60 d after AI during summer heat stress. Perry *et al*. (2005) reported that GnRH-induced ovulation of small follicles (< 11.3 mm) in beef cows was associated with decreased pregnancy rates and decreased circulating concentrations of E2 at AI and greater pregnancy loss, although pregnancy outcome following spontaneous estrus and ovulation was not affected by follicle diameter. These data are consistent with the concept that greater circulating E2 near AI may reduce pregnancy loss at the later embryonic stages (between 32 to 60 d after AI). However, it should be noted that response to treatments may be affected by season of the year.

Collectively for this and the previous section, the improved fertility with a longer time period from induction of luteolysis with PGF until induction of synchronized ovulation could be related to greater time for CL regression and thus lower P4 at FTAI or, alternatively, greater time for follicle growth in the absence of P4, increased ovulatory follicle size, and increased E2 before synchronized ovulation.

P4 priming during early diestrus

The pre-implantation window of gestation in cattle (i.e., three first weeks) is greatly susceptible to failure (Sartori *et al*. 2002b, Santos *et al*. 2004, Diskin *et al*. 2012). During this period, the conceptus relies on endometrial histotrophic nutrition to develop (Spencer & Gray 2006, Bazer *et al*. 2011). The endometrial transcriptome, secretome and, consequently, production and composition of histotroph are regulated by P4 concentrations in the pre-implantation period (Forde *et al*. 2009). Indeed, supplementation of P4 after ovulation stimulates conceptus elongation (Carter *et al*. 2008), and reduction in P4 concentrations causes the opposite effect (Forde *et al*. 2011). As mentioned previously, intense steroid catabolism, associated with high dry matter intake needed to support milk production of the dairy cow, acts to decrease concentrations of P4 at this critical window and thereby contributes to the low fertility (Demetrio *et al*. 2007). Thus, it is rational that strategies to supplement P4 post-ovulation may benefit P/AI.

A number of strategies to supplement P4 post-ovulation have been attempted, and effects on fertility are variable. Strategies include inducing the ovulation of the first wave follicle to cause the formation of an accessory CL to provide additional P4, treating cows with exogenous sources of P4 after AI, and stimulating the P4 secreting ability of the original CL. Regarding the formation of accessory CL, Santos *et al*. (2001) administered hCG to dairy cows 5 d after AI and reported increased P/AI as compared to controls (45.8% *vs*. 38.7%). More recently, Nascimento *et al*. (2013b), found a positive effect on P/AI in primiparous cows treated with hCG 5 d after AI (49.7% *vs*. 39.5%) but not multiparous (35.7% *vs*. 36.0%, respectively). Vasconcelos *et al*. (2011) evaluated the effects of treatments with hCG or GnRH at 7 d after induced ovulation on reproductive performance of lactating dairy cows submitted to FTAI or ET.

Cows were assigned randomly to receive, on d 7, either no additional treatments (Control; FTAI: n = 156; TET: n = 126), a 100 μg i.m. injection of GnRH (GnRH; FTAI: n = 155; TET: n = 124), or a 2,500 IU i.m. injection of hCG (hCG; FTAI: n = 151; TET: n = 122). There were effects of post-breeding treatments on the percentages of pregnant cows at TET on d 28 (Control: 38.1%; GnRH: 52.4%; hCG: 45.1%) and on d 60 (Control: 32.5%; GnRH: 41.1%; hCG: 38.5%), but no effects of post-breeding treatments were detected on the percentages of pregnant cows at FTAI on d 28 (Control: 30.1%; GnRH: 32.2%; hCG: 32.4%) nor on d 60 (Control: 25.6%; GnRH: 27.1%; hCG: 29.8%). It was concluded that treatment with GnRH or hCG at 7 d after induced ovulation increased conception rates in lactating dairy cows submitted to TET, but not in cows submitted to FTAI. Collectively, there is no consistently positive response to the additional P4 provided by the CL resulting from the ovulation of the first wave dominant follicle. Responses vary according to day after induction of ovulation in which P4 increases relative to non-treated controls and parity. Specifically, if the additional P4 becomes available at later moments (e.g., such as when hCG is injected at day 7 after induced ovulation), it will affect the uterus at a stage in which it may no longer be limiting to pregnancy success. In fact, Demetrio *et al*. (2007) reported that there was a positive association between P4 concentrations on d 7 after AI and P/AI for lactating cows but there was no association between P4 concentrations on that same day for cows receiving embryo transfer. More recently, researchers started to target supplementation of P4 at earlier days after AI.

Some studies have supplemented cows with exogenous P4 and observed an improvement in P/AI. For example, Holstein cows had 48% P/AI, when a CIDR was used between d 3.5 and 10 after AI as compared to 35% when cows were not supplemented (Larson *et al*. 2007). Likewise, Monteiro Jr *et al*. (2014) supplemented P4 between d4 and 17, with either a single CIDR or a single CIDR with an additional CIDR added at day 7 and both groups having device (s) withdrawn at the end of D17. Supplementation failed to alter P/AI at either day 34 or day 62. However, a single CIDR inserted at day 4 and removed at day 17 increased P/AI in cows that were FTAI but not in cows that were inseminated at detected estrus. The two supplemental CIDR sequence did not influence P/AI in cows inseminated at detected estrus or FTAI. However, with an E2/P4 based protocol, Monteiro Jr *et al*. (2013) did not detect improvement in P/AI after CIDR supplementation between d 3 and 17 after AI in Holstein and cross-bred (Holstein x Gir) cows. Causes for the lack of clear effects of supplementation of P4 through exogenous sources on P/AI are not known. One might expect that the extra P4 could affect original CL formation and function, but this was not supported by the data of Monteiro Jr *et al*. (2013) and Souza *et al*. (2013) for dairy cattle, nor from Pugliesi *et al*. (2014) for beef cattle. In the study of Pugliesi *et al*. (2014), P4 supplementation via intramuscular injection of 300 mg P4 in a slow release formulation given two days after ovulation, induced functional and structural regression of the CL 4 days earlier than vehicle-injected controls. It seems there is a paradoxical effect of supplementing P4, as described by O'Hara *et al*. (2014). On one hand P4 induces alterations in histotroph composition and quantity to stimulate conceptus growth. On the other hand, P4 supplementation advances luteolysis leading to embryo mortality. The outcome of this dilemma is the inconsistent results of experiments in terms of P/AI in response to P4 supplementation.

One final strategy to supplement P4 post AI is through the stimulation of the secretory ability of the CL originated from the synchronized ovulation. A recent paper from Maillo *et al*. (2013) reported that an injection of hCG 2 d after estrus increased the area of the original CL as well as its ability to produce P4 from days 6 to 11, in comparison to placebo injected controls. Although promising, this strategy has not been tested in fertility trials yet. Additional circulating P4 during this window may be beneficial to conceptus development and survival. Finally, strategies to effectively reduce P4 concentrations at AI and to stimulate E2 priming during proestrus-estrus, such as described in the previous sections, stimulate growth of the

pre-ovulatory follicle and growth and function of the subsequent CL (Vasconcelos *et al*. 2001). Furthermore, such strategies result in increased P/AI.

"Pro-fertility" protocols

According to what was discussed above, an ideal FTAI protocol should attend to all the premises to provide adequate hormonal milieu for oocyte quality, ovulation, gamete transport as well as embryo development. To serve as a reference, we suggest "pro-fertility" protocols that incorporate the concepts described in this paper, and compare them to other FTAI protocols (Fig. 1 and Table 1). One of them is the Double-Ovsynch protocol, described by Souza *et*

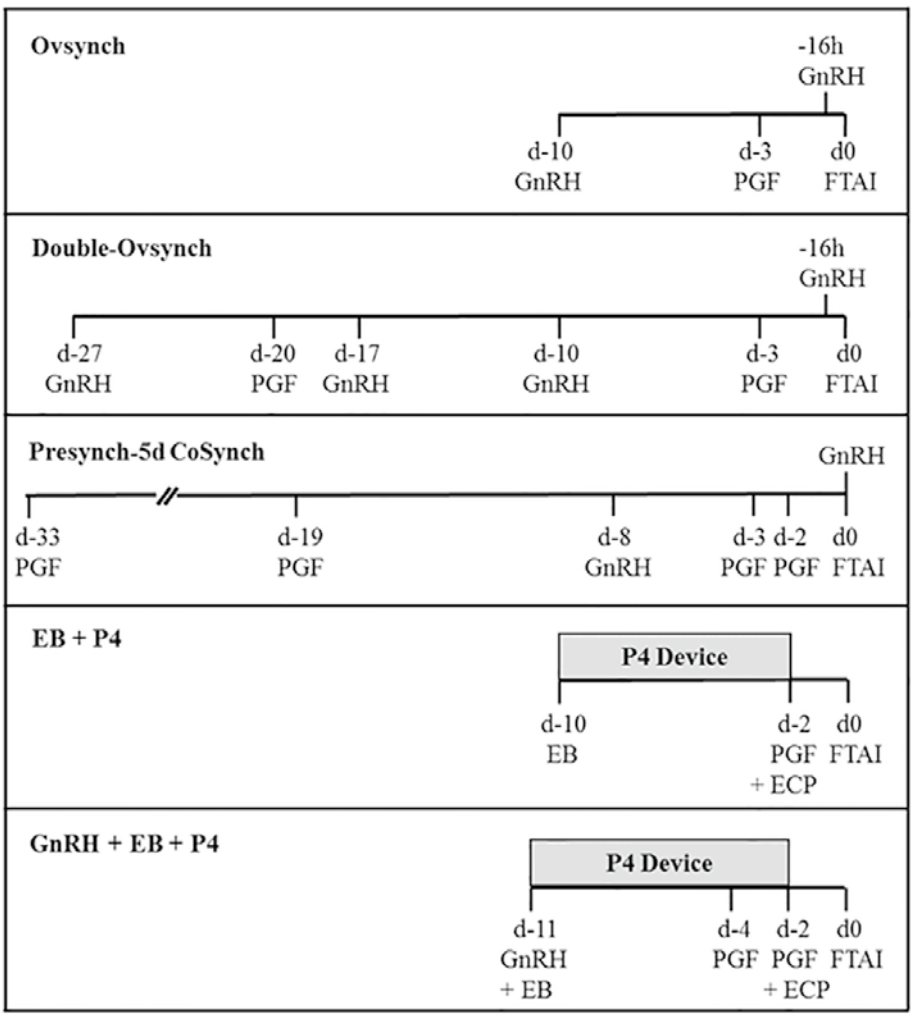

Fig. 1. Schematic diagrams of FTAI protocols for dairy cows (please see text for details). GnRH = Gonadotropin-releasing hormone; PGF = Prostaglandin F2α; EB = Estradiol benzoate; ECP = Estradiol cypionate; P4 device = Intravaginal device for controlled progesterone release; FTAI = Fixed-time artificial insemination.

Table 1. Analysis of Ovsynch (Pursley *et al.*, 1995), Double-Ovsynch (Souza *et al.* 2009), Presynch-5d CoSynch (Santos *et al.*, 2010), EB (estradiol benzoate) + P4 (Souza *et al.*, 2009) and GnRH + EB + P4 (Pereira *et al.* 2013c) protocols based on concepts applied to novel protocols for lactating dairy cows.

	Protocols				
	Ovsynch	Double-Ovsynch	Presynch-5d Cosynch	EB + P4	GnRH + EB + P4
Shorter follicle dominance period		X	X	X	X
Higher P4 during follicle development		X	X		X
Total reduction of P4 concentration prior to AI			X		X
Estradiol priming of sufficient duration and intensity				X	X
Early and prominent post-ovulatory rise in P4*					

*None of the above protocols provides an ideal post-ovulatory rise in P4 for adequate embryo development in the high-producing dairy cow.

al. (2008), whose advantages were very well discussed by Wiltbank *et al.* (2011). Another protocol that has been evaluated with similar results to the Double-Ovsynch is the Presynch-5d Cosynch (Santos *et al.* 2010; Ribeiro *et al.* 2012). Recently, we developed the GnRH + EB + P4 protocol, described by Pereira *et al.* (2013c). This protocol presents some extra features that make it very attractive, such as the association of GnRH with EB at the beginning, resulting in better wave emergence synchronization, increased P4 concentrations and proportion of cows with CL at PGF. The addition of an intravaginal P4 device, not only increases synchronization, but also contributes to more circulating P4 during follicle development. Finally, the use of two PGF injections would result in lower P4 at AI, increasing fertility in lactating dairy cows. In fact, Pereira *et al.* (2013c) performed a study with 1642 lactating Holstein cows and observed more circulating P4 during pre-ovulatory follicle development, higher percentage of cows that had luteolysis, and higher synchronization by adding GnRH at the onset of the protocol and an extra PGF treatment at the end of the protocol as compared to the Control protocol (EB + P4). The GnRH protocol (described as GnRH + EB + P4 in Fig. 1 and Table 1) improved P/AI at 32 (40 *vs.* 32%) and 60 d (33 *vs.* 26%), compared to Control.

Table 1 provides a subjective analysis comparing FTAI protocols regarding their efficiency on applying the concepts described in this review article. In this sense, the protocols that best fit these concepts are the Presynch-5d Cosynch and the GnRH + EB + P4. Although some of these protocols are having promising results, none of them adequately meets the need to provide enough circulating P4 post AI to compensate for the high clearance in high-producing cows (Table 1).

Conclusions

Current concepts, as well as future concept development, on pharmacological manipulation of the estrous cycle to synchronize ovulations must take in consideration the endocrinology of particular animal categories. For the high yield dairy cow, intense liver sex steroid catabolism results in an endocrine environment that is sub-optimal to fertility. Protocols that provide steroid supplementation at critical portions within the synchronization regime were beneficial and increased P/AI. To conclude, evidence is accumulating to support the concept that programming and coordination of the continuum of endocrine events from preovulatory follicle development to post-AI P4 effects is necessary to increase P/FTAI and reduce embryo losses.

Acknowledgements

Brazilian granting agencies FAPESP, CAPES and CNPq for research grant support, Zoetis and Ourofino for pharmaceutical products, students and staff at the University of São Paulo and São Paulo State University for technical support, owners and staff of collaborating dairies for the use of animals and facilities and Drs. William W. Thatcher and Milo C. Wiltbank for their long term support to our programs.

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