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Aspects and mechanisms of low fertility in anovulatory dairy cows

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Abstract

Postpartum anovulation is a natural process that is observed in most mammals, including women. In lactating dairy cows, the interval from calving to first ovulation typically averages 4 to 5 weeks, but a substantial proportion of cows have not resumed estrous cyclicity by 60 days postpartum. Extended delay in resumption of first postpartum ovulation is known to exert long-lasting detrimental effects on fertility in dairy cows including the lack of spontaneous estrus and subsequent timely insemination postpartum, but when anovular cows have the estrous cycle synchronized for artificial insemination (AI), still pregnancy per AI is reduced and the risk of pregnancy loss increased. Many risk factors exist for extended postpartum anovulatory periods such as negative nutrient balance and diseases, and these risk factors are also known to depress fertility by themselves. A key feature in anovular cows when inseminated is that they develop the ovulatory follicle under subluteal or low concentrations of progesterone. Progesterone from the corpus luteum is pivotal for follicle development, oocyte competence, embryo growth, and endometrial function; however, many of these effects exerted by progesterone are mediated either by secretion of gonadotropins influencing follicular function and oocyte competence or by endometrial histotroph secretion influencing embryo/conceptus growth and developmental biology. Sub-optimal concentrations of progesterone during follicle growth in anovular cows affect morula and early blastocyst quality, alter conceptus gene expression and affect endometrial function increasing the synthesis of prostaglandin F2α. When anovular cows receive sufficient supplemental progesterone during the antral stages of development of the ovulatory follicle, then pregnancy per AI is reestablished and resembled that of estrous cyclic cows that developed the follicle during diestrus. Preliminary data suggest that a minimum concentration of progesterone during follicle growth is needed to optimize fertility in anovular cows, at least 2.0 ng/ml.

Keyword: anovular, dairy cow, fertility, progesterone.

Introduction

Lactating dairy cattle are notorious for having less than adequate fertility as measured by suboptimal pregnancy per artificial insemination (P/AI) or by the increased pregnancy loss during the first months of gestation (Santos *et al.*, 2004). Recent data from the United States' dairy herd indicate that P/AI has remained somewhat stable in the last 10 years, at around 33%, whereas pregnancy rate, which is the rate at which cows become pregnant measured at 21-days intervals, increased and calving interval declined substantially (Bisinotto *et al.*, 2014). It is thought that the improvements observed in reproductive performance in dairy herds in the United States are, in part, the result of better implemented reproductive programs. However, expressive gains in genetics for daughter fertility have also been achieved and likely P/AI will increase in the near future.

One of the impediments for proper fertility in dairy cows is delayed resumption of estrous cyclicity during the first 2 months postpartum (Santos *et al.*, 2009). The lack of estrous cyclicity, a phenomenon also denoted as anovulation (Wiltbank *et al.*, 2002), usually affects 25% of dairy cows by approximately 65 days in milk (Walsh *et al.*, 2007; Santos *et al.*, 2009). Nevertheless, some herds might have up to 40% prevalence of anovular cows by the end of the voluntary waiting period when poor peripartum health and/or poor nutritional management are in place (Walsh *et al.*, 2007; Santos *et al.*, 2009). Anovular cows subjected to synchronization programs for AI on estrus or timed AI usually have reduced P/AI (Santos *et al.*, 2009; Bisinotto *et al.*, 2010) and increased pregnancy loss compared with estrous cyclic herdmates (Santos *et al.*, 2004; Bisinotto *et al.*, 2010). Similar to anovular cows, those cows that develop the ovulatory follicle during the last week of growth under low concentrations of progesterone have P/AI as low as that of anovular cows (Bisinotto *et al.*, 2010). Such response suggests that one of the culprits for low fertility in anovular cows is likely mediated by the lack of adequate concentrations of progesterone controlling the final stages of follicle growth and/or affecting the endocrine milieu during proestrus and the uterine function during the post-ovulatory period (Cerri *et al.*, 2011a, b; Shaham-Albalancy *et al.*, 1997, 2001).

A strategy to counteract the negative effects of low endogenous concentrations of progesterone on fertility has been the supplementation with exogenous sources at strategic times in an attempt to improve fertility in dairy cows. Nevertheless, responses to progesterone supplementation are variable (Bisinotto and Santos, 2012; Wiltbank *et al.*, 2012b) and oftentimes reproductive physiologists and veterinary practitioners have taken the simplistic tactic of "one approach fits

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all", which does not seem to have been very effective (Bisinotto and Santos, 2012; Wiltbank *et al.* 2012b). The questions that need to be asked and eventually answered are what cows require progesterone supplementation, when should progesterone be supplemented, and how much progesterone needs to be delivered to optimize establishment and maintenance of pregnancy in lactating dairy cows. Another and more long-term approach is to genetically select cows based on genomic markers linked to improved resumption of ovulation postpartum or shorter anovulatory intervals, so the prevalence of anovular cows by the end of the voluntary waiting period is reduced.

The role of progesterone in controlling reproduction

The steroid hormone progesterone is synthesized by follicles and the corpus luteum (**CL**; Mason and Savard, 1964), placenta (Conley and Ford, 1987), suprarenal glands (Wagner *et al.*, 1972), and the central and peripheral nervous systems (Schumacher *et al.*, 2004), with that of luteal origin having the greatest importance for regulation of reproductive events in cattle. Therefore, progesterone is considered one of the key ovarian steroids regulating the estrous cycle in females. Furthermore, inadequate concentrations during distinct phases of the cycle and during pregnancy are known to impair fertility in dairy cattle.

Luteal progesterone was determined to be a key regulator of reproduction in females after the observation that the presence of CL was required for the establishment and maintenance of pregnancy (Fraenkel and Cohn, 1901; Magnus, 1901; Corner and Allen, 1929). Subsequent studies described the complex mechanisms through which progesterone stimulates the secretion of nutrients and growth factors by the endometrial glands, collectively referred to as histotroph (Bazer *et al.*, 2008). Recent studies have documented and emphasized the role of progesterone in priming the uterus for proper bovine conceptus elongation after hatching from the zona pellucida (Brandão *et al.*, 2004; Alexopoulos *et al.*, 2005), underlying the importance of progesterone stimulation of uterine gland secretion for successful establishment and maintenance of pregnancy in cattle.

Nevertheless, the importance of progesterone regulating reproduction in dairy cattle goes beyond its role as coordinating uterine functions during peri-fertilization and early embryonic periods. Progesterone plays important roles in coordinating antral follicle grew and oocyte quality through its effects on luteinizing hormone (LH) pulsatility. Lactating cows classified as having low progesterone concentrations in the week preceding spontaneous estrus had less P/AI than herdmates with high concentrations of progesterone during the same period (Folman *et al.*, 1973). Similarly, cows in which the ovulatory follicle developed in the absence of a mature CL had reduced fertility compared with those in which follicles grow during diestrus (Bisinotto *et al.*, 2010). These differences in fertility in cows with low compared with moderate to high concentrations of progesterone during the development of the ovulatory follicle are, in part, attributed to the permissive role of progesterone regulating follicle growth and oocyte maturation.

Progesterone exerts a regulatory effect on the hypothalamus-pituitary-gonadal axis (Clarke and Pompolo, 2005) and differences in progesterone concentrations in plasma affect follicular fluid composition (Cerri *et al.*, 2011a), cumulus expansion and oocyte competence (Fair and Lonergan, 2012), embryo quality (Rivera *et al.*, 2011), and uterine function during the subsequent estrous cycle (Shaham-Albalancy *et al.*, 1997, 2001; Cerri *et al.*, 2011a). It has been estimated that the CL of dairy cows produce large quantities of progesterone (Wiltbank *et al.*, 2012a), but extensive catabolism by splanchnic tissues in high-producing cows results in concentrations in blood that are often considered inadequate for optimum reproduction (Wiltbank *et al.*, 2006).

Little is known about the optimum concentrations of progesterone pre- and post-insemination that maximizes fertility in dairy cows. Numerous examples exist of failures in response to supplemental progesterone to improve fertility in cattle (Monteiro Jr *et al.*, 2014; Wiltbank *et al.*, 2012b; 2014). In some cases, failures occurred because the delivery systems used were either inappropriate or less than ideal from the biological point of view. In other cases, even a depression in fertility was observed with supplemental progesterone after breeding in cows receiving embryo transfer (Monteiro Jr *et al.*, 2015).

Low progesterone affects follicle and embryo quality and influences endometrial function

Progesterone, although critical for establishment and maintenance of pregnancy, is known to exert little if any direct effect on oocytes and early embryo development (Lonergan *et al.*, 2016). The impacts of progesterone mediating embryo quality and conceptus elongation are indirect and likely caused by a combination of effects on LH pulse frequency during follicle development and on endometrial function during the post-ovulatory period (Lonergan *et al.*, 2016; Santos *et al.*, 2016).

Bovine follicular cells such as those of the cumulus-oocyte complexes are responsive to progesterone and differences exist in steroidogenic activity between dominant follicles of the first compared with those of the second follicular wave (Badinga *et al.*, 1992). First wave dominant follicles, which develop under low concentrations of systemic progesterone have high aromatase activity and concentrations of progesterone within the follicular fluid is rather high compared with those in blood plasma (Badinga *et al.*, 1992; Cerri *et al.*, 2001a). In fact, manipulating concentrations of progesterone in plasma did not alter the concentrations of progesterone within the follicular fluid in dairy cows (Cerri *et al.*, 2001a). Therefore, the effects of low concentrations of progesterone in plasma on follicular function very likely are mediated by changes in LH pulsatility, and not by a direct effect of progesterone on follicular cells.

It is well described that progesterone receptors are present in the arcuate nucleus of the hypothalamus in kiss-releasing neurons and activation of those receptors attenuates kisspeptin release which depresses gonadotropin-releasing hormone (GnRH**)** pulsatility (Clarke and Pompolo, 2005). Progesterone influences the release of LH, which can be noted by the reduction in LH pulse frequency during the luteal phase compared with the follicular phase of the estrous cycle in the sheep (Clarke, 1995) and during metestrus compared with diestrus in dairy cows (Endo *et al.*, 2012). In fact, treatment with exogenous progesterone decreased the frequency of LH pulses in ovariectomized ewes (Goodman and Karsch, 1980; Goodman *et al.*, 1982) and postpartum dairy cows (Nation *et al.*, 2000). Nevertheless, data suggest that progesterone does not affect the amount of GnRH receptors in the pituitary gland (Schoenemann *et al.*, 1985) and the negative feedback of progesterone on LH secretion is observed primarily at the level of the hypothalamus (Clarke and Pompolo, 2005), not the pituitary (Lane *et al.*, 2009). Because GnRH neurons do not express progesterone receptors (Herbison *et al.*, 1996; Skinner *et al.*, 2001), the action of progesterone on GnRH secretion is mediated by changes in upstream release of kisspeptin. When cows are exposed to low concentrations of progesterone, LH concentrations increase, follicle growth is accelerated, and follicular composition is altered (Cerri *et al.*, 2001a, b). Although these changes are unlikely to resemble those depicted in models for prolonged follicular dominance, the alterations in LH pulsatility elicited by low concentrations of progesterone likely influence oocyte quality by effects on gap junctions, protein phosphorylation and reactivation of meiosis from the diplotene stage of prophase I (Santos *et al.*, 2016). The advancement in oocyte maturation induced by increased LH pulsatility when concentrations of progesterone are low compromises early embryonic development and conceptus elongation (Wiltbank *et al.*, 2011).

Wiltbank *et al.* (2011) assigned cows to start the Ovsynch timed AI protocol on day 7 of the estrous cycle, but manipulated concentrations of progesterone such that in one treatment, cows developed the pre-ovulatory follicle under low concentrations of progesterone and in another treatment under high concentrations of progesterone. Treatments were achieved by administration of prostaglandin (PG) F2α concurrent with the initiation of the Ovsynch protocol to regress the pre-existing CL on day 7 of the estrous cycle. By doing that, the authors created two treatments, one in which the pre-ovulatory follicle developed only with the newly formed CL (low progesterone) and another treatment in which the pre-ovulatory follicle developed with both, the pre-existing CL and the newly formed CL (high progesterone). Oocytes-embryos were collected by flushing the uteri of cows on day 7 after AI. A total of 81 oocytes-embryos were collected from 168 cows flushed. Fertilization was similar between treatments, but embryo quality was markedly depressed in cows in the low progesterone treatment (Table 1). Similar depression in embryo quality has been observed when lactating dairy cows subjected to superstimulatory treatments initiated the follicle stimulating hormone (FSH) treatment under low concentration of progesterone in early metestrus (Rivera *et al.*, 2011). Interestingly, in the embryo donor experiment, supplementing progesterone during early metestrus with two intravaginal inserts reestablished concentrations of progesterone similar to those observed in cows in which the FSH started in early diestrus and rescued embryo quality (Rivera *et al.*, 2011).

Table 1. Effect of concentration of progesterone during follicle development on embryo quality in lactating dairy cows[1].

	Treatment[2]		
Item	Low progesterone	High progesterone	P-value
Fertilization, % (n/n)	78.8 (26/33)	77.1 (37/48)	0.43
Mean grade quality, 1 to 4	2.4	1.5	0.01
Grade 1, % (n/n)	34.6 (9/26)	67.6 (25/37)	0.01
Grade 2, % (n/n)	26.9 (7/26)	18.9 (7/37)	0.29
Grade 1 and 2, % (n/n)	61.5 (16/26)	86.5 (32/37)	0.02
Grade 4, % (n/n)	34.6 (9/26)	8.1 (3/37)	0.01

[1]Data from Wiltbank *et al.* (2011). [2]Cows were synchronized to start the Ovsynch timed AI protocol (GnRH, 7 days PGF2α, 56 h GnRH, 16 h AI) starting on day 7 of the estrous cycle. Low progesterone cows received an injection of PGF2α 1 day after initiating the Ovsynch timed AI protocol to regress the pre-existing CL, whereas high progesterone cows received no further hormonal treatment.

It is interesting that the negative effects of developing the ovulatory follicle under low concentrations of progesterone are not limited to reduced embryo quality. Anovular cows and those estrous cyclic initiating the timed AI protocol under low concentrations of progesterone have increased risk of pregnancy loss (Bisinotto *et al.*, 2010). It is well established that progesterone in the preceding estrous cycle influences endometrial function in the subsequent cycle (Shaham-Albalancy *et al.*, 1997, 2001). Cows that

develop the ovulatory follicle under low concentrations of progesterone have an earlier upregulation of endometrial expression of estrogen receptor-α protein in the postovulatory period and produce more PGF2α following an oxytocin challenge than cows that develop the ovulatory follicle under high concentrations of progesterone (Cerri *et al.*, 2011a). In fact, these cows are more likely to suffer from short luteal phases (Cerri *et al.*, 2011a), which is devastating to maintenance of pregnancy in cattle.

Although early embryo quality is compromised in anovular cows, many still present an elongated conceptus by day 15 of gestation. Nevertheless, conceptuses from anovular cows have marked changes in transcriptome indicating molecular changes that can compromise subsequent survival and explain the increased pregnancy loss later in gestation. Ribeiro *et al.* (2016c) identified 500 transcripts differently expressed between anovular and estrous cyclic lactating Holstein cows. Of those, 262 were upregulated and 238 were downregulated in anovular cows. Many of those genes were related to the process of transitioning from a tubular to a filamentous conceptus in dairy cattle (Ribeiro *et al.*, 2016a). Functional analysis of transcriptome data evaluated by Ribeiro *et al.* (2016c) observed that apoptosis, 14-3-3 signaling pathway, and autophagy were predicted to be increased in conceptus from anovular compared with those from estrous cyclic cows. These data suggest that conceptuses that survive to day 15 of gestation have altered molecular signatures that might favor mechanisms of cell death.

It is important to emphasize that one cannot ignore that anovulation is linked to numerous events that take place in early lactation such as excessive negative nutrient balance and diseases. Catabolism induced by negative energy balance and associated peripartum diseases are known to affect reproduction in dairy cows and to predispose them to have extended periods of anovulation (Ribeiro *et al.*, 2016a, b; Santos *et al.*, 2009). Diseases of inflammatory nature such as those that affect the reproductive tract and the mammary gland have catastrophic links with fertility in dairy cows. Cows that develop diseases are more likely to remain anovular and both influence fertility in dairy cattle. In fact, disease in early lactation leave long-lasting imprinting marks in the molecular features of conceptus (Ribeiro *et al.*, 2016b), which likely respond for some of the reduced P/AI observed in anovular cows.

Identification of anovular cows and those that respond to progesterone supplementation

As in most biological systems, there is likely an ideal concentration of each hormone that would likely maximize pregnancy in dairy cattle. For progesterone, this concentration or range of concentration remains unknown and likely varies with the stage of the reproductive cycle. Nevertheless, it is clear that anovular cows and those that develop the ovulatory follicle with low concentrations of progesterone have marked reductions in P/AI (Bisinotto *et al.*, 2010; Wiltbank *et al.*, 2012b, 2014).

One of the challenges with anovular cows is that absence of a CL following an ultrasonographic examination of the ovaries or a single measurement of progesterone concentration in blood plasma or serum are likely to inflate the prevalence of the problem. Cows in proestrus, estrus and metestrus usually will have either a small CL or no visible CL at all, and concentrations of progesterone will be low, typically below 1.0 ng/ml. This is why most studies characterizing the prevalence of anovular cows use two sequential ultrasound examinations of the ovaries 7 to 14 days apart or two sequential measurements of progesterone concentrations in blood in the same interval (Santos *et al.*, 2009). In most cases, this approach to diagnosing anovular cows is not practical under field conditions because of the labor involved, particularly in large farms.

One approach that has been used effectively is a single ultrasonographic examination of the ovaries at a strategic time, when low progesterone is known to depress fertility (Silva *et al.*, 2007). In fact, a single ultrasound examination at the beginning of the synchronization protocol is capable of identifying not only most of the anovular cows, but also cohort of cows known to have low P/AI (Bisinotto *et al.*, 2010, 2013). A similar method has been the norm of identification of low fertility cows in grazing farms in New Zealand and elsewhere (Rhodes *et al.*, 2003). In fact, estrous cyclic cows that received the timed AI program initiated during proestrus, estrus, or metestrus supposedly ovulated the first wave follicle when inseminated and had P/AI that was similar to that observed in anovular cows (Bisinotto *et al.*, 2010). The observation that the presence of CL and not estrous cyclic status has the greatest impact on P/AI is critical considering that approximately 25% of the cows receiving the first AI postpartum (Santos *et al.*, 2009) and 22 to 46% of those receiving resynchronized AI (Fricke *et al.*, 2003; Silva *et al.*, 2009) lack a CL when the synchronization protocol is initiated. Therefore, the use of a single ultrasonographic examination of the ovaries when the synchronization protocol is initiated is suggested as the method of choice to select cows for therapy with supplemental progesterone.

Supplementation with progesterone

The concentration of progesterone during diestrus in dairy cows is determined by the rates of luteal steroidogenesis and clearance from the circulation (Wiltbank *et al.*, 2012a). Therefore, more extensive catabolism usually results in reduced concentrations of progesterone in blood. The same concept would apply when cows receive exogenous progesterone. Treatment of growing heifers with progesterone inserts usually result in changes in plasma concentrations much greater than those typically observed when the same insert is used in high-producing dairy cows (Macmillan *et al.*, 1991; Cerri *et al.*, 2009), which is typically attributed to the more extensive catabolism of steroids by the splanchnic tissues in cows of increased nutrient intake and hepatic metabolism. Thus, when the same delivery

method is used in cows with distinct metabolic rates, it is not a surprise that concentrations of progesterone in plasma will greatly differ.

The fact that concentrations of progesterone vary with the type of cattle receiving a given intravaginal insert has major implications to designing supplementation systems because practitioners usually have available methods to deliver progesterone that are designed for a given type of cattle, but eventually use in all categories of cows and heifers in the farm. An example is the controlled internal drug-release system designed by Welch *et al.* (1984) as a vaginal device for use in sheep that became an alternative to the nylon sponges that were formerly used to deliver progesterone (Welch, 1984). This system later adapted to be used in cattle (Macmillan *et al.*, 1990), but it was designed to be used in heifers to facilitate adoption of AI (Macmillan *et al.*, 1991). Later, the same technology was adopted for use in lactating cows of moderate frame size and low genetic potential for production (Macmillan and Peterson, 1993). Use of the same delivery system in high-producing lactating dairy cows typically result in concentrations of progesterone in plasma that are only 20 to 30% of those observed in growing heifers or small-frame low producing cows (Cerri *et al.*, 2009).

Plasma concentrations during use of intravaginal inserts

For the subsequent discussion, the example of the controlled internal drug-release (**CIDR**) insert will be used because of familiarity with the literature, although the information herein would likely apply to any intravaginal insert that releases 80 to 90 mg of progesterone daily.

The original CIDR insert developed for cattle that still is marketed in many countries contains 1.9 g of progesterone. When used in ovariectomized heifers, it resulted in progesterone concentrations of 5.6 ng/ml for a period of 12 days, and the concentrations ranged from 8.7 ng/ml in the first hours after placement of the device in the vagina to 2.5 ng/ml at device removal on day 12 (Macmillan *et al.*, 1991). These are typical concentrations of heifers in early to mid diestrus and more than enough to block estrus, LH surge and ovulation. In fact, when applied to intact heifers in diestrus, the concentrations of progesterone increased 5 to 6 ng/ml within the first 24 h of treatment (Macmillan *et al.*, 1991). On the other hand, the same insert used in dry grazing New Zealand cows that had the CL regressed by PGF2α resulted in a mean concentration of progesterone of 2.8 ng/ml for a period of 10 days (McMillan *et al.*, 1991). Concentration was highest on the day of device insertion (4.1 ng/ml) and slowly declined to 1.9 ng/ml after 10 days of use. It is known from *in vitro* and *in vivo* drug release assessments that the CIDR insert releases on average approximately 89 mg of progesterone daily (Rathbone *et al.*, 2001, 2002) and the release is dependent primarily on the surface area of the device in contact with the mucosa of the vagina. However, it is unlikely that the delivery system is stable and not variable throughout the treatment period. In fact, although concentrations of progesterone in high-producing lactating dairy cows average 1 ng/ml when 89 mg of progesterone is delivered daily by the intravaginal insert, considerable cow to cow variability exist (Cerri *et al.*, 2009), either because of the pharmacokinetics vary among cows or because the delivery is not constant in all inserts. Likely both occur and explain the variability in blood progesterone responses when lactating dairy cows are treated with intravaginal inserts.

The re-engineered CIDR that is marketed in the United States and other countries contains 1.38 g of progesterone, but it is supposed to release the same amount daily as the original device containing 1.9 g (Rathbone *et al.*, 2001; 2002). Cerri *et al.* (2009) evaluated the concentrations of progesterone when estrous cyclic high-producing Holstein lactating cows received a new (1.38 g of progesterone) or a 7-day used CIDR insert after regressing the CL. The authors showed that a device releasing 89 mg of progesterone daily (Rathbone *et al.*, 2001, 2002) increased concentrations in plasma by approximately 0.8 to 1.0 ng/ml (Cerri *et al.*, 2009). Concentrations increased in the first 15 min and reached a plateau by 90 min after insertion of the device. Similar to the findings of Macmillan *et al.* (1991), concentrations declined over the course of use of the device, but in the lactating Holstein cow, they dropped to 0.5 to 0.7 ng/ml after 7 days of use. These concentrations of progesterone in dairy cows are sufficient to block estrus and the LH surge and ovulation, but not ideal to improve fertility when the goal is to supplement progesterone (Bisinotto and Santos, 2012; Bisinotto *et al.*, 2015a). This probably explains why previous studies in which a single CIDR was incorporated into timed AI programs demonstrated inconsistent responses in anovular cows (Bisinotto and Santos, 2012). The incremental progesterone from a single insert is likely insufficient to optimize follicle or oocyte maturation during the final stages of development before AI, or even to prime the endometrium for proper post-insemination function during conceptus development and maintenance of pregnancy.

Because release of progesterone from intravaginal devices is dependent primarily on the surface area in contact with the vaginal mucosa (Rathbone *et al.*, 2001, 2002), it is not a surprise that addition of multiple devices increases progesterone in plasma in a parallel manner to the number of inserts used (Macmillan *et al.*, 1991; Lima *et al.*, 2009). This is important because in many countries approval of new devices is costly, but opportunities exist for extra-label use of current devices to target individuals that might require daily doses of progesterone of at least 180 to 200 mg such as high-producing anovular dairy cows.

Incorporation of supplemental progesterone in synchronization programs

Programs for synchronization of ovulation and timed AI have been implemented worldwide as a management tool for the systematic control of reproduction in dairy herds. Timed AI programs allow for submission of all eligible cows to insemination with

satisfactory P/AI, which typically improves pregnancy rates especially when detection of estrus is inefficient (Tenhagen *et al.*, 2004) or when replacing breeding by natural service (Lima *et al.*, 2012). Fertility of estrous cyclic and anovular lactating dairy cows induced to ovulate the first-wave dominant follicle is usually compromised (Bisinotto *et al.*, 2010). Our work has clearly demonstrated that first-wave follicles that develop concurrently with the CL and, therefore, under low concentrations of progesterone result in alterations in the follicular fluid composition (Cerri *et al.*, 2011a), alterations in endometrial function (Cerri *et al.*, 2011a), reduced embryo quality (Rivera *et al.*, 2011; Wiltbank *et al.*, 2011), and compromised P/AI (Bisinotto *et al.*, 2010). More importantly, our work has shown that progesteron is likely to mediated these changes in reproductive responses of cows ovulating the dominant follicle of the first follicular wave (Bisinotto *et al.*, 2013). In fact, reduction in progesterone concentraiton during development of the second wave follicle markedly reduced embryo quality in single ovulating dairy cows (Table 1; Wiltbank et al., 2011).

Timed AI programs provide a unique platform for the manipulation of the ovulatory follicle in order to improve P/AI in dairy cows. One of these opportunities is the supplementation of progesterone to cows that are identified as being anovular or those in which the stage of the cycle results in low concentration of progesterone during the final phase of follicle development. Because lack of a CL when the timed AI protocol is initiated is predictive of low fertility, it then becomes logical that identification of cows without CL would be one of the targeted populations to receive supplemental progesterone (Bisinotto *et al.*, 2013, 2015a).

It is important to emphasize that synchronization programs based on the use of estrogens such as those with estradiol benzoate do require a source of exogenous progesterone (Baruselli *et al.*, 2004), otherwise cows without a CL when estrogens are administered or those that regress the CL after the treatment with estrogens will not have a properly synchronized estrus or ovulation.

Supplemental progesterone during the timed AI protocol according to presence of CL

Numerous studies have evaluated the impact of supplementing exogenous progesterone during timed AI protocols on fertility of dairy cows. Bisinotto *et al.* (2013, 2015a) evaluated the effect of supplementing progesterone in GnRH-PGF2α based synchronization protocols. The authors showed that increasing progesterone in blood above 2.0 ng/ml with use of two intravaginal inserts restored fertility in cows without CL similar to that of cows in diestrus. The data of Bisinotto *et al.* (2015a) suggested that a minimum of approximately 2.0 ng/ml was needed during the development of the ovulatory follicle to optimize fertility in high-producing dairy cows (Fig. 1). Such response likely explains the lack of benefit of a single intravaginal insert that results in 0.8 to 1.0 ng/ml in cows without a CL (Bisinotto and Santos, 2012). In fact, when progesterone was supplemented to cows without a CL with the use of 2 intravaginal inserts (Bisinotto *et al.*, 2013, 2015a), to supply approximately 180 mg of progesterone released per day (Rathbone *et al.*, 2001, 2002), then the P/AI was similar to that of cows in diestrus when the timed AI protocol was initiated (Table 2). The increments observed were of approximately 10 percentage units in P/AI (Bisinotto *et al.*, 2013, 2015a; Lima *et al.*, 2009).

Most experiments evaluating the use of supplemental progesterone were not necessarily designed considering that the intravaginal device might not supply the amounts needed to improve fertility in dairy cow. Because of that, it is then not surprising that the responses to supplemental progesterone during synchronization programs have been equivocal (Bisinotto and Santos, 2012). The inconsistency in results led us to conduct a systematic review of the literature with the objective to evaluate whether progesterone supplementation using a single intravaginal insert during timed AI programs benefit fertility in lactating dairy cows (Bisinotto *et al.*, 2015b). A total of 25 randomized controlled experiments including 16,683 dairy cows, half supplemented and half untreated controls were included in the meta-analysis. A portion of the studies, 21 experiments including 13,762 cows (82.5% of the all cows) had information whether they were in diestrus or did not have a CL when the timed AI protocol was initiated. Additional information collected included detection or no detection of the estrus during the timed AI protocol and if cows had or did not have the estrous cycle pre-synchronized before enrollment in the timed AI program. The meta-analysis revealed that progesterone supplementation increased P/AI on day 32 and 60 after insemination by 8 (relative risk [RR] = 1.08; 95% confidence interval [CI] = 1.03 to 1.17) and 10% (RR = 1.10; 95% CI = 1.03 to 1.17), respectively. Interestingly, the benefit of treating cows with progesterone during the timed AI protocols was greater in cows without CL (P/AI on day 60: RR = 1.18; 95% CI = 1.07 to 1.30) than those in diestrus (RR = 1.06; 95% CI = 0.99 to 1.12). Also, progesterone supplementation benefited P/AI only when all cows were subjected to timed AI. When detection of estrus was performed throughout the synchronization protocol, and cows could be inseminated if observed in estrus, then progesterone did not increase P/AI. Collectively, these results clearly demonstrate that cows without CL benefit from progesterone supplementation, but delivering 80 to 90 mg of progesterone/day to high-producing Holstein cows is not ideal. For instance, the 16% increase in RR of P/AI on day 60 after insemination when anovular cows received supplemental progesterone with a single insert to deliver 90 mg/day translated into an increment of 6.0 percentage units (from 27.3 to 33.3%) in P/AI (Bisinotto et al., 2015b), still less than the value typically observed when anovular cows received two inserts to deliver twice the progesterone (Table 2). The results of the meta-analysis also demonstrate that one of the benefits of progesterone supplementation is to better synchronize estrus/ovulation in these programs because detection of estrus and insemination during the protocol abolished the positive effects of supplementation on fertility.

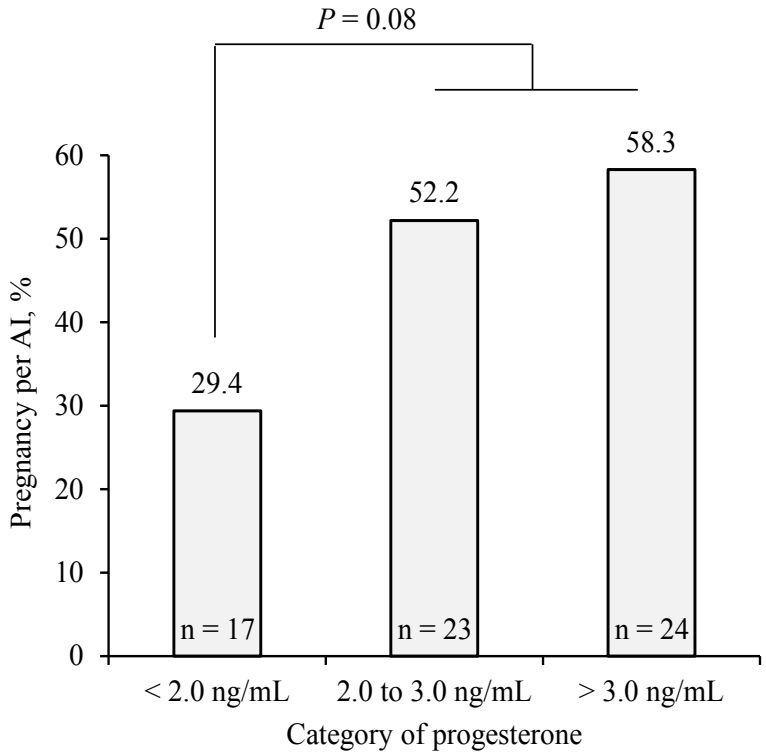

Figure 1. Pregnancy per AI on day 32 after insemination based on the concentrations of progesterone during follicle growth in cows receiving 2 intravaginal inserts containing progesterone during follicle growth in the Ovsynch protocol. Cows were categorized as having progesterone < 2.0 ng/mL (1.66 ± 0.32 ng/ml), from 2.0 to 3.0 ng/ml (2.53 ± 0.26 ng/ml), or > 3.0 ng/ml (3.97 ± 0.89 ng/ml) between the day of the first GnRH and that of the PGF2α. Data from Bisinotto *et al.* (2015a).

Table 2. Effect of presence of corpus luteum (CL) and progesterone supplementation for cows without CL at the initiation of the timed AI protocol on fertility responses.

	Treatment[1]		
	No CL control	No CL and progesterone	Diestrus
		% (n.)	
Estrus at AI			
Bisinotto *et al.* (2013)	34.2 (234)	36.2 (218)	35.0 (946)
Bisinotto *et al.* (2015a)	35.8 (652)	39.6 (635)	30.6 (640)
Mean estrus at AI	35.4	38.7	33.2
Pregnant day 60			
Bisinotto *et al.* (2013)	28.6 (234)	43.7 (215)	47.3 (941)
Bisinotto *et al.* (2015a)	28.9 (642)	37.2 (630)	33.9 (633)
Lima *et al.* (2009)	23.0 (87)	32.9 (85)	35,9 (334)
Mean pregnancy day 60	28.3	38.4	40.9
Pregnancy loss			
Bisinotto *et al.* (2013)	6.9 (72)	5.1 (99)	4.7 (467)
Bisinotto *et al.* (2015a)	8.5 (208)	11.4 (260)	8.8 (231)
Lima *et al.* (2009)	16.7 (24)	9.7 (31)	8.4 (131)
Mean pregnancy loss	8.9	9.7	6.4

[1]All cows were subjected to the 5-days timed AI protocol (Bisinotto *et al.*, 2013), Ovsynch-56 protocol (Bisinotto *et al.*, 2015a) or Heatsynch protocol (Lima *et al.*, 2009). No CL control = cows without a CL on the day of the first GnRH that received no supplemental progesterone; No CL progesterone = cows without a CL on the day of the first GnRH that received two intravaginal inserts containing each 1.38 g of progesterone; Diestrus = cows with CL on the day of the first GnRH of the timed AI protocol.

Conclusion

Delayed resumption of ovulation beyond 60 days postpartum affects a large proportion of dairy cows. Development of the ovulatory follicle under low concentrations of progesterone is the hallmark in anovular cows when subjected to synchronized inseminations. Suboptimal concentration of progesterone during follicle growth is one of the impediments for adequate fertility and markedly decreases P/AI in cows subjected to synchronization of estrus and ovulation. The compromised fertility observed in anovular cows is attributed to changes in the follicle/oocyte which carryover to the developing embryo, but also legacy effects on the conceptus and uterus that influence receptivity to pregnancy and maintenance of the CL. Supplementing progesterone to high-producing dairy cows has not always improved fertility in a consistent manner. In many cases, the inability of progesterone to improve P/AI is attributed to the delivery method that has not always been ideal for the type of cow under question. When sufficient progesterone is supplied to anovular cows and those without a CL at the initiation of the timed AI protocol, then fertility is restored similar to that of cows in diestrus. Based on the limited data available, it is suggested that a minimum of 2.0 ng/ml is needed during follicle development to improve fertility in dairy cows. Reaching such concentrations with supplemental progesterone increased P/AI by approximately 10 percentage units, equalizing that of cows in diestrus. On the other hand, when supplemental progesterone increases plasma concentrations by only 0.8 to 1.0 ng/ml then, although benefits were also observed, they usually ranged from 3 to 5 percentage units increment in P/AI, which is not sufficient to reach the values of P/AI observed in cows in diestrus.

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